

# Sox9: A Master Regulator of the Pancreatic Program

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## ■ Abstract

Over the last decade, it has been discovered that the transcription factor Sox9 plays several critical roles in governing the development of the embryonic pancreas and the homeostasis of the mature organ. While analysis of pancreata from patients affected by the Sox9 haploinsufficiency syndrome campomelic dysplasia initially alluded to a functional role of Sox9 in pancreatic morphogenesis, transgenic mouse models have been instrumental in mechanistically dissecting such roles. Although initially defined as a marker and maintenance factor for pancreatic progenitors, Sox9 is now considered to fulfill additional indispensable functions during pancreogenesis and in the postnatal organ through its interactions with other transcription factors and signaling path-

ways such as Fgf and Notch. In addition to maintaining both multipotent and bipotent pancreatic progenitors, Sox9 is also required for initiating endocrine differentiation and maintaining pancreatic ductal identity, and it has recently been unveiled as a key player in the initiation of pancreatic cancer. These functions of Sox9 are discussed in this article, with special emphasis on the knowledge gained from various loss-of-function and lineage tracing mouse models. Also, current controversies regarding Sox9 function in healthy and injured adult pancreas and unanswered questions and avenues of future study are discussed.

**Keywords:** Sox9 · pancreas development · beta-cell · multipotent progenitor cell · Fgf · Pdx1 · Notch · Hes1 · transgenic mouse · molecular marker

## 1. Introduction

Over the preceding 50 years, much effort has been expended in deciphering the mechanisms governing morphogenesis of the mammalian pancreas and, in particular, the insulin-producing  $\beta$ -cell, loss or dysfunction of which manifests in diabetes mellitus. Acquiring mechanistic insight into  $\beta$ -cell neogenesis during pancreas morphogenesis is obviously of potential benefit in the derivation of cell-based diabetes therapies. Firstly, such knowledge may be applied in the optimization of *in vitro* protocols for driving differentiation of functional, glucose-responsive  $\beta$ -cells from either human embryonic or induced pluripotent stem cells. Secondly, insight into  $\beta$ -cell neogenesis may be used to stimulate this process *in*

*situ* in the adult diabetic pancreas to restore functional  $\beta$ -cell mass. Associated with this goal, recent years have seen a resurgence in the study of traditional pancreatic injury models such as partial duct ligation (PDL) [1, 2] or cerulein treatment [3], both of which induce pancreatitis.

In concert with modern inducible genetic lineage tracing of various pancreatic cell populations, these models have been employed to examine whether inflammatory stimuli are able to induce differentiation of new  $\beta$ -cells and to identify the source of these new  $\beta$ -cells, which can either be existing non- $\beta$ -cell pancreatic cell types or a putative facultative adult progenitor. Efforts to mechanistically dissect  $\beta$ -cell neogenesis would benefit greatly from the development of molecular markers enabling identification of  $\beta$ -cell-competent pancreatic

progenitors throughout the duration of pancreas development and, potentially, in the adult.

Since the mid 1990s, studies of knockout and mutant mice have identified crucial roles for a slew of transcription factors in pancreas and  $\beta$ -cell development. Strikingly, many of these factors, including *Isl1*, *NeuroD*, *Nkx2.2*, and *Nkx6.1* are expressed during central nervous system (CNS) development [4], highlighting the conserved transcriptional mechanisms governing morphogenesis in both systems.

The sex-determining region Y (*Sry*)-box-containing (*Sox*) factors are a structurally-related family of developmentally-regulated transcription factors belonging to the high-mobility group (HMG) superfamily; members of this family are united by their highly-conserved HMG-box, a 79-amino acid DNA-binding domain [5-7]. Currently, 20 *Sox* factors have been identified in mammals [8]. These 20 members have each been assigned to one of eight subgroups, A-H on the basis of similarity between HMG-box sequence and protein structure; members sharing more than 80% HMG domain sequence identity occupy the same group [9], and due to biochemical similarity, often play parallel functional roles [10].

The study of *Sox* genes originated in 1990 with the identification of *Sry*, the mammalian testis-determining factor and founding member of the family [5, 6]. Subsequently, crucial roles for *Sox* factors were described in the maintenance of tissue-specific stem/progenitor cells in the central and peripheral nervous system [11-14]. Given the conserved roles of transcription factors regulating neuronal and pancreatic developmental programs [4], attention soon turned in the early 2000s to examining putative conserved roles of *Sox* transcription factors in pancreas and  $\beta$ -cell development. These efforts were spurred on by the report of pancreas dysmorphogenesis in human patients affected by the lethal *SOX9* haploinsufficiency syndrome campomelic dysplasia [15]. This study provided the first tantalizing insight into a role for *SOX9/Sox9* in governing pancreas development, and pointed to the general involvement of *Sox* genes in regulating the pancreatic program.

Evidence, acquired primarily from studying knockout and transgenic mouse models over the past decade, has revealed that *Sox9* serves as a marker and critical maintenance factor for pancreatic progenitors [16-19]. While this role has, by popular perception, defined the function of *Sox9* during pancreatic organogenesis ("pancreogenesis"), mounting evidence suggests that this factor

#### Abbreviations:

ADPKD - autosomal dominant polycystic kidney disease  
 BAC - bacterial artificial chromosome  
 BCIP - 5-bromo-4-chloro-3-indolyl phosphate  
 bHLH - basic helix-loop-helix  
 Bmp - bone morphogenetic protein  
 CAII - carbonic anhydrase II  
 CD - campomelic dysplasia  
 ChIP - chromatin immunoprecipitation  
 CNS - central nervous system  
 Cpa1 - carboxypeptidase A1  
 DAPT - *N*-(*N*-(3,5-difluorophenacetyl)-L-alanyl)-*S*-phenylglycine *t*-butyl ester  
 DCC - *N,N*-dicyclohexylcarbodiimide  
 Dll1 - delta-like ligand 1  
 E - embryonic day  
 ER - estrogen receptor  
 Fgf10 - fibroblast growth factor 10  
 Fgfr - fibroblast growth factor receptor  
 Foxa2 - forkhead-box protein a2  
 GFP - green fluorescent protein  
 GSI-IX - gamma-secretase inhibitor-IX  
 Hes1 - hairy and enhancer of split 1  
 hESC - human embryonic stem cell  
 HMG - high-mobility group  
 Hnf1beta - hepatocyte nuclear factor 1beta (aka Tcf2)  
 HPD - hepatopancreatic ductal system  
 IAPP - islet amyloid polypeptide  
 IF - immunofluorescence  
 iPSC - induced pluripotent stem cell  
 IRES - internal ribosome entry site  
 ISH - *in situ* hybridization  
 Isl1 - ISL LIM homeobox 1  
 LacZ -  $\beta$ -galactosidase-encoding gene  
 Mash1 - mammalian achaete scute homolog 1  
 MODY - maturity-onset diabetes of the young  
 MPC - multipotent pancreatic progenitor cell  
 mRNA - messenger ribonucleic acid  
 NBT - nitro blue tetrazolium chloride  
 NeuroD - neuronal differentiation 1 (aka Beta2)  
 Ngn3 - neurogenin 3  
 NICD1 - Notch 1 intracellular domain  
 Nkx6.1 - Nk6 homeobox protein 1  
 Onecut1 - one cut homeobox 1 (aka Hnf6)  
 PanIN - pancreatic intraepithelial neoplasia  
 PC1/3 - proprotein convertase 1  
 PCFU - pancreatic colony-forming unit  
 PDAC - pancreatic ductal adenocarcinoma  
 PDL - partial duct ligation  
 Pdx1 - pancreatic and duodenal homeobox 1  
 PNS - peripheral nervous system  
 PP - pancreatic polypeptide  
 Ptf1a - pancreas transcription factor 1 subunit alpha  
 Rbpj - recombination signal binding protein for the immunoglobulin kappa J region  
 RT-PCR - reverse transcription polymerase chain reaction  
 Sox9 - sex-determining region Y (*Sry*) box 9  
 STZ - streptozotocin  
 Tcf2 - transcription factor 2 (aka Hnf1 $\beta$ )  
 UTR - untranslated region  
 Vegf - vascular endothelial growth factor  
 Wnt - wingless-type MMTV integration site family  
 YFP - yellow fluorescent protein

performs additional, equally important functions in both the developing and mature pancreas. These functions include the induction of endocrine differentiation [20, 21] and maintenance of the embryonic and adult ductal state [20, 22].

While two recent reviews have covered the roles of Sox9 in regulating pancreatic as well as either hepatic [23] or duodenal [24] programs, the present review focuses exclusively on the former to enable a more comprehensive examination of the multiple roles of Sox9 in the pancreas. This review aims to describe the roles of Sox9 during pancreas development with an emphasis on mouse models, placing major findings in the context of pancreas developmental biology from a historical perspective. In the second part of this review, focusing on the postnatal pancreas, recent contradictory findings from pulse-chase labeling of *Sox9*<sup>+</sup> cells in both healthy and injured conditions are discussed. Finally, recently uncovered roles for Sox9 in the initiation of pancreatic cancer are summarized.

## 2. Pancreas morphogenesis in the mouse

### 2.1 Origins of the pancreas and its differentiated cell types

In the mouse, the pancreas arises as two opposing dorsal and ventral “buds” of the foregut endoderm (**Figure 1A**). While these buds become morphologically discernible at around embryonic day (E) 8.75-9.0, pancreatic fate is specified in undifferentiated foregut endoderm (coined the “primary transition” [25]) several hours prior to overt bud emergence [26]. This prepancreatic endoderm is demarcated by expression of the transcription factors Pdx1 and Ptf1a ventrally from E8.5 (8-10 somites) and dorsally from E8.5-8.75 (10 somites) [27, 28]. A third pancreatic transcription factor, Nkx6.1, is expressed in prospective ventral and dorsal pancreatic endoderm from E8.75 and E9.0, respectively [27]. Pdx1, Ptf1a and Nkx6.1 continue to mark the pancreatic endoderm which, until E12.5 when the two buds fuse (**Figure 1B**), is comprised predominantly of early multipotent pancreatic progenitor cells (MPCs) [29, 30]. *Pdx1-Cre* [29] or *Ptf1a<sup>Cre</sup>* [30] mediated lineage tracing have revealed that, at the population level, MPCs give rise to all mature cell types in the adult organ, including:

1. Exocrine cells: the digestive enzyme-producing acinar cells and the cells of the

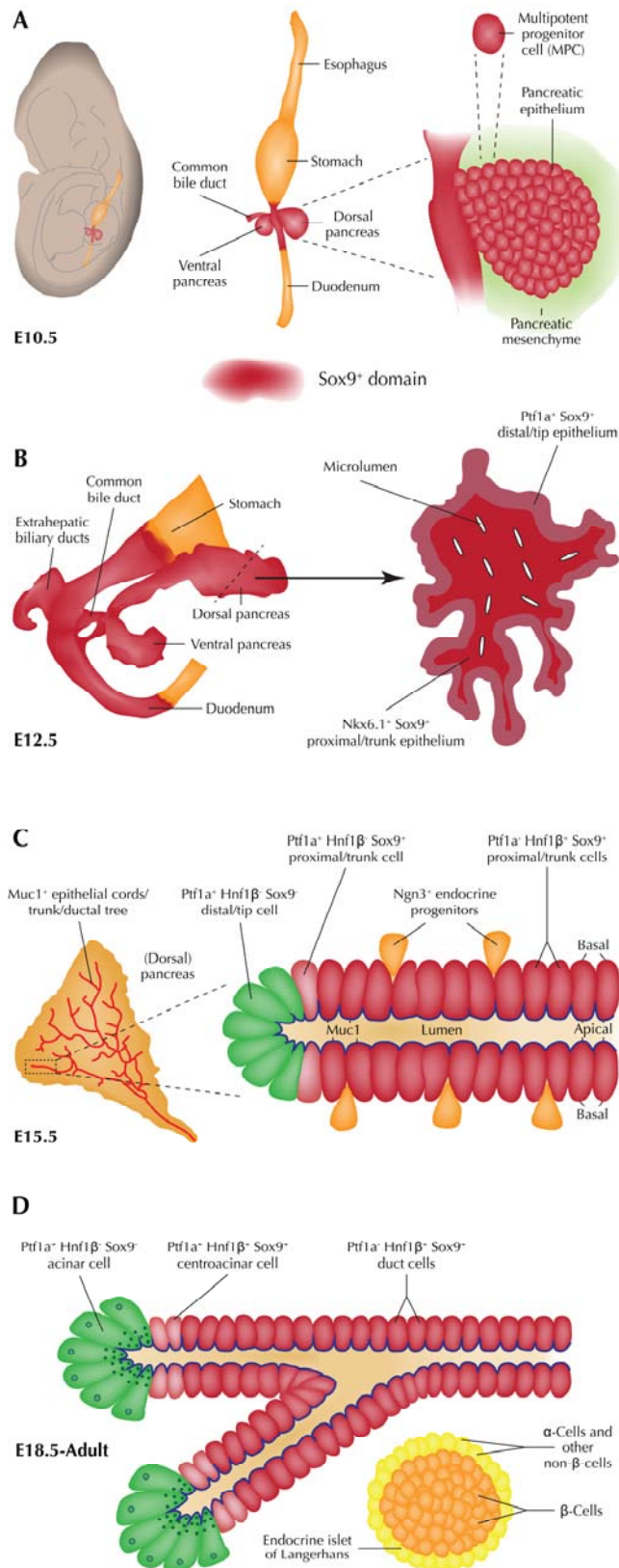
ducts that transport these enzymes to the duodenum.

2. Endocrine cells of the islets of Langerhans (**Figure 1D**).

Notably, lineage tracing has shown that, in addition to Pdx1 and Ptf1a, MPCs can be similarly defined by their expression of the transcription factor Hnf1 $\beta$  (Tcf2) [31], the Notch downstream effector Hes1 [32], and carboxypeptidase A1 (Cpa1) [33]. Nkx6.1 undoubtedly marks this population even though this is not yet formally demonstrated because of the lack of suitable genetic tools. The five endocrine cell types derived from such progenitors comprise  $\beta$ -cells,  $\alpha$ -cells,  $\delta$ -cells, PP-cells, and  $\epsilon$ -cells: respectively, they produce and secrete insulin, glucagon, somatostatin, pancreatic polypeptide (PP), or ghrelin. All endocrine cells arise in turn from a common, transient endocrine progenitor marked by expression of the transcription factor Ngn3 [29] which is both necessary [34] and sufficient [35-38] for endocrine differentiation. *Ngn3* expression in MPCs results in temporally-distinct competence windows during which given endocrine cell types arise [39], commencing at E9.5 with the appearance of glucagon<sup>+</sup> cells in the dorsal pancreas [40]. Insulin<sup>+</sup>  $\beta$ -cells are not detected until E12.5; thereafter, from E14.5, their numbers expand exponentially [40] through the “secondary transition” [25], a major window of pancreatic differentiation. Somatostatin<sup>+</sup> cells are detectable next, followed by PP-cells late in gestation [40]. Cells expressing ghrelin, the latest endocrine cell type to be discovered, can be detected as early as E10.5 and peak in number during the secondary transition [41].

### 2.2 Pancreatic progenitor expansion

After evaginating, the pancreatic buds rapidly expand and branch, concurrently with the synchronous extension and remodeling of a multi-lumen tubular plexus into a contiguous ductal tree [42, 43]. Expansion of MPCs is driven by both progenitor-intrinsic cues and extrinsic signals from the enveloping pancreatic mesenchyme (**Figure 1A**). Since final pancreas size is dictated by initial MPC pool size [44], the absence of such signals typically impacts upon progenitor proliferation, reducing progenitor numbers, manifesting in pancreatic hypoplasia. The progenitor-intrinsic transcription factors Pdx1 [45, 46] and Ptf1a [30, 47] as well as the Notch ligand Dll1 and effector Hes1 [35, 48, 49] all maintain MPC numbers, and are thus crucially required for pancreatic epithelial



**Figure 1. The dynamic spatiotemporal pattern of Sox9 expression in the developing and adult mouse pancreas.** **A:** By E10.5, the foregut Sox9 expression domain encompasses the endoderm of the dorsal and ventral pancreatic buds as well as the antral (posterior) stomach, common bile duct (CBD), and anterior duodenum. In both pancreatic buds, Sox9 is confined to the epithelium, which at E10.5 is predominantly comprised of multipotent pancreatic progenitor cells (MPCs). **B:** At E12.5, Sox9 expression continues to demarcate the anterior stomach, CBD, extrahepatic biliary ducts and anterior duodenum as well as the fused dorsal and ventral pancreatic buds. By this stage, the pancreas is actively growing and is clearly lobulated; remodeling of microlumens to form a contiguous tubular plexus is also underway. These processes are concurrent with patterning of MPCs into a Ptf1a<sup>+</sup> acinar-fated distal "tip" domain surrounding an Nkx6.1<sup>+</sup> bipotent ductal-/endocrine-fated proximal "trunk" domain. Sox9 expression at this stage is highly dynamic: while Sox9 mRNA is receding from the tips at E12.5, Sox9 protein remains coexpressed with both Ptf1a and Nkx6.1 in the tips and the trunk, respectively. **C:** By E15.5, the mid-point of the secondary transition, Sox9 expression defines polarized Muc1<sup>+</sup> epithelial cords of the proximal trunk, the predecessor of the ductal tree. Sox9 marks both Ptf1a<sup>+</sup> Hnf1β<sup>+</sup> cells comprising the majority of the trunk as well as Ptf1a<sup>+</sup> Hnf1β<sup>+</sup> acinar cells "capping" the cords are devoid of Sox9 expression. Similarly, Ngn3<sup>+</sup> endocrine progenitors delaminating from the Sox9<sup>+</sup> trunk rarely coexpress high levels of Sox9 and are instead intercalated amongst Sox9<sup>+</sup> cells. **D:** By late gestation (E18.5), Sox9 expression becomes confined to a subpopulation of ductal cells and centroacinar cells which are also defined by their expression of Hnf1β. Sox9 is excluded from the Ptf1a<sup>+</sup> acinar cells as well as all endocrine cells of the islets of Langerhans.

expansion: hypoplasia of one or both pancreatic buds ensues when MPCs are devoid of any of these factors. Likewise, deficiency for certain pancreatic mesenchyme-derived signaling cues results in hypoplasia of the pancreatic epithelium: Wnt [50, 51], Bmp [52], and, most prominently, Fgf10 [53] signaling have all been shown to stimulate proliferation of epithelial MPCs.

### 2.3 Proximodistal patterning

Concurrently with their expansion from E10.5 onwards, initially multipotent pancreatic progenitors become physically segregated by the secondary transition in terms of their developmental competency. MPCs in the distal “tip” domain marked by Pdx1, Ptf1a, and Cpa1 [33], gradually become acinar-fated while those progenitors left in their wake in the proximal “trunk”, expressing Pdx1, Nkx6.1 (and its paralog, Nkx6.2), and Hnf1 $\beta$  [54], form both the ductal tree and endocrine cells and are thus “bipotent progenitors” [33] (**Figure 1B, C**). This compartmentalization is established through the cross-antagonism between Ptf1a and Nkx6.1/Nkx6.2; while initially co-expressed in early progenitors, by E14.5 they resolve into distinct Ptf1a<sup>+</sup> distal tip and Nkx6.1<sup>+</sup> proximal trunk domains [55]. More recently, a crucial requirement for Notch signaling has been demonstrated in the patterning of the trunk domain [56, 57].

### 2.4 The secondary transition and pancreatic differentiation

During the secondary transition, Ngn3<sup>+</sup> endocrine progenitors appear in an intercalated pattern between the cells of the trunk, which comprises a primitive ductal epithelium marked by Hnf1 $\beta$  [54, 58], as well as the transcription factors Onecut1 (previously Hnf6) [59, 60] and Foxa2 (formerly Hnf3 $\beta$ ) [61]. It has long been assumed that endocrine progenitors originate from the secondary transition ductal epithelium [54]. However, the relative paucity of exclusive markers of this domain and, hence, suitable genetic tools has meant that it is only quite recently, with their advent, that it has become feasible to test this hypothesis formally. The strictly mutually-exclusive expression domains of Ngn3 and Hes1 within the trunk have led to the widely-held notion that endocrine progenitors are specified through the process of Notch-mediated lateral inhibition [35, 62] (**Figure 1C**). As endocrine progenitors differentiate into the five endocrine cell types, they coalesce to form polyclonal islets [63]. In the mouse, islets display a

**Table 1.** Qualitative relative expression of Sox factors in developing pancreas and adult islets of the mouse

Group	Member	E12.5	E15.5	Islet
SoxA	Sry	-	-	
SoxB1	Sox1	-	-	
	Sox2	-	-	-
	Sox3	-	-	
SoxB2	Sox14			
	Sox21			
SoxC	Sox4	+++	++	++
	Sox11	+	+	-
	Sox12	+	-	-
SoxD	Sox5	+	+	-
	Sox6			
	Sox13	++	+	++
SoxE	Sox8	+	+	-
	Sox9	+++	+++	+/-*
	Sox10	+	+	-
SoxF	Sox7	+	+	
	Sox17	+	+	
	Sox18	+	+	
SoxG	Sox15	-	+	
SoxH	Sox30	+	+	

**Legend:** Data indicate the qualitative representation of relative abundance of Sox family member expression in whole embryonic mouse E12.5 and E15.5 pancreata and isolated adult islets, as surveyed by RT-PCR [66, 67]. *Symbols:* -: not detected; +, ++, +++: detected in increasing abundance, respectively. Absence of a symbol indicates expression not examined [66, 67]. \*+: Sox9 transcripts initially detected in isolated adult islets [66], but (-) not in a subsequent study [16].

highly stereotyped cytoarchitecture comprising a central core of insulin<sup>+</sup>  $\beta$ -cells and a peripheral mantle composed predominantly of glucagon<sup>+</sup>  $\alpha$ -cells as well as the other three scarcer endocrine cell types (**Figure 1D**). It is only with the onset of postnatal life that the  $\beta$ -cells become fully-functional, capable of sensing glucose and modulating their secretion of insulin accordingly [64]. Concomitantly and during the preceding period, the pancreas grows rapidly, principally through the proliferation of acinar cells [65].

## 3. Expression of Sox factors in the developing and adult pancreas

The first comprehensive survey of Sox family gene expression in the mammalian pancreas [66] revealed that transcripts for a dozen Sox genes are detectable in the developing mouse pancreas: Sox11, Sox4, Sox13, Sox5, Sox9, Sox8, Sox10, Sox7, Sox17, Sox18, Sox15, and Sox30, three of which (Sox4, Sox9, and Sox13) were reported to be addi-

tionally detected in mature islets (**Table 1**). Notwithstanding the additional detection of *Sox2* and *Sox12* in developing mouse pancreas, findings of a similar analysis were in close concordance with this outcome [67] (**Table 1**). Of these dozen *Sox* genes expressed during mouse pancreogenesis, orthologs of five (*SOX4*, *SOX9*, *SOX10*, *SOX11*, and *SOX12*) have been shown to be enriched in the developing human pancreas versus adult islets [68], testament to the conserved nature of the pancreatic programs in mouse and man.

Via a more in-depth *in situ* hybridization (ISH) expression survey by Sox group, the Sander group [66] showed group C member *Sox11* to be expressed predominantly in early pancreatic mesenchyme, then exclusively within some epithelial cells by E12.5, while *Sox4* (also group C) expression is widespread in pancreatic epithelium from E12.5; both factors become islet-localized by birth with *Sox4* being expressed in all mature islet cell types [67]. Corresponding with a pro-endocrine role for *Sox4*, explanted E11.5 *Sox4*<sup>-/-</sup> pancreata exhibit reduced numbers of insulin<sup>+</sup> and glucagon<sup>+</sup> cells [67]. Given the lack of quantification of Ngn3<sup>+</sup> endocrine progenitors in *Sox4*-deficient explants coupled with no detectable effect of *Sox4* deletion on either proliferation or survival of insulin<sup>+</sup> cells [67], the underlying cause of endocrine hypoplasia in *Sox4*<sup>-/-</sup> mice remains obscure. Zebrafish studies, however, provide some insight. The *Sox4* ortholog, *sox4b*, is expressed in endocrine precursors and glucagon<sup>+</sup> cells in the developing zebrafish pancreas; when its expression is downregulated, glucagon<sup>+</sup> cells are specifically lost, revealing a role in  $\alpha$ -cell differentiation [69]. While the absence of an effect on insulin<sup>+</sup> cell numbers suggests their differentiation to be *sox4b*-independent in zebrafish, specific effects on a single endocrine cell type suggest *sox4b* to either function downstream of a common endocrine precursor or to impact endocrine precursor numbers specifically during the window of  $\alpha$ -cell neogenesis.

Of the group D factors, *Sox13* is expressed at E10.5 in some, then by E14.5, in almost all pancreatic epithelial cells [66]. Likewise, L-*Sox5* is first detectable at E12.5 in some pancreatic epithelial cells and is broadly epithelially expressed by the secondary transition. After this point, both factors are rapidly downregulated, becoming undetectable by birth. In contrast, *Sox8* and *Sox10*, two of the three group E factors, are expressed in dispersed glucagon-negative cells at the epithelial periphery by E10.5 through E12.5, paralleling the pattern of *Sox8* expression during chick pancreo-

genesis [70]. By birth, expression of both factors is confined to cells enveloping the islets [66]. Given that in the peripheral nervous system (PNS), both Sox factors are expressed in glial cells with *Sox10* being required for their development [71, 72] and glia have been reported [73, 74] to ensheath the pancreatic islets, it was posited [66] that these *Sox8*<sup>+</sup>/*Sox10*<sup>+</sup> cells are islet glial cells. Despite the unique peri-islet expression patterns of *Sox8* and *Sox10*, analysis of pancreata from *Sox8*<sup>-/-</sup> or *Sox10*<sup>-/-</sup> mice revealed no gross dysgenesis of either the endocrine or exocrine compartments, indicating these factors to be dispensable for normal pancreatic morphogenesis [66]. While the precise function of peri-islet glia remains unknown, it has been proposed that they may provide neurotropic support to islet-innervating nerves and endocrine cells, and also play a role in neuroendocrine regulation [75]. While the possible function of *Sox10* and peri-islet glia in adult endocrine function warrants closer examination, the perinatal lethality of *Sox10*<sup>-/-</sup> mice will dictate the requirement for a conditional *Sox10* ablation model.

#### 4. Sox9 expression in the developing pancreas

Alongside *Sox8* and *Sox10*, *Sox9* completes the Sox group E trifecta. Of the seven Sox factors, whose pancreatic expression patterns were comprehensively assessed by Lioubinski *et al.* [66], *Sox9* proved the most intriguing. *Sox9* was found to be highly expressed in both emerging pancreatic buds at E9.5 when they comprise almost exclusively of multipotent progenitors, hinting at a role in this population [66]. While *Sox9* ISH signal remains elevated throughout E12.5 pancreatic epithelium, some diminution is evident at the periphery, denoting *Sox9* withdrawal from the distal tips: concordantly, *Sox9* becomes confined to trunk epithelium by the secondary transition. In line with this proximal expression pattern, the distribution of *Sox9* ISH signal at birth was interpreted as marking a subset of ductal cells, islets, and a few acinar cells [66]. At the time of publication a decade ago, proximodistal patterning and secondary transition bipotent ductal/endocrine progenitors had yet to be conceptualized and thus, their markers identified. Notwithstanding, embryonic ductal *Sox9* expression was deemed significant, given the assumption that endocrine progenitors arise from this domain during the secondary transition [36].

To better characterize dynamic spatiotemporal *Sox9* expression during mouse pancreogenesis and

to circumvent limited ISH resolution, co-immunofluorescence (IF) analysis was performed with a well-characterized Sox9-specific antiserum [76]. While colocalizing with the MPC marker Pdx1 in early (by E9.0) dorsal and ventral pancreatic endoderm (**Figure 1A**), Sox9 was excluded from glucagon<sup>+</sup> endocrine cells [16, 77]. Strikingly, while at E12.5 *Sox9* mRNA has receded from the epithelial tips [66], Sox9 protein is retained throughout the entire E12.5 pancreatic epithelium (excluding endocrine cells) [16], a finding validated by later studies [19] (**Figure 1B**). While implying Sox9 to be a novel pancreatic progenitor marker, these analyses also indicated that concomitant with proximodistal patterning, *Sox9* expression is highly dynamic. Notably, hinting at evolutionarily-conserved roles for Sox9 in vertebrate pancreatic morphogenesis, this pattern of Sox9 expression in the early pancreatic rudiment is recapitulated in the frog *Xenopus laevis*, zebrafish, and human. *Sox9* expression, within a broader *Pdx1*<sup>+</sup> endodermal domain, specifically demarcates dorsal and ventral prepancreatic endoderm in *X. laevis* from its initial specification and continues to define the pancreatic buds through the point at which they fuse [78]. Similarly, the *Sox9* paralog *sox9b* is expressed in the early zebrafish hepatopancreatic endoderm, in the anterior *Pdx1*<sup>+</sup> domain, marking both the base of the dorsal pancreas and the prospective ventral pancreas [79]. A very recent immunohistochemistry-based expression analysis [80] has provided unprecedented (and beautiful) insight into the expression patterns of a number of pancreatic transcription factors (including SOX9) during the earliest steps of human pancreatic morphogenesis. This study shows that in humans, SOX9 is weakly coexpressed with PDX1 in prospective duodenal-pancreatic endoderm by Carnegie Stage (CS) 12 (29-31 days post-conception, dpc), equivalent to ~E9-9.5 in mouse embryogenesis [80]. By CS13 (30-33 dpc ≈E9.5-10 in mouse), SOX9 is strongly expressed in the readily-discernible dorsal and ventral pancreatic buds and is maintained through CS16 (37-40 dpc ≈E12.25-12.75) in the branched pancreatic epithelium [80], in agreement with earlier work [15].

Consistent with withdrawal of Sox9 from the murine pancreatic tip domain after E12.5, IF showed Sox9 to be restricted by E15.5 to a subpopulation of Pdx1<sup>+</sup> cells comprising secondary transition pancreatic “epithelial cords” [16, 77] (**Figure 1C**). Such cords, historically posited to give rise to all differentiated cell types arising during the secondary transition [81] are now known as the proximal trunk domain. Notably, the proc-

ess of segregating SOX9 to the proximal trunk appears to occur relatively late during the human pancreatic program, between 10-14 weeks post-conception (wpc) [80].

Supporting a continuing progenitor role through the secondary transition, a large proportion (~40%) of Sox9<sup>+</sup> cells in the E15.5 mouse pancreas are both proliferative and Notch-responsive (Hes1<sup>+</sup>) [16]. In contrast, little overlap is evident between strongly Sox9-stained (Sox9<sup>hi</sup>) cells and those expressing the endocrine progenitor marker Ngn3; Ngn3<sup>+</sup> cells tend instead to be intercalated between Sox9<sup>hi</sup> trunk cells [16, 21, 77] (**Figure 1C**). Similarly, in the developing human pancreas, cells expressing high levels of the Ngn3 ortholog NEUROG3 occur in juxtaposition with SOX9<sup>+</sup> cells, concurrently with endocrine differentiation at 10 wpc [80]. Discordance between the reported extent of Sox9/Ngn3 colocalization in the embryonic mouse pancreas [16, 77] triggered a more comprehensive examination. This study concluded that, while only a small proportion (13.1%) of Ngn3<sup>+</sup> cells are Sox9<sup>hi</sup>, the vast majority (74.1%) are weakly Sox9-immunoreactive (Sox9<sup>lo</sup>), with the remaining 12.8% of Ngn3<sup>+</sup> cells being Sox9<sup>+</sup>, consistent with Sox9<sup>+</sup> cells initiating Ngn3 coexpression then extinguishing Sox9 followed by Ngn3 expression before commencing endocrine hormone production [21]. In accordance with Ngn3<sup>+</sup> cells arising from the Sox9<sup>+</sup> trunk then downregulating Sox9 expression, Sox9 rarely colocalizes with late endocrine progenitor markers and is excluded from all differentiated endocrine and acinar cells [16] (**Figure 1D**). This is consistent with Sox9 being enriched in pancreatic progenitors while being largely excluded from endocrine progenitors and differentiated endocrine and acinar cells. Confirming this notion, pancreatic Sox9 expression is unaffected in embryos lacking the endocrine differentiation/maturation factors *Ngn3* or *Nkx6.1*, showing *Sox9* to be upstream of both [16]. Cementing its characterization as a pancreatic progenitor marker, expression of Sox9 is maintained throughout pancreata from *Pdx1-Fgf10* mice in which the MPC mitogen Fgf10 is forcibly expressed throughout the pancreatic epithelium under the governance of the *Pdx1* promoter, arresting pancreatic cells in the progenitor state and abrogating differentiation [16, 82].

## 5. Lineage tracing *Sox9*<sup>+</sup> cells during pancreas development

### 5.1 *Sox9* marks MPCs during the primary transition

While *Sox9* coexpression with known MPC markers and regulation consistent with being a progenitor-expressed factor both infer such a role, these lines of evidence by themselves are insufficient to prove this. Such incontrovertible evidence for *Sox9* defining MPCs can only be provided by genetic lineage tracing of the pancreatic *Sox9*<sup>+</sup> population. Using this approach, the de Crombrughe group [18] was the first to show indisputably that expression of *Sox9* defines multipotent progenitors not only for the pancreas but other organs as well. The authors generated a *Sox9*<sup>IRES-Cre</sup> knock-in line through the ingenious stratagem of inserting the *Cre* transgene into the 3'-UTR of mouse *Sox9*; in theory, *Cre* expression faithfully recapitulates endogenous *Sox9* expression while maintaining *Sox9* dosage integrity [18]. Notably, such recapitulation of whole-organism expression domains is of particular relevance to *Sox9*, given that three separate *long-range* (251 kb 5' to 95 kb 3') upstream and downstream enhancers have been demonstrated to govern distinct organ-specific subsets of the complex spatiotemporal *Sox9* expression domain [83]. Analysis of embryos carrying both *Sox9*<sup>IRES-Cre</sup> and the *Rosa26R*<sup>lacZ</sup> *Cre* reporter allele [84] revealed that by either E17.0 [18] or postnatal day (P)1 [17], the entire pancreatic (exocrine and endocrine) epithelium comprised descendants of *Sox9*<sup>+</sup> cells as marked by heritable  $\beta$ -gal expression resulting from *Sox9*<sup>IRES-Cre</sup>-mediated *Rosa26R*<sup>lacZ</sup> recombination (**Figure 2A**). This genetic lineage tracing study showed *Sox9* to mark progenitors not only of the pancreas, but also those of testis, intestine and spinal cord which were all similarly uniformly  $\beta$ -gal-labeled in E17.0 *Rosa26R*<sup>lacZ</sup>; *Sox9*<sup>IRES-Cre</sup> embryos [18].

### 5.2 *Sox9* continues to define multipotent progenitors throughout pancreogenesis

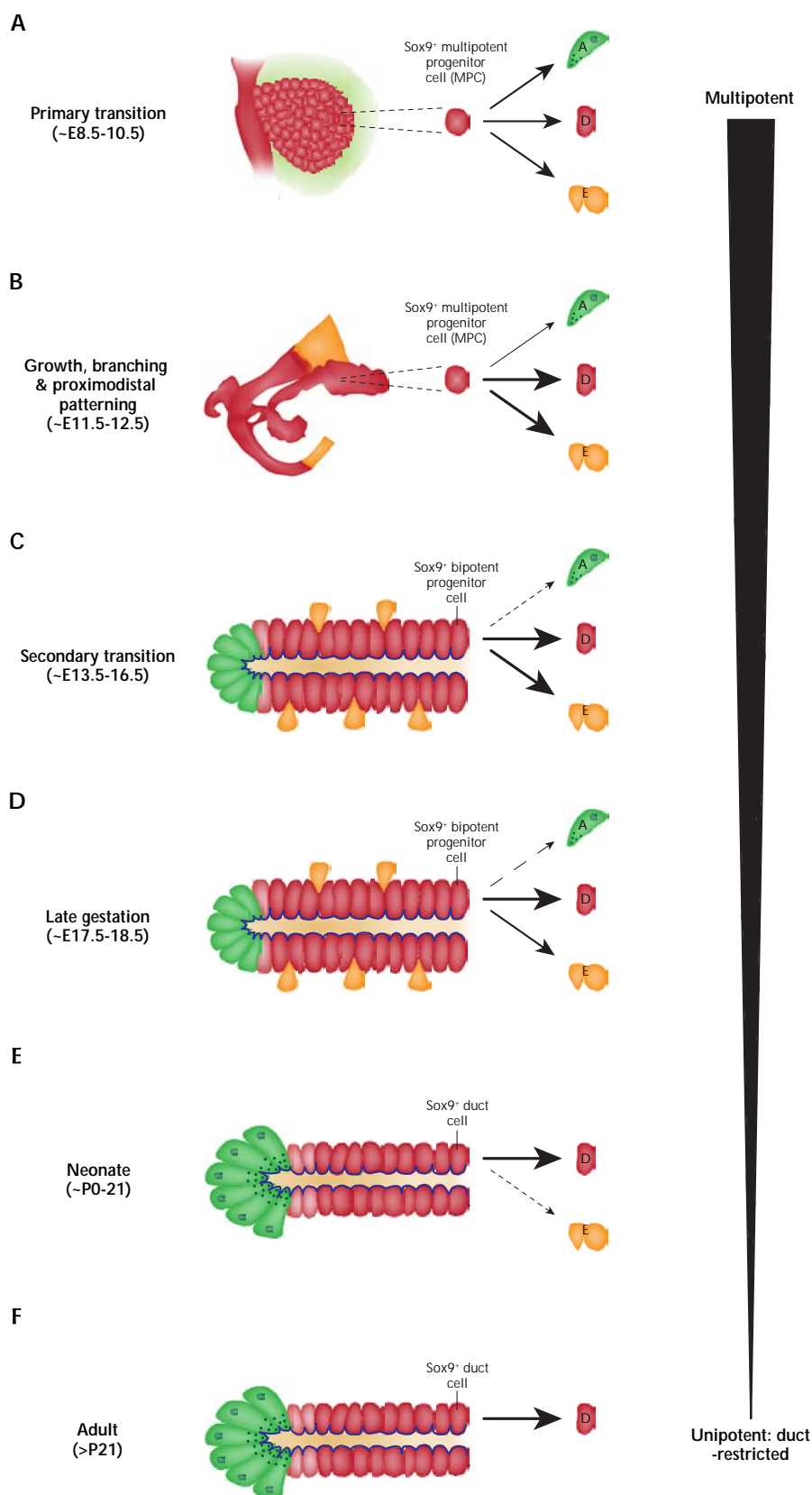
Since in *Rosa26R*<sup>lacZ</sup>; *Sox9*<sup>IRES-Cre</sup> embryos, *Sox9*<sup>+</sup> cells (and their progeny) are labeled from the onset of pancreatic *Sox9* expression, this model cannot be used to trace the descendants of *Sox9*<sup>+</sup> cells at later time-points. To circumvent this issue in the absence of purpose-made genetic tools (namely, a *Sox9-CreER* line), “pseudo short-term” lineage tracing of *Sox9*<sup>+</sup> cells was performed by exploiting

the perdurance of *Sox9* promoter-driven eGFP over endogenous *Sox9* in the *Sox9-eGFP* BAC transgenic line [85]. As a consequence of the ~26-hour half-life of eGFP [86], retention of eGFP label for 1-2 days past the point of *Sox9* promoter inactivity allows descendants of *Sox9*<sup>+</sup> cells to be followed through this window. Consistent with *Sox9* marking early pancreatic progenitors, *Sox9*-eGFP colocalized with *Sox9* in almost all cells of the early (E10.5) pancreatic epithelium, including *Sox9*<sup>+</sup> progenitor-derived glucagon<sup>+</sup> endocrine cells [21]. Concordantly, it has recently been demonstrated that *ex vivo*, when diluted to clonal density, isolated *Sox9*-eGFP<sup>+</sup> cells from E11.5 pancreata are capable of undergoing an estimated >8-9 doublings and consequently expand to form clonal epithelial “pancreatic spheres” containing several endocrine cell types, including glucose-responsive insulin-secreting cells [87]. Thus, primary *Sox9*<sup>+</sup> cells from early pancreatic rudiments are capable of self-renewal and multilineage differentiation, hallmarks of multipotent progenitor cells. Later in pancreogenesis, during the secondary transition (E15.5), short-term lineage tracing showed *Sox9*-eGFP, mirroring *Sox9*, to be restricted to the epithelial cords/trunk [21]. Strikingly, until E17.5, eGFP was also detected in a proportion of *Sox9*<sup>+</sup> Ngn3<sup>+</sup> endocrine progenitors, lending further credence to the notion that endocrine progenitors arise from *Sox9*<sup>+</sup> cells. Consistent with both this and the transient ~18-hour existence of Ngn3<sup>+</sup> cells [88], eGFP was detected in some insulin<sup>+</sup> and glucagon<sup>+</sup> cells at E15.5 and birth. Together, these findings imply that endocrine cells arise from *Sox9*<sup>+</sup> progenitors through ~E14-18 (**Figure 2C, D**). Like endocrine cells, at E15.5, acinar cells abutting the distal tips of the *Sox9*<sup>+</sup> trunk also bore eGFP label, inferring that they also arise from *Sox9*<sup>+</sup> cells (**Figure 2C**). However, since no eGFP label was evident in acinar cells at birth, this suggests that acinar neogenesis ceases in late embryogenesis (**Figure 2D**) and proliferation becomes the predominant method of acinar expansion from then onwards. In sum, the results of this “pseudo short-term” lineage tracing study imply that at the population level, *Sox9*<sup>+</sup> cells are multipotent during the secondary transition, capable of giving rise to both endocrine and exocrine cells. Unarguably, colocalization upon which this approach is based cannot substitute fully for genetic lineage tracing, since the identity of labeled and traced or “chased” cells cannot be fully defined and to this end has (justifiably) invoked criticism [31]. Notwithstanding its caveats however, subsequent



**Figure 2. Developmental potency of Sox9<sup>+</sup> cells becomes progressively restricted during pancreogenesis.**

**A:** Genetic lineage tracing has shown that during the primary transition, Sox9 defines multipotent MPCs (also marked by *Pdx1* and *Ptf1a*) comprising the pancreatic epithelium which give rise to all three pancreatic compartments: exocrine acinar (A), ductal (D), and endocrine (E) cells (via an intermediate *Ngn3*<sup>+</sup> endocrine progenitor cell). **B:** Concurrently with branching and proximodistal patterning of the pancreatic epithelium between approximately E11.5-12.5, Sox9 continues to mark MPCs with an increased propensity to give rise to ductal and endocrine cells at the expense of acinar cells. **C:** During the secondary transition, Sox9 expression defines bipotent progenitor cells which almost exclusively yield ductal or endocrine cells; Sox9<sup>+</sup> cells at the extreme distal ends of the trunk can, however, yield acinar cells. **D:** By late gestation, Sox9<sup>+</sup> cells predominantly contribute to the ductal compartment; they can, however, still yield endocrine cells and, very rarely, acinar cells. **E:** During the first three weeks of life, Sox9<sup>+</sup> cells almost exclusively yield ductal cells; endocrine neogenesis can, however, still rarely occur from the Sox9<sup>+</sup> compartment. **F:** In contrast, in later adulthood, Sox9<sup>+</sup> cells are unipotent, giving rise only to ductal cells. Thickness of arrows denotes relative propensity to yield cells of each of the three pancreatic compartments.



tamoxifen-inducible genetic lineage tracing of *Sox9*<sup>+</sup> cells during pancreogenesis using BAC transgenic *Sox9-CreER*<sup>T2</sup> mice [19] bore out the main conclusions of this earlier study, validating the efficacy of this approach. In these mice, administration of tamoxifen results in nuclear translocation of *Sox9* promoter-driven *CreER*<sup>T2</sup>, which remains active and capable of recombination ~12-36 hours following tamoxifen injection [33]. Thus, in mice carrying both *Sox9-CreER*<sup>T2</sup> and *Rosa26R*<sup>lacZ</sup> alleles, a single injection of tamoxifen will result in a “pulse” of *CreER*<sup>T2</sup>-mediated  $\beta$ -gal labeling of all cells in which *Sox9* is active during this 12-36-hour post-injection window such that these cells and their progeny can then be traced or “chased”.

Replicating the results of labeling *Sox9*<sup>+</sup> cells from the onset of pancreogenesis [18], administering tamoxifen to *Sox9-CreER*<sup>T2</sup>; *Rosa26R*<sup>lacZ</sup> embryos at E8.5 resulted in  $\beta$ -gal labeling of all three pancreatic compartments—acinar, ductal, and endocrine—by three weeks of age [19] (**Figure 2A**). Strikingly, and largely corresponding with the preceding *Sox9-eGFP* analysis, exposure to tamoxifen between E12.5-18.5 similarly resulted in *Sox9*<sup>+</sup> cells contributing to all three compartments [19], showing that the pancreatic *Sox9*<sup>+</sup> population serves as progenitors, giving rise to ductal, endocrine, and acinar cells, throughout embryogenesis (**Figure 2B-D**). Quantification of the relative distributions of  $\beta$ -gal-labeled cells between ducts, islets, and acini revealed that with progressively later tamoxifen administration, relative contribution to the ducts increased at the expense of the acini [19]. However, as stressed by Kopp and colleagues [19], since mitotic indices differ between endocrine, ductal, and acinar cells, quantification of such genetic pulse-chase labeling cannot provide a direct index of their relative rates of neogenesis. Notwithstanding this caveat, the decline in acinar labeling with progressively later pulses implies that the competence of *Sox9*<sup>+</sup> progenitors to give rise to acinar cells decreases as pancreogenesis proceeds (**Figure 2A-D**). This caveat might also conceivably account for the discrepancy in late acinar cell labeling between the pseudo short-term and genetic lineage-tracing studies (**Figure 2D**): similar rates of acinar neogenesis from *Sox9*<sup>+</sup> cells during late embryogenesis might result in different labeling outcomes in both. While *Sox9-eGFP* in a small proportion of progenitor-derived acinar cells might be diluted out by subsequent cell division, the extent of heritable *Sox9-CreER*<sup>T2</sup>-mediated  $\beta$ -gal labeling in the same proportion of

acinar cells might be amplified by rapid proliferative expansion of acini in the immediate postnatal period [65] comprising the three-week chase.

Findings from an additional genetic lineage-tracing study [17], while generally in agreement with *Sox9-eGFP*- and *Sox9-CreER*<sup>T2</sup>-mediated tracing, further emphasize how varying proliferative rates of the pancreatic compartments confound interpretation of pulse-chase labeling data. Using an analogous approach to their earlier generation of *Sox9*<sup>RES-Cre</sup> knock-in mice, Furuyama and colleagues [17] generated a *Sox9*<sup>RES-CreERT2</sup> knock-in line by inserting *CreER*<sup>T2</sup> into the 3'-UTR of mouse *Sox9*. Analysis of *Sox9*<sup>RES-CreERT2</sup>; *Rosa26R*<sup>lacZ</sup> mice at eight weeks of age revealed that following tamoxifen administration at E16.5,  $\beta$ -gal labeling was observed in ducts and islets, although the largest proportion of all  $\beta$ -gal-labeled cells (73%) were acinar cells [17]. While qualitatively this study validates that of Kopp *et al.* [19] in showing that in late embryogenesis, *Sox9*<sup>+</sup> cells continue to beget endocrine and acinar cells, the protracted chase period confounds interpretation of labeling patterns. Following tamoxifen administration at E16.5, *Sox9-CreER*<sup>T2</sup>-mediated labeling (as a proportion of total labeled cells) of ducts at three weeks of age is just over twice that of acini [19] while tamoxifen exposure at the same stage in embryos carrying *Sox9*<sup>RES-CreERT2</sup> results in duct labeling being a little over three-fold less than in acinar cells when analyzed at eight weeks of age [17]. Given the irrelevance of tamoxifen dosage on such relative quantifications and recombination of the same *Rosa26R*<sup>lacZ</sup> allele being examined, other factors must account for this large discrepancy in the ratio of ductal:acinar labeling between the two studies. The rapid proliferative expansion of the acini in the immediate postnatal period [65] renders it impossible to distinguish relative proportions of acinar cells arising through either: 1) neogenesis from *Sox9*<sup>+</sup> progenitors or 2) proliferation of labeled acinar cells. Hence, the longer chase period in the study by Furuyama and coworkers [17] might conceivably account for a greater degree of “amplification” of initial numbers of  $\beta$ -gal-labeled acinar cells through their proliferation, skewing the labeling ratio towards such cells. While plausible, other factors might equally account for the discrepancy between lineage tracing data generated using the two *CreER*<sup>T2</sup> lines, which will be speculated upon later.

While the findings of these *Sox9*<sup>+</sup> cell lineage tracing analyses differ in their details, their main conclusions are unified in showing that *Sox9* ex-

pression in the developing pancreas defines a multipotent progenitor population which gives rise to ductal, endocrine, and acinar cells throughout the duration of pancreogenesis (**Figure 2A-D**). The multipotency of *Sox9*<sup>+</sup> progenitors declines progressively however: while at the population level early MPCs are “tripotent”, capable of giving rise to endocrine, acinar and ductal cells (**Figure 2A-C**), perinatal *Sox9*<sup>+</sup> progenitors are bipotent, giving rise to mostly ductal, and a few endocrine, cells (**Figure 2D**). By adulthood, *Sox9*<sup>+</sup> cells become “locked” into a ductal identity (**Figure 2F**). It should be noted that, while such “tripotency” of secondary transition trunk *Sox9*<sup>+</sup> cells holds true at the population level, it most likely does not apply to individual cells. Despite its caveats, short-term *Sox9-eGFP* tracing yielded insight at single-cell resolution into lineage events immediately preceding examination. Such analysis revealed that *Sox9-eGFP*-labeled acinar cells arise from only the (relatively sparse) cells at the extreme distal tips of the *Sox9*<sup>+</sup> trunk, while the remaining majority of *Sox9*<sup>+</sup> trunk cells give rise to cells of the ductal tree or endocrine cells (*via* an *Ngn3*<sup>+</sup> intermediate) [21]. It remains unknown whether this acinar-endocrine differentiation bias is governed by a proximal-distal distribution of fate determinants within the trunk or instead is dictated by relative proximity to the surrounding stroma and associated signaling cues. Comparison of the transcriptional signatures of proximal *versus* distal *Sox9*<sup>+</sup> trunk cells will help resolve this question. Since the majority of cells marked by *Sox9* in the secondary transition pancreas are capable of giving rise either to duct or endocrine cells, at this stage *Sox9*<sup>+</sup> cells are commonly referred to as “bipotent progenitors”. Several groups in the field are currently working on deciphering the molecular mechanisms underlying the endocrine-*versus*-duct fate decision at the level of a single bipotent progenitor cell.

Strikingly, parallel lineage tracing studies using *Hnf1β-CreERT<sup>2</sup>* mice have revealed that mirroring the *Sox9*<sup>+</sup> domain, *Hnf1β*<sup>+</sup> cells exhibit a remarkably similar pattern of diminishing potency during pancreogenesis: *Hnf1β* marks MPCs during early organogenesis (E11.5-13.5), duct-endocrine bipotent progenitors during the secondary transition (E13.5-16.5), and then duct (including terminal duct or centroacinar) cells from late gestation (E16.5) onwards [31]. This is not unexpected given that *Sox9* and *Hnf1β* expression is almost perfectly colocalized throughout pancreas development and beyond, in the adult organ [19], the possible functional significance of which is worthy of specula-

tion. Intriguingly however, the *Sox9*<sup>+</sup> and *Hnf1β*<sup>+</sup> domains diverge during the secondary transition: while *Sox9* expression extends the entire length of the *Nkx6.1*<sup>+</sup> *Pdx1*<sup>+</sup> epithelial trunk to the most distal trunk cells at the tip-trunk interface which are, in addition, marked by *Ptf1a*, *Hnf1β* expression is extinguished in these distal-most trunk cells [19] (**Figure 1C**). Thus, since exclusion of *Ptf1a* from bipotent progenitors is associated with their loss of acinar competence, and MPCs are marked by expression of *Sox9*, *Nkx6.1*, and *Ptf1a* [55], the retention of *Ptf1a* in *Nkx6.1*<sup>+</sup> *Sox9*<sup>+</sup> distal trunk cells suggests that they retain multipotency and the ability to give rise to acinar as well as duct and endocrine cells. This notion has been borne out by a recent *Ptf1a*<sup>CreERTM</sup>-mediated lineage tracing study, showing that rare multipotent progenitors persist late into the secondary transition [89]. Expression of *Sox9* but not *Hnf1β* in rare distal trunk *Ptf1a*<sup>+</sup> *Nkx6.1*<sup>+</sup> “late MPCs” is consistent with the observation of *Sox9-eGFP*-labeled acinar cells at the very distal tips of the secondary transition trunk [21]. This finding also explains why lineage tracing reveals *Sox9*<sup>+</sup> cells to give rise to all three pancreatic cell types, including acinar cells, throughout pancreogenesis [19], while *Hnf1β*<sup>+</sup> cells are unable to yield acinar cells beyond E13.5 [31]: *Hnf1β*<sup>+</sup> cells lose their multipotency prior to *Sox9*<sup>+</sup> cells during pancreogenesis. On account of their highly defined tip-trunk junction localization and unique transcriptional signature, it has been proposed [90] that these rare *Ptf1a*<sup>+</sup> *Sox9*<sup>+</sup> *Hnf1β*<sup>+</sup> distal tip cells represent the earliest precursors of terminal duct/centroacinar cells which reside at the duct-acinus interface (**Figure 1C, D**). However, for lack of centroacinar cell-specific markers, this remains to be formally tested through genetic lineage tracing. Partly on account of their retention of a multipotent transcriptional signature (*Sox9*<sup>+</sup> *Hnf1β*<sup>+</sup> *Ptf1a*<sup>+</sup> *Hes1*<sup>+</sup>), such centroacinar cells have been proposed to serve as multipotent progenitors in the adult pancreas (reviewed in [90]). Concordantly, isolated centroacinar cells are able to function as progenitors *ex vivo*, giving rise to self-renewing “pancreatospheres” capable of endocrine and exocrine differentiation [91]. It remains to be seen whether centroacinar cells can similarly function as progenitors *in vivo* in the adult pancreas.

## 6. Sox9 expression in adult pancreas

Following the secondary transition, *Sox9* expression in embryonic mouse pancreas is progressively down-regulated, becoming restricted to a

subpopulation of ductal cells and centroacinar cells in the adult [16] (**Figure 1D**). Mirroring this, SOX9 is restricted to duct-like cells by 14 wpc in the developing human pancreas and by birth is similarly restricted to only ductal and centroacinar cells [15, 16, 80]. This is particularly poignant since, as mentioned above, it has been widely posited that a subset of pancreatic ductal cells, including centroacinar cells, might function as facultative progenitor cells in adulthood [90, 92]. It is equally intriguing since it shows Sox9, like the other early MPC markers Pdx1 and Ptf1a, to become confined in adulthood to a single compartment. While Sox9 becomes postnatally restricted to ducts, Pdx1 becomes confined to endocrine ( $\beta$ - and  $\delta$ -) cells [93] and Ptf1a becomes restricted to the acini [94]. It is quite worthy of speculation why these three MPC markers become exclusively confined to distinct pancreatic compartments.

Notably, in contrast to previous reports of *Sox9* expression in mouse islets (as detected by ISH or RT-PCR), it was subsequently reported that Sox9, as in embryonic endocrine cells, was not immunodetectable in the adult islet [16] (**Figure 1D**). In conjunction with the failure to amplify *Sox9* mRNA from isolated islets [16], this constitutes clear evidence that neither Sox9 RNA nor protein is expressed in endocrine cells. Leaching of BCIP/NBT used to visualize *Sox9* ISH probe from ducts to adjacent islets is likely to have caused an apparent *Sox9* mRNA signal in E18.5 islets [66]. Similarly, detection of *Sox9* transcripts in adult islets in the same study can be attributed to residual genomic DNA or ductal/centroacinar cell contamination of isolated islet preparations. A factor that further confounds IHC detection of Sox9 in endocrine cells and one that we have experienced first-hand is the variable pancreatic Sox9 staining patterns resulting from IHC using a range of Sox9-specific antisera (personal communication). In addition to the *bona fide* ductal and centroacinar cell Sox9-specific staining typically observed in adult mouse pancreas, several commonly-used anti-Sox9 antisera yield either  $\alpha$ -cell-like cytoplasmic staining or pan-endocrine perinuclear staining patterns. Such non-specific signal is not to be confused with that which might result from sera cross-reactivity with Sox8 and/or Sox10 in the peripheral islet glial sheath outside the islet cells proper. However, both staining patterns are consistent with the notoriously immunogenic nature of pancreatic islets,  $\alpha$ -cells in particular [95]. Accordingly, preadsorption of such Sox9 antisera with the immunizing peptide typically abolishes

ductal and centroacinar IHC signal while the non-specific islet staining persists (personal communication). Thus, while it is now clearly apparent that Sox9 is not expressed in endocrine cells in the developing or adult pancreas, this notion continues to be perpetuated in the literature.

## 7. Functional roles of Sox9 in the developing pancreas

### 7.1 *Sox9* maintains pancreatic progenitors through Notch-dependent and -independent mechanisms

While its expression defines pancreatic progenitors, it does not necessarily follow that Sox9 serves a functional role in this population. However, amassing evidence prior to 2007 increasingly hinted at this being likely given the requirement for *Sox9* in maintaining progenitors in the neural crest [13], intestinal epithelium [96] and hair bulge [97] during embryogenesis. Since neonatal lethality of *Sox9*<sup>-/-</sup> mice [98] precludes breeding *Sox9*-null embryos, *Pdx1-Cre*-mediated conditional *Sox9* ablation in the pancreas was used to test for a role of this factor in pancreas development. Deletion of Sox9 in *Sox9*<sup>fl/fl</sup>; *Pdx1-Cre* pancreata, while rapid and efficient, only occurred once specification and outgrowth of the pancreatic buds was complete, precluding testing a requirement for *Sox9* in these processes [16]. Dramatic hypoplasia of both pancreatic buds became apparent almost immediately after *Sox9* deletion due to the loss of Pdx1<sup>+</sup> MPCs, revealing a requirement for Sox9 in early pancreatic growth [16]. This strong phenotype indicates an apparent lack of functional redundancy in pancreogenesis between *Sox9* and the additional SoxE subgroup members *Sox8* and *Sox10*. This is consistent with the fact that, while members of the same Sox subgroup often play parallel functional roles [10], the pancreatic expression domains of *Sox8* and *Sox10* do not overlap with that of *Sox9* [66]. Moreover, given such a crucial role for Sox9 in maintaining the early pancreatic endoderm, it is tempting to speculate whether Sox9 is required for its initial specification and, if so, how this is achieved. This will require the generation and analysis of mice in which *Sox9* is ablated in the gut endoderm preceding the initiation of pancreogenesis.

Consistent with final pancreas size being constrained by the initial number of MPCs specified between ~E8.5-12.5 [44], the pancreas remained hypoplastic at birth in *Sox9* mutants, causing

perinatally-lethal hyperglycemia due to a near-absence of all endocrine cells. Lineage tracing at birth revealed an over-representation of the progeny of unrecombined (*Sox9*<sup>+</sup>) progenitors, suggesting that the mutant pancreas becomes repopulated by unrecombined MPCs. Thus, *Sox9*-deleted progenitors bear a reduced capacity to contribute to the growing organ. A mechanistic dissection attributed this to a 50% reduction in proliferation, increased apoptosis, and precocious differentiation of *Sox9*-deleted progenitors. Notably, subsequent examination of *Sox9*<sup>fl/+</sup>; *Pdx1-Cre* embryos revealed a 25% reduction in progenitor proliferation in the *Sox9*-haploinsufficient pancreas [21]. Thus, MPC proliferation is exquisitely *Sox9* dosage-sensitive with loss of each functional *Sox9* allele conferring a 25% decrease in mitotic index. Previous reports of similar pancreas hypoplasia in mice lacking the Notch effector *Hes1* due to both precocious progenitor differentiation [48] and reduced MPC proliferation [49, 99] prompted an analysis of *Hes1* expression in *Sox9*-deleted pancreata. This revealed an almost two-fold reduction in *Hes1*<sup>+</sup> progenitors at E10.5. *Sox9* thus plays a crucial role in driving early pancreatic growth by maintaining MPC pool size through promoting the survival, proliferation, and undifferentiated status of progenitors (**Figure 3A**). Given that *Hes1*-deficient pancreatic progenitors exhibit reduced proliferation and precocious differentiation but not decreased survival, it is highly plausible that *Sox9* maintains the MPC pool through both Notch-dependent and -independent mechanisms (**Figure 3A**).

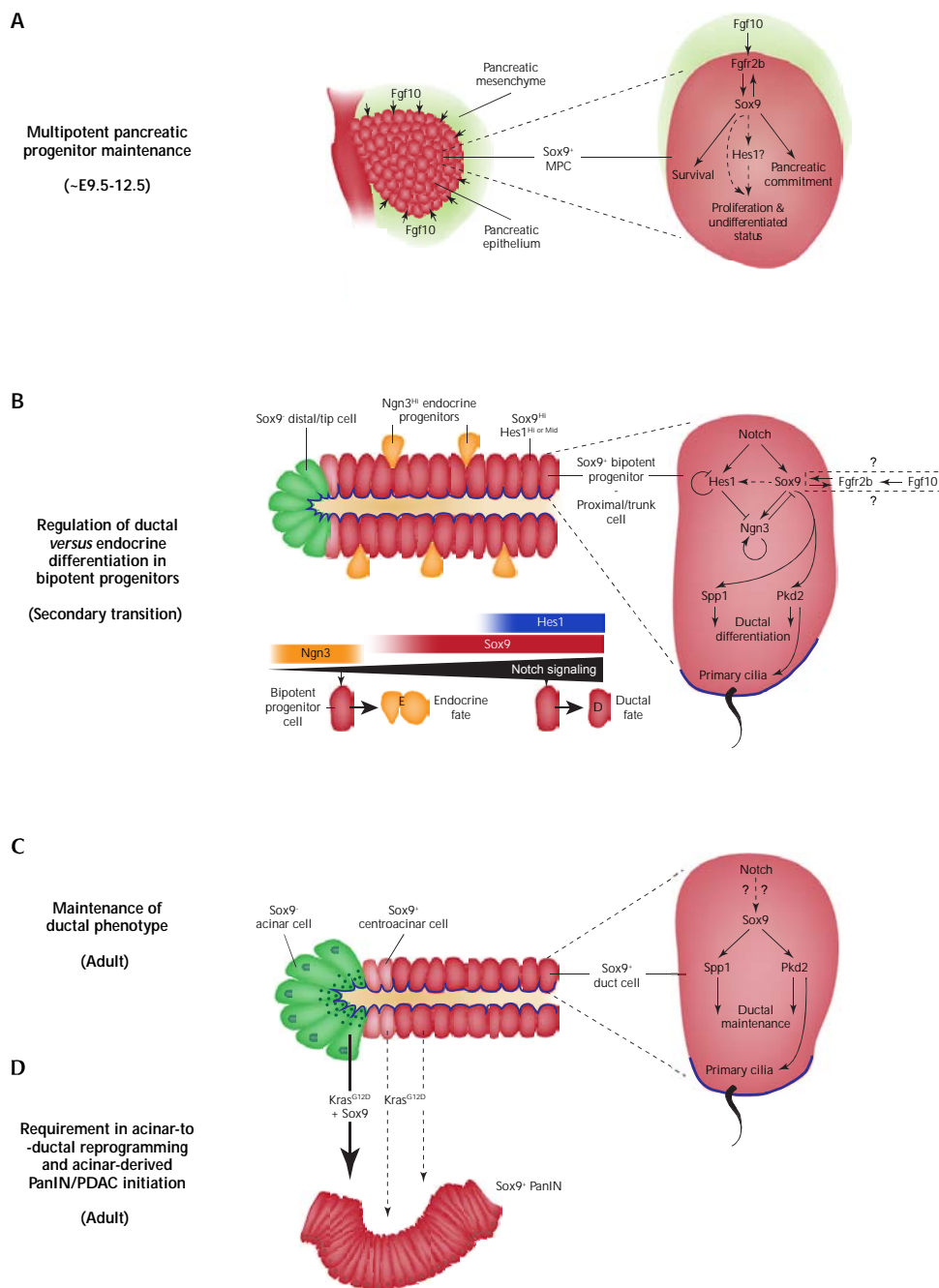
### 7.2 *Sox9* interacts with the *Fgf* pathway to reinforce pancreatic fate commitment

Recently, mouse genetic studies provided deeper mechanistic insight into how *Sox9* governs MPC proliferation, and in so doing, revealed that in addition to Notch, *Sox9* interacts with the *Fgf* signaling pathway to maintain both expansion and organ identity of early MPCs [100]. Transcriptional profiling of early *Sox9*<sup>fl/+</sup>; *Pdx1-Cre* pancreata uncovered upregulation of liver markers such as AFP and albumin, borne out by IF studies confirming their widespread expression in *Pdx1*<sup>+</sup> *Sox9*-deleted MPCs by E11.5 [100]. When deprived of *Sox9* up until ~E12.5, pancreas-fated progenitors adopt an alternative hepatic program. MPCs are labile in their organ commitment through this window, requiring *Sox9* for reinforcing their pancreatic commitment [100]. Such aberrant hepatic differentiation in the early pancreas mirrors that

in *fgf10* mutant zebrafish [101]. In such mutants, the hepatopancreatic ductal system (HPD), which connects the ducts of the proximal pancreas and liver to the intestine, is dysmorphic, with some HPD cells aberrantly adopting pancreatic and hepatic fates; similarly, some cells in the proximal pancreas and liver misdifferentiate into hepatic and pancreatic cells, respectively [101].

As mentioned, in mice, *Fgf10* from the pancreatic mesenchyme serves a crucial mitogenic role in driving pancreatic progenitor proliferation [53] (**Figure 3A**). Growth arrest of MPCs therefore results in mice lacking either *Fgf10* [53] or its receptor *Fgfr2b* [102-104] expressed in early pancreatic epithelium [53, 105, 106], resulting in a mirroring of the pancreatic hypoplasia seen in *Sox9*-deficient mice. *Fgfr2b* and *Sox9* almost perfectly colocalize within MPCs between E9.0-12.5, concurrently with mesenchymal *Fgf10* expression and ablation of *Sox9* results in an almost immediate extinguishing of *Fgfr2b* with only unrecombined *Sox9*<sup>+</sup> progenitors retaining its expression [100]. *Sox9* therefore cell-autonomously maintains *Fgfr2b* expression in MPCs and thus their *Fgf10* receptivity (**Figure 3A**). While *Pdx1* also regulates *Fgfr2b* expression in MPCs [107], the synchronous downregulation of *Sox9* and *Fgfr2b* in *Pdx1*-deficient pancreata suggests that loss of *Fgfr2b* in *Pdx1*<sup>-/-</sup> mice is *Sox9*-dependent [100]. As predicted by their downregulation of *Sox9*, MPCs in *Pdx1*-null embryos also undergo hepatic fate conversion [100], inferring *Sox9* to be dominant over *Pdx1* in maintaining MPC pancreatic commitment. Together with the finding that SU5402-mediated *Fgfr* inhibition in E9.5 foregut explants downregulates *Sox9* and induces AFP in the pancreas, these data support a model in which *Fgfr*-transduced *Fgf10* signaling maintains *Sox9* and so *Fgfr2b* expression in MPCs [100]. Completing this model, *Sox9* and *Fgfr2b* are synchronously downregulated in MPCs of E9.5 *Fgf10*<sup>-/-</sup> embryos [100], which is consistent with *Fgf10* maintaining *Sox9* expression in progenitors, as seen in *Pdx1-Fgf10* pancreata [16, 82]. As predicted by their losing *Sox9* and *Fgfr2b* expression, *Fgf10*-deficient MPCs also undergo hepatic reprogramming [100]. This study therefore supports the operation of an *Fgf10/Fgfr2b/Sox9* feed-forward loop in early pancreatic progenitors that maintains both their proliferation and pancreatic commitment (**Figure 3A**).

Mesenchymal *Fgf10* maintains *Sox9* expression in epithelial progenitors, which in turn, maintains their expression of *Fgfr2b*, and thus, their receptivity to mitogenic, pro-pancreatic *Fgf10* signaling. Abrogation of any component of this loop manifests



**Figure 3. Multiple crucial roles of Sox9 in the developing and adult pancreas. A:** Fgf10 from the pancreatic mesenchyme is transduced by Fgfr2b to maintain Sox9 expression in early (E9.5-12.5) multipotent pancreatic progenitor cells (MPCs), comprising the pancreatic epithelium. Sox9 in turn maintains Fgfr2b expression. This Sox9/Fgf10/Fgfr2b feed-forward loop maintains both proliferation and pancreatic fate commitment of MPCs. Also, Sox9 is required for both MPC survival and maintenance of their undifferentiated state. Sox9 acts as a modulator of Hes1 expression in MPCs and possibly regulates MPC proliferation and differentiation status in a Hes1-dependent manner. **B:** In ductal-/endocrine-fated bipotent progenitors comprising the majority of the proximal trunk in the secondary transition pancreas, graded Notch signaling regulates ductal versus endocrine fate commitment by controlling the cellular equilibrium between Sox9 and Hes1. Notch signaling promotes the expression of Sox9 which cell-autonomously activates Ngn3 which in turn positively auto-regulates itself. This cascade enables an Ngn3<sup>Hi</sup> state to be attained. However, high-level Notch activity induces the expression of both Sox9 and the Ngn3 repressor Hes1, preventing Ngn3 induction and endocrine differentiation. At intermediate levels of Notch signaling, only Sox9 is induced,

initiating Ngn3 expression. Endocrine differentiation then requires cell-autonomous repression of Sox9 by Ngn3 to repress the ductal program. Bipotent progenitors remaining Sox9<sup>+</sup> and Hes1<sup>+</sup> adopt a ductal fate by default as Sox9 positively regulates expression of ductal genes including *Spp1* and *Pkd2*. Therefore, *Pkd2* and *Sox9* are required for maintaining primary cilia. Continued Fgfr2b expression in secondary transition Sox9<sup>+</sup> cells raises the possibility of a Sox9/Fgf feed-forward loop functioning in bipotent progenitors as in MPCs. **C:** Analogously to its role in embryonic bipotent progenitors, Sox9 maintains ductal identity in the adult by maintaining expression of both *Spp1* and *Pkd2* in mature ductal cells; it is therefore required for maintaining primary cilia on ductal cells. **D:** Genetic lineage tracing following induction of the oncogenic constitutively active *Kras*<sup>G12D</sup> allele reveals ductal and centroacinar cells to be relatively refractory to oncogenic transformation into PDAC precursor lesions called PanINs (dashed arrows). In contrast, acinar cells readily form duct-like premalignant lesions following *Kras*<sup>G12D</sup> expression; while ectopic induction of *Sox9* is required for this reprogramming, coexpression of *Sox9* with *Kras*<sup>G12D</sup> accelerates PanIN formation (broad, solid arrow).

in MPC growth-arrest and pancreatic hypoplasia, as well as loss of pancreatic commitment and the adoption of an alternative hepatic program. This mechanism unifies the pancreatic hypoplasia observed in mice lacking *Sox9*, *Pdx1*, *Fgf10* or *Fgfr2b* [16, 45, 46, 53, 103, 104, 108]. Significantly, Sox9 positively regulates *Fgfr2* expression in other developmental contexts such as testes [109] and ventral prostate [110]. Whether this regulation is direct remains unknown and will require chromatin immunoprecipitation (ChIP) studies to elucidate. Similarly, while Fgf10-Fgfr2 signaling promotes Sox9 expression in developing lung [111], this study is the first demonstration of the complete Fgf10/Fgfr2b/Sox9 loop in the same context. It is highly likely that such a loop maintains the expansion and/or fate commitment of progenitors in other developmental and regenerative contexts. In the case of early pancreogenesis, further work will be required to mechanistically dissect how the activity of this loop maintains MPC proliferation while repressing the hepatic differentiation program. Such studies will undoubtedly provide insight into downstream effects of Fgf-Sox9 signaling in other tissue progenitors.

Strikingly, recent genetic studies indicate the Sox9-Fgf relationship to be evolutionarily conserved in zebrafish hepatopancreatic patterning, although its pro-pancreatic influence applies only to the pancreas. During zebrafish development, the paralog *sox9b* is expressed in the hepatopancreatic endoderm and subsequently in developing intrapancreatic and intrahepatic ducts as well as in the HPD connecting them to the intestine [79, 112]. In *sox9b* mutants, these structures are dysmorphic with ectopic hepatic and pancreatic cells appearing within the HPD, proximal pancreas (hepatic), and liver (pancreatic) [79, 112], phenocopying zebrafish *fgf10* mutants [101]. Consistent with this, early expression of *sox9b* in the hepatopancreatic endoderm is lost in compound mutants lacking both *fgf10* and *fgf24*, although not in single *fgf10* mutants [79], which is consistent with *fgf10* acting redundantly with *fgf24* in specification of the zebrafish pancreas [113]. These findings imply that *sox9b* functions downstream of Fgf signaling to pattern the zebrafish gut. Intriguingly, in the same study [79], loss of *sox9b* expression was observed in hepatopancreatic endoderm following morpholino-mediated *bmp2a* knockdown. Thus, multiple cues undoubtedly govern the induction and/or maintenance of *sox9b/Sox9* expression in the gut endoderm.

While there are parallels in Sox9/Fgf-governance of gut endoderm patterning between

mouse and fish, it is unclear whether a similar feed-forward loop functions in the latter. Although *fgfr2* is expressed in zebrafish anterior embryonic endoderm concurrently with hepatopancreatic patterning [101], its expression has not been examined at single organ resolution. Furthermore, *fgfr2* expression has yet to be assayed in *sox9b* mutants. It thus remains to be seen whether *sox9b* likewise maintains expression of *fgfr2* in zebrafish gut endoderm cells, completing such a regulatory loop.

Finally, given the central role of Sox9 in early MPCs in modulating the Notch effector Hes1 and maintaining receptivity to Fgf10, Sox9 appears to provide a nexus between the Fgf and Notch pathways during early pancreas development (**Figure 3A**). Several studies have unambiguously shown that persistent Fgf10 expression during pancreogenesis increases proliferation and arrests pancreatic cells in a progenitor state and is associated with their maintained Notch activation, as indicated by maintained *Notch1*, *Notch2* and *Hes1* expression [82, 114]. While not demonstrated incontrovertibly, two facts strongly implicate this progenitor arrest occurring within MPC-stage progenitors: *Fgf10* expression being *Pdx1* promoter-driven (so MPC-expressed) and maintained *Notch1/Notch2* co-expression with the Notch ligands *Jagged1* and *Jagged2* in later ectopic *Fgf10*-expressing cells as in normal (E12.5) MPCs [82]. In agreement, abrogation of Notch signaling in explanted E10.5 pancreatic epithelium is able to suppress the proliferative and progenitor-maintaining effects of Fgf10 treatment [115]. Together, these studies imply that Notch signaling is an obligate downstream effector of Fgf10 signaling in MPCs. However, exactly how Fgf10 modulates Notch activity in progenitors remains obscure. As Sox9 serves both as a downstream effector of Fgf10 signaling and as an upstream modulator of Hes1 in MPCs, it is conceivable that Sox9 acts as the Fgf10-Hes1/Notch conduit (**Figure 3A**). This notion is made even more plausible by the recent finding that another pancreatic progenitor maintenance factor, Ptf1a, maintains Hes1 expression in MPCs *via* the Notch ligand Dll1 [49]. Given the central role that modulation of Fgf and Notch signaling pathways plays in protocols for differentiating hESC/iPS cells into insulin<sup>+</sup> cells *in vitro* [116], establishing whether Sox9 is the point of Fgf10-Hes1/Notch cross-talk is likely to provide insights to aid their optimization. Dissecting this regulatory network should drive the development of protocols aimed at specifically expanding hESC/iPS-derived cells at the pancreatic progenitor stage.

### 7.3 *Sox9* is required in a dosage-dependent manner for endocrine differentiation

At birth, the hypoplastic pancreas in *Sox9*<sup>fl/fl</sup>; *Pdx1-Cre* mice is cystic, comprising dispersed acini, a poorly-branched ductal tree, and almost no endocrine cells [16]. Thus, while *Sox9* is required for pancreatic growth, the greater perturbation of endocrine than exocrine morphogenesis reveals that differentiation of endocrine cells is more *Sox9* dosage-sensitive than that of exocrine cells. Accordingly, islet-specific defects occur in *Sox9*<sup>fl/+</sup>; *Pdx1-Cre* mice deficient for one functional copy of *Sox9* in the pancreas [21]. Size and gross morphology of the pancreas are unperturbed by such pancreatic *Sox9* haploinsufficiency [21], in a way consistent with no detectable impact on the morphogenesis of exocrine tissue which comprises 98-99% of the adult mouse pancreas [26]. However, while exocrine morphogenesis is insensitive to a halving of *Sox9* gene dosage, the number of endocrine cells (and both  $\alpha$ - and  $\beta$ -cell mass) is halved at birth, with all islet cell types being equally affected [21]. As both *pro rata*  $\beta$ -cell insulin content and expression of mature  $\beta$ -cell markers (such as *Glut2*, *PC1/3* and islet amyloid polypeptide - IAPP) are unperturbed in *Sox9*<sup>fl/+</sup>; *Pdx1-Cre* mice [21], this suggests that terminal differentiation of  $\beta$ -cells is unhampered by *Sox9* haploinsufficiency. This endocrine hypoplasia phenocopies that reported by the Hanley group [15] in human patients affected by the lethal *SOX9* haploinsufficiency syndrome campomelic dysplasia (CD; OMIM 114290) characterized by severe skeletal dysplasia and variable autosomal XY sex reversal [117, 118]. Notably, this study was the first to hint at a potential functional role for *Sox9/SOX9* in pancreatic morphogenesis. Histological examination revealed two of three perinatal pancreata from severe, perinatally-lethal CD cases to display hypoplastic islets with variable hormone expression as well as reduced expression of *PC1/3* and IAPP [15]. While this discrepancy in marker expression between  $\beta$ -cells of *Sox9*-haploinsufficient mouse and human pancreata might reflect minor deviations in their pancreatic programs, it is more likely to be attributable to divergent tissue fixation given the more challenging nature of preserving human autopsy-derived tissues.

Given that in *Sox9*-haploinsufficient mice endocrine differentiation is unperturbed and islet hypoplasia affects all cell types equally, this suggests that such hypoplasia results from an upstream defect impacting endocrine progenitor specification.

Confirming this notion, *Ngn3*<sup>+</sup> endocrine progenitor cell numbers are halved in *Sox9*<sup>fl/+</sup>; *Pdx1-Cre* embryos [21]. In the light of lineage tracing data that support one *Ngn3*<sup>+</sup> progenitor giving rise to one endocrine cell [119], this halving of *Ngn3*<sup>+</sup> cells is entirely consistent with the halving of islet cell numbers. Therefore, islet hypoplasia in *Sox9*-haploinsufficient mice and, presumably, humans, occurs through impaired endocrine neogenesis, revealing *Sox9* to directly govern the initiation of endocrine differentiation. Further supporting this role, while inducible deletion of *Sox9* immediately prior to the secondary transition only negligibly impacts pancreatic growth and exocrine morphogenesis, *Ngn3*<sup>+</sup> endocrine progenitors and, therefore, endocrine cells are almost completely ablated [20]. Lineage tracing reveals that *Sox9*-deleted progenitors exhibit a reduced capacity to give rise to *Ngn3*<sup>+</sup> progenitors and endocrine cells but their probability of yielding acinar or ductal cells is unaffected [20]. Thus, *Sox9* is required cell-autonomously for initiating the endocrine program. ChIP studies have provided mechanistic insight into the molecular basis underlying this cell-autonomous role. ChIP performed both in primary embryonic (E15.5) mouse pancreata [21] and the mouse pancreatic duct cell line mPAC L20 [77, 120] has revealed *Sox9* occupancy on the *Ngn3* promoter. Furthermore, *Sox9* has been demonstrated to directly stimulate *Ngn3* promoter activity by the finding that it can bind to the promoter of human *NEUROG3* and increase its expression [77]. Knockdown of *Sox9* expression in mPAC L20 cells in the same study was demonstrated to abrogate expression of *Ngn3* induced by adenoviral expression of the bHLH protein *Mash1* [77]. Together, these studies show that, while exocrine differentiation is *Sox9*-independent, *Sox9* is absolutely required for initiating endocrinogenesis by inducing *Ngn3* expression (**Figure 3B**) and furthermore, *Sox9* regulates this process in a dosage-sensitive, cell-autonomous manner.

### 7.4 Discrepancies between *in vitro*- and *in vivo*-identified progenitor transcriptional networks

Having shown that *Sox9* regulates *Ngn3* expression *in vitro*, Lynn *et al.* [77] sought to understand how *Sox9* induces *Ngn3* expression in a portion of progenitor cells only. Using mPac L20 duct cells, they attempted to characterize the position of *Sox9* in the transcriptional network governing progenitor maintenance *versus* differentiation.



Sox9 was found to bind to the promoters of the pancreatic transcription factors *Hnf1 $\beta$* , *Onecut1*, and *Foxa2* [77], all previously implicated in governing *Ngn3* expression and coexpressed with Sox9 in MPCs and bipotent progenitors [121]. Furthermore, partial (~50%) knockdown of Sox9 expression was demonstrated to result in a 20-30% decrease in expression of *Onecut1* and *Foxa2* and a 50% increase in *Hnf1 $\beta$*  expression [77].

A parallel approach saw *Hnf1 $\beta$*  knockdown modestly reduce expression of *Foxa2* without affecting that of *Onecut1* while knockdown of *Foxa2* diminished expression of Sox9 without affecting that of either *Hnf1 $\beta$*  or *Onecut1* [77]. It was concluded from these results that *Foxa2* is required for maintaining the expression of Sox9 in a transcriptional network of endocrine differentiation genes including *Hnf1 $\beta$* , *Onecut1*, and *Foxa2*, which is required for proper pancreatic morphogenesis. However, as this *in vitro* study is based upon the mPAC L20 duct cell line, it is unclear whether the proposed transcriptional network is conserved in progenitors of the developing pancreas *in vivo* and, if so, given the coexpression of these factors in MPCs and later bipotent progenitors, to which progenitor population(s) it is applicable. To resolve this issue, Dubois and colleagues [121] assessed the expression of the same endocrine differentiation genes in *Sox9*-deficient pancreata. *Sox9* ablation by E10.5 in *Sox9<sup>fl/fl</sup>*; *Pdx1-Cre* embryos, or at E13 or E15 in *Sox9<sup>fl/fl</sup>*; *Rosa26-CreER* embryos exposed to tamoxifen at E12.5 or E14.5 respectively failed to perturb pancreatic expression of *Hnf1 $\beta$* , *Onecut1* or *Foxa2* [121].

Thus, contrary to *in vitro* studies, Sox9 is dispensable for the expression of all three factors during pancreatic organogenesis. In contrast however, *Pdx1* expression was strongly downregulated following *Sox9* ablation [121], uncovering a requirement for Sox9 in maintenance of *Pdx1* expression in pancreatic progenitors. As *Pdx1* is crucially required for endocrinogenesis and regulates expression of *Ngn3* [122], this would argue that Sox9-dependent regulation of *Pdx1* expression in secondary transition bipotent progenitors is required for the major wave of  $\beta$ -cell neogenesis from the secondary transition until birth. This is consistent with the loss of  $\beta$ -cells seen following *Sox9* deletion at E13 or E15 [121]. While Sox9 therefore induces expression of *Ngn3* both directly as well as indirectly through *Pdx1*, it is arguably loss of the former which predominantly accounts for endocrine hypoplasia in *Sox9*-haploinsufficient mice as their levels of pancreatic *Pdx1* expression remain unperturbed. Further work will be required to resolve

whether or not Sox9 regulates *Pdx1* expression directly in bipotent progenitors. The fact that *Pdx1* expression is maintained in MPCs immediately following *Pdx1-Cre*-mediated *Sox9* ablation [16] and is only downregulated at E12.5 [121] suggests that Sox9 does not directly regulate *Pdx1* during early pancreogenesis and/or that Sox9 regulates *Pdx1* via distinct mechanisms in MPCs versus bipotent progenitors. Given that in humans mutations in *Pdx1* manifest in MODY (maturity-onset diabetes of the young) 4 [123], the maintenance of *Pdx1* by Sox9 during the major wave of  $\beta$ -cell neogenesis implicates *SOX9* loss-of-function mutations being a putative causative factor of diabetes in humans. While *SOX9*-haploinsufficiency is associated with lethal CD as mentioned above, mutations affecting the long-range E1 enhancer driving pancreas-specific *SOX9* expression [83] might conceivably manifest in human diabetes. Although the majority of MODY gene products function in  $\beta$ -cells, *Hnf1 $\beta$ /Tcf2*-mutations of which are associated with MODY5 [124]—is excluded from such cells, only being expressed like Sox9 in the MPCs and bipotent progenitors from which they arise [31, 54]. This sets a precedent for dysfunction of pancreatic progenitor genes to manifest in diabetes independently of  $\beta$ -cell dysfunction, presumably as a consequence of endocrine dysgenesis instead. Thus, mutations reducing *Sox9/SOX9* dosage might conceivably manifest in MODY, consistent with glucose intolerance in *Sox9*-haploinsufficient adult mice [121] as mentioned below. Whether *SOX9* mutations are associated with human diabetes remains to be seen.

### 7.5 Notch activity governs ductal-versus-endocrine fate choice through *Sox9* and *Hes1*

While Sox9 is required to induce pro-endocrine *Ngn3* expression in secondary transition bipotent progenitors, this contradicts with the Sox9<sup>+</sup> population being Notch-active at this time, as evidenced by widespread Sox9/*Hes1* colocalization at E15.5 [16] (Figure 3B). Sox9 inducing *Ngn3* is discordant with it also positively regulating *Hes1* expression [16] since to activate *Ngn3*, Sox9<sup>+</sup> progenitors must escape from the *Ngn3*-repressing influence of *Hes1* [125]. Recently, an attractive model was posited to account for how such “escape” might occur, reconciling these apparently contradictory Sox9 functions [20]. Confirming secondary transition Sox9<sup>+</sup> bipotent progenitors to be Notch-active [16], Shih and colleagues [20] showed a subpopulation of E15.5 Sox9<sup>+</sup> progenitors to express high levels of the Notch target *Hes1* and

Notch receptor Notch2 as well as lower levels of Notch1. Concurrently, these cells also expressed NICD1 and NICD2. This was further supported by expression of the Notch ligands Dll1 and Jag1 and the Notch mediator Rbpj in the Sox9<sup>+</sup> trunk. Thus, Sox9<sup>+</sup> progenitors appear to be Notch-regulated during pancreogenesis as they are in the embryonic liver [112, 126].

In confirmation of previous findings [16, 21, 127], while elevated Ngn3 expression was seen in a small proportion of Sox9<sup>+</sup> cells, it was never detected in Hes1<sup>+</sup> cells (**Figure 3B**). Combined with previous reports of NICD1 and Sox9 downregulation in Ngn3<sup>+</sup> cells [128], this indicates that Ngn3 induction and endocrine progenitor delamination is associated with diminished Notch signaling [20]. Apparent Notch governance of Sox9<sup>+</sup> bipotent progenitors coupled with Hes1 being expressed in only a proportion of them led to the hypothesis that Sox9 and Hes1 are Notch-responsive at distinct thresholds. By culturing pancreatic explants in graded concentrations of the Notch inhibitor  $\gamma$ -secretase inhibitor-IX (GSI-IX), Shih *et al.* [20] found that at low (1  $\mu$ M) inhibitor concentrations, expression of Hes1 is reduced while that of Sox9 is unaffected while at higher (5 or 10  $\mu$ M) concentrations, expression of both factors is abrogated. Thus, Sox9, like Hes1, is a Notch target: it remains to be resolved however whether this regulation is direct as during liver development when NICD1 directly binds the *Sox9* promoter [126]. While both are Notch targets, Hes1 and Sox9 respond to Notch at distinct thresholds: both are expressed in Notch<sup>hi</sup> progenitors, but “intermediate” Notch activity results in Sox9 expression only, accounting for the fact that only a subset of Sox9<sup>+</sup> progenitors is Hes1<sup>+</sup> (**Figure 3B**). The resulting prediction that Sox9 should be more sensitive to increased Notch activity was proved by showing that *Pdx1-Cre*-driven NICD misexpression induced a far greater upregulation of Sox9 than Hes1 in bipotent progenitors [20]. As Notch inhibition induces both Ngn3 expression and endocrine differentiation [128, 129], the authors tested whether this induction is Sox9-dependent. As GSI-IX fails to induce Ngn3 expression in *Sox9*-deleted pancreatic explants, this suggests that Sox9 is an obligatory inducer of *Ngn3* [20]. It would appear therefore that Notch signaling induces both activators and inhibitors of endocrine differentiation, and competition between the two maintains the undifferentiated *status quo* (**Figure 3B**).

To better investigate the interaction between Sox9 and Ngn3, Shih and colleagues [20] misex-

pressed *Ngn3* throughout MPCs. The resulting repression of Sox9 in progenitors shows Sox9 and Ngn3 to participate in a cell-autonomous negative-feedback loop (**Figure 3B**). As Sox9 loss in Notch-inhibited *Ngn3*-null pancreatic explants excludes Ngn3 repression of *Sox9* accounting for Sox9 loss following Notch inhibition, this shows that *via* Sox9, Notch signaling induces expression of Ngn3, which, at high levels, then represses Sox9. This finding is consistent with the earlier demonstration of persistent Sox9 mRNA [130] and protein [128] in Ngn3-deficient pancreatic cells activating the *Ngn3* promoter. Contradicting reports of Ngn3 repressing itself [131], Shih *et al.* [20] then went on to show that *Ngn3*<sup>+</sup> (eGFP<sup>+</sup>) cells in the pancreata of *Ngn3*<sup>eGFP/eGFP</sup> (functionally *Ngn3*-nullizygous) embryos display reduced eGFP intensity compared with those in *Ngn3*<sup>eGFP/+</sup> (*Ngn3*-haploinsufficient) siblings, a result which is conserved following Notch inhibition of explanted pancreata. Taken together, these findings suggest that during pancreogenesis, *Ngn3* is positively autoregulated in a Notch-independent fashion so that once Sox9-induced *Ngn3* expression is initiated, a positive feedback loop enables an Ngn3<sup>hi</sup> state to be achieved, ensuring endocrine commitment (**Figure 3B**). If this state is not attained, a bipotent progenitor adopts a ductal fate, as illustrated by the persistent expression of bipotent progenitor/ductal genes (such as Sox9, Hes1 and Hnf1 $\beta$ ) in eGFP<sup>+</sup> cells of *Ngn3*<sup>eGFP/eGFP</sup> embryos [20]. While this suggests that Ngn3 represses the ductal program, Sox9 in contradiction is directly required for differentiation and maintenance of pancreatic ducts. Inducible *Sox9* deletion immediately prior to the secondary transition manifests in dysgenic ducts with dilated lumina at E15.5 and a cystic phenotype after birth which is mirrored by conditional *Sox9* ablation in adulthood [20]. Expression profiling revealed ductal dysgenesis to be associated with downregulation of ductal genes including *Spp1* [132], a known Sox9 target [133] and *polycystin 2* (*Pkd2*), mutations of which are associated with autosomal dominant polycystic kidney disease (ADPKD), an inherited systemic disorder characterized by the development of renal, hepatic, and pancreatic cysts [134, 135]. Further supporting a requirement for Sox9 in maintaining ductal *Pkd2* expression, primary cilia, which *Pkd2* maintains [136], are lost from the apical membrane of duct cells in embryonic and adult pancreas following *Sox9* ablation, showing Sox9 to be required for primary cilia formation in the pancreas [20] (**Figure 3B**).

Finally, Shih *et al.* examined in more detail the relationship between Notch and Sox9 in regulating the ductal program [20]. Not normally expressed in endocrine progenitors, *Ngn3-Cre*-driven expression of NICD in such cells induced expression of Sox9 and Hes1 as well as ductal markers such as *Spp1* and *Pkd2* and, as shown [137, 138], perturbed endocrine differentiation [20]. On a *Sox9*-deficient background, Hes1 expression was maintained in NICD-expressing *Ngn3<sup>+</sup>* cells, confirming that Sox9 serves as a modulator of Hes1 expression [20]. Mirroring *Ngn3<sup>+</sup>* cells expressing both NICD and Sox9, endocrine differentiation was still abrogated in *Sox9*-deleted mice [20]. This failure of high Notch activity to rescue endocrinogenesis in the absence of *Sox9* is consistent with an absolute requirement for *Sox9* in this process. Likewise, in the *Sox9*-deficient state, NICD misexpression was insufficient to induce a ductal program in bipotent progenitors, showing Sox9 to be an obligatory effector of Notch-induced ductal differentiation [20].

Together, the findings of this intricate study by Shih and colleagues point to the fate of secondary transition bipotent duct/endocrine progenitors being governed by a Notch-dependent molecular network [20] (**Figure 3B**). According to their model, given that Hes1 expression in bipotent progenitors represses expression of *Ngn3* and Sox9 induces it, intermediate levels of Notch activity favor Sox9 over Hes1 expression, promoting *Ngn3* induction. Positive autoregulation of *Ngn3* ensures that once induced, progenitors are able to rapidly attain an *Ngn3<sup>hi</sup>* state, committing them to the endocrine program. *Ngn3* then cell-autonomously represses *Sox9*, and thus, ductal development, enabling endocrine differentiation to occur. Thus, different gradations of Notch activity are able to specify distinct endocrine or ductal fates within the same bipotent progenitor population.

Studies in zebrafish point towards this Notch-Sox9 interaction being conserved in mid-pancreogenesis. Treatment of late zebrafish embryos with the  $\gamma$ -secretase inhibitor DAPT between three and four days post-fertilization results in both loss of *sox9b* expression and excess endocrine differentiation in the intrapancreatic ducts [79], showing Notch activity to be required for maintaining ductal *sox9b* expression as it is in the mouse. Notably, this effect is recapitulated in the extrapancreatic duct by treatment with the Fgf receptor inhibitor SU5402 between two and three days post-fertilization [79]. Thus, Fgf, like Notch signaling, is required to maintain ductal *sox9b* expression in zebrafish. The conservation of relationships between Fgf, Notch and Sox9 during mouse

and zebrafish pancreogenesis invites speculation as to whether a Sox9-Fgf loop is active in bipotent progenitors in the same way as it is in MPCs (**Figure 3B**). In support of this contention, as well as being expressed in MPCs, *Fgfr2b* expression persists in Sox9<sup>+</sup> bipotent trunk progenitors during the secondary transition [100], revealing that this population remains Fgf10-receptive. While *Fgf10* is downregulated in mouse pancreatic mesenchyme after E12.5 [53], it is purportedly weakly expressed in the ductal tree of the secondary transition pancreas [114, 139]. *Fgf10* is also detectable in the pancreas by RT-PCR throughout embryogenesis [140].

Additional studies will be required to ascertain whether physiological levels of Fgf10 are expressed in the secondary transition pancreas and if so, whether Fgf10 interacts with *Fgfr2b* and Sox9 to maintain trunk progenitors during this window as it does MPCs in earlier development. If so, this immediately invokes the question of how Notch and Fgf cues interact to govern the adoption of ductal *versus* endocrine fates in bipotent progenitor cells. As mentioned above, on account of the integral role of Fgf and Notch modulation in hESC/iPS cell to insulin<sup>+</sup> cell differentiation protocols [116], the molecular dissection of such putative Fgf-Notch cross-talk is likely to prove instrumental in guiding the optimization of such protocols.

## 8. Roles of Sox9 in the postnatal pancreas

As mentioned above, by birth, expression of Sox9 in both mouse and human pancreas is confined to ductal and centroacinar cells [15, 16, 80] (**Figure 1D**). While *Sox9* expression is required in adult as well as in developing ductal cells to maintain ductal integrity and primary cilia formation [20] (**Figure 3C**), this requirement - unlike that for endocrine differentiation - does not appear to be dosage-sensitive, as a halved complement of pancreatic *Sox9* gene dosage does not manifest in detectable ductal dysmorphogenesis [121]. In fact, despite two-fold reduced islet size and  $\beta$ -cell mass at birth [21], while *Sox9*-haploinsufficient (*Sox9<sup>fl/+</sup>*; *Pdx1-Cre*) mice are glucose-intolerant, this fails to progress to overt diabetes, most likely as a consequence of a compensatory increase in  $\beta$ -cell proliferation, and hence, mass, in the early postnatal period [121]. Superficially, this would seem to imply that homeostasis of the adult pancreas is relatively insensitive to changes in *Sox9* dosage. However, genetic lineage tracing studies have revealed

that this is not necessarily the case, as will be expanded upon below.

Given the role of Sox9 as a maintenance factor for progenitors in the developing pancreas [16] and the commonly held belief that in adulthood facultative progenitors reside in the pancreatic ducts [92], an additional role for Sox9 in tissue homeostasis in the healthy and/or injured pancreas was heavily anticipated. Several studies over the last few years have begun to offer insight into the functions of this factor in a number of pathological states including neonatal growth, pancreatitis-induced regeneration, and pancreatic cancer.

### 8.1 *Sox9<sup>+</sup> cells retain some bipotency in the immediate postnatal period*

Given that *Sox9<sup>+</sup>* cells retain the potential to give rise to duct, acinar, and endocrine cells as late as E18.5 in embryogenesis [19], genetic lineage tracing was conducted neonatally to ascertain whether they retain such multipotency after birth. While tamoxifen administration to five-day-old, *Sox9-CreER<sup>T2</sup>*-expressing mice did not result in any acinar labeling, scarce labeling was detected in small endocrine cell clusters, with non- $\beta$ -cells labeling more frequently than insulin<sup>+</sup> cells [19]. While inappropriate transgene activation cannot be fully excluded, such labeling implies that endocrine neogenesis continues, albeit at a negligible rate, from the *Sox9<sup>+</sup>* domain in the immediate postnatal period (**Figure 2E**). However, despite intensive regimens of tamoxifen administration at three weeks of age, no evidence was obtained in this analysis [19] to support endocrine (or acinar) neogenesis from *Sox9<sup>+</sup>* cells beyond the immediate postnatal stage, implying that *Sox9<sup>+</sup>* cells become progressively “locked in” to a ductal fate beyond birth (**Figure 2F**).

### 8.2 *Neogenic potential of Sox9<sup>+</sup> cells in the mature pancreas is contentious*

In stark contrast to the mature pancreatic *Sox9<sup>+</sup>* cell pulse-chase findings of Kopp and co-workers [19], tamoxifen-induced marking of *Sox9<sup>+</sup>* duct and centroacinar cells in two-month-old *Sox9<sup>JRES-CreERT2</sup>; Rosa26R<sup>lacZ</sup>* mice surprisingly resulted in progressively increased labeling of the acinar compartment over the following months such that almost all exocrine (duct, centroacinar, and acinar), but no endocrine, cells were labeled after a 12-month chase [17]. This pronounced acinar labeling was recapitulated following a tamoxifen pulse to E16.5 *Sox9<sup>JRES-CreERT2</sup>; Rosa26R<sup>lacZ</sup>*

embryos [17] but was not evident in *Sox9-CreER<sup>T2</sup>; Rosa26R<sup>lacZ</sup>* mice pulsed at the same stage [19]. This dramatic, postnatal acinar repopulation by descendants of labeled *Sox9<sup>+</sup>* ductal and/or centroacinar cells is consistent with continuous acinar replenishment by *Sox9<sup>+</sup>* progenitors during normal homeostasis. In attempting to resolve these conflicting studies, it is conceivable that this discordance with *Sox9-CreER<sup>T2</sup>*-mediated tracing stems from an acinus-supplying centroacinar subpopulation being differentially-labeled by the two different *CreER<sup>T2</sup>* lines. A side-by-side survey of the exact adult ductal sub-populations targeted by the two lines will be required to test this hypothesis.

A second, alternative explanation for these discordant findings is, however, equally plausible. While *Sox9-CreER<sup>T2</sup>* mice were derived through BAC transgenesis, the *Sox9<sup>JRES-CreERT2</sup>* line was generated by knock-in of *IRES-CreER<sup>T2</sup>* into the *Sox9* 3'-UTR. Such regions modulate eukaryotic gene expression by governing mRNA translation, stability, and subcellular localization [141] and, in accordance with such crucial roles, comprise some of the most conserved elements within the mammalian genome [142]. As 3'-UTR-mediated post-transcriptional regulation is necessary for the appropriate spatial and temporal expression of the mRNA-encoded protein [143], knock-in into the 3'-UTR is sometimes known to disrupt expression levels of the targeted gene, often in quite unpredictable fashion. In illustration, knock-in of *lacZ* into the 3'-UTR of mouse *Vegf* (encoding vascular endothelial growth factor A) has been reported to result in either increased [144] or decreased [145] *Vegf* expression levels, contingent upon the exact insertion site. Given the exquisite *Sox9* dosage-sensitivity of pancreatic morphogenesis, it is plausible that even slight perturbations in *Sox9* expression level caused by disruption of the *Sox9* 3'-UTR might impact *Sox9<sup>+</sup>* cell behavior. Indeed, lending credence to this notion, deletion of a recently-discovered enhancer located 70 kb upstream of mouse *Sox9*, produces an 18-37% decrease in *Sox9* expression levels in a number of organs including pancreas, phenocopying (albeit to a lesser degree) islet hypoplasia resulting from *Sox9*-haploinsufficiency [146]. This complex transcriptional regulation of *Sox9* is further compounded by its positive autoregulation as implied by a previous study [77]: Sox9 dimers bind multiple recognition sites within the enhancer region and are both necessary and sufficient for its activity [146]. Thus, together, even relatively minor reductions in *Sox9*

expression levels are sufficient to affect the neogenic potential of *Sox9*<sup>+</sup> pancreatic cells.

Confirming the notion that *IRES-CreER*<sup>T2</sup> insertion into its 3'-UTR impacts *Sox9* dosage, while the level of endogenous pancreatic (and hepatic) *Sox9* expression is unaffected in *Sox9*<sup>IRES-CreERT2</sup> neonates, it is decreased in adult mice [23], implying divergent transcriptional mechanisms regulating *Sox9* expression in development versus adulthood. Such postnatal *Sox9* hypomorphism is likely to affect the neogenic competence of *Sox9*<sup>+</sup> ductal (including centroacinar) cells, rendering them more plastic [23] and able to give rise to acinar cells which repopulate the acinar compartment through self-duplication [147]. This hypothesis might be easily tested by pulse-chasing *Sox9*<sup>+</sup> cells in adult *Sox9-CreER*<sup>T2</sup>; *Rosa26R*<sup>lacZ</sup> mice on *Sox9*<sup>fllox/+</sup> (*Sox9*-haploinsufficient) versus *Sox9*<sup>+/+</sup> backgrounds. While reduced *Sox9* expression in adult pancreatic cells might conceivably result in them yielding acinar cells, how such labeled acinar cells populate the entire acinar compartment is a matter of contention. This phenomenon might result from labeled acinar cells having some selective advantage and "outcompeting" unlabeled cells in the same acinus. However, it may also result from previously unappreciated mechanisms of cell renewal in pancreas homeostasis such that acinar cells juxtaposing terminal duct/centroacinar cells are predisposed to repopulate the entire acinus at intervals, consistent with individual pancreatic acini being clonal [148]. While unintentional, this tracing of *Sox9*-hypomorphic adult duct cells [17] has deftly revealed that *Sox9* dosage regulates neogenic potential of *Sox9*<sup>+</sup> cells in adult as well as embryonic pancreas. It is clear that further work will be required to determine how exactly *Sox9* dosage modulates adult cell plasticity and how this finding might be exploited for therapeutic gain.

### 8.3 Pancreatic injury invokes incomplete endocrine differentiation of *Sox9*<sup>+</sup> cells

Despite their discrepancies, the aforementioned pulse-chase studies [17, 19] are unanimous in presenting no evidence for endocrine neogenesis from *Sox9*<sup>+</sup> cells in the mature pancreas during normal tissue homeostasis. However, injury to the adult organ might invoke the necessary stimuli required to induce endocrine differentiation from a putative facultative adult ductal progenitor [92]. Over the last decade, the study of pancreatic regeneration initiated in the nineteenth century has undergone a major revival, which is in large part due to the advent of modern molecular techniques and suit-

able transgenic tools. Historically, a range of insults has been exploited experimentally to induce pancreatic regeneration including partial pancreatectomy [149, 150], cerulein-induced pancreatitis [3, 151] and  $\beta$ -cell ablation - either pharmacologically using streptozotocin (STZ) or alloxan [152] or genetically in conjunction with diphtheria toxin [153, 154]. However, the partial duct ligation (PDL) model [1, 2] has garnered most attention in recent years, largely owing to the publication of a high-profile study by the Heimberg group demonstrating that PDL stimulates *Ngn3*-dependent  $\beta$ -cell neogenesis from adult pancreatic ducts [155].

Following ligation of the duct leading to the "tail" (the splenic portion) of the pancreas, pancreatitis leads to rapid acinar apoptosis in the tail while the "head" (the duodenal portion) of the pancreas remains unaffected.  $\beta$ -cell mass in the tail was reported to more than double one week post-PDL, concomitantly with strong, tail-specific upregulation of *Ngn3* expression [155]. Abrogation of this *Ngn3* induction via lentiviral knockdown significantly diminished the PDL-induced increase in  $\beta$ -cell mass showing at least a portion of this increase to be *Ngn3*-dependent. Following PDL in mice expressing *Ngn3* promoter-driven *lacZ*,  $\beta$ -gal<sup>+</sup> cells were detected specifically in the ligated tail, many residing within ducts. Akin to *Sox9-eGFP* pseudo short-term tracing,  $\beta$ -gal perdurance [156] allowed *Ngn3*<sup>+</sup> cells to be traced and, consistent with an endocrine progenitor identity, a third of  $\beta$ -gal<sup>+</sup> cells expressed islet hormones one week post-PDL. As well as being ultrastructurally similar to embryonic *Ngn3*<sup>+</sup> endocrine progenitors, when PDL-induced adult *Ngn3*<sup>+</sup> cells were grafted into *Ngn3*-null embryonic pancreas explants, they could be induced to differentiate into endocrine cells, including glucose-responsive  $\beta$ -cells. Together, these observations infer activation of  $\beta$ -cell neogenesis from *Ngn3*<sup>+</sup> endocrine progenitors induced in ducts by PDL-mediated adult pancreatic injury.

This contention is one of the most controversial topics in current diabetes biology. Several groups have argued that, while PDL *does* induce ductal *Ngn3* expression, absolute  $\beta$ -cell *number* in the ligated tail remains unchanged, although  $\beta$ -cell *mass* (i.e. calculated morphometrically as proportional area of insulin-immuno-positive pancreas tissue  $\times$  pancreatic wet weight) increased as an artifact of the ~70% decrease in total pancreatic tail area seen one week after PDL due to extensive acinar apoptosis [19, 32, 157]. While pancreatic edema, fatty degeneration, and fibrosis resulting from ligation-induced pancreatitis contribute to

wet weight, such tissue regions will be excluded from morphometric area measurements, artificially inflating calculated  $\beta$ -cell mass in ligated pancreata [19]. However, in conflict with this and in validation of their initial findings, Heimberg and colleagues recently demonstrated a post-PDL doubling of total  $\beta$ -cell volume and islet number in the tail, the latter being especially attributable to an increase in small  $\beta$ -cell clusters [158].

Furthermore, pulse-chase labeling of *Ngn3*<sup>+</sup> cells in *Ngn3*<sup>CreERT</sup>; *Rosa26R*<sup>YFP</sup> mice following PDL revealed YFP label in duct-associated insulin<sup>+</sup> cell clusters as well as islets, which is consistent with induction of ductal *Ngn3*<sup>+</sup> endocrine progenitors,  $\beta$ -cell neogenesis, and their contribution to existing islets [158]. Interpretation of *Ngn3*<sup>+</sup> cell lineage tracing is, however, confounded by persistent low-level endogenous *Ngn3* expression in mature adult endocrine cells, including those in duct-ligated pancreata [19, 159]. Thus, genetic lineage tracing of  $\beta$ -cells from pancreatic ducts post-PDL would constitute the most incontrovertible evidence to support  $\beta$ -cell neogenesis in this injury model. To this end, pulse-chase duct cell labeling has been undertaken following PDL in several transgenic lines in which CreER expression is duct-specific in the adult pancreas, including *CAII* (*carbonic anhydrase II*)-*CreER*<sup>TM</sup> [160], *Hnf1 $\beta$* -*CreER*<sup>T2</sup> [31] and *Hes1*<sup>CreERT2</sup> [32] mice. The outcomes of these studies are, however, conflicting as only the first study shows labeling in insulin<sup>+</sup> cells following ligation [160]. While such  $\beta$ -cell labeling is consistent with  $\beta$ -cell neogenesis from ducts following PDL, it might conceivably also arise from non-ductal CreER expression, given the low-frequency endocrine and acinar labeling seen in control animals [160]. Equally, the lack of contribution of labeled cells to islets following PDL in *Hnf1 $\beta$* -*CreER*<sup>T2</sup> or *Hes1*<sup>CreERT2</sup> mice might reflect heterogeneity in labeling ductal subpopulations so that a rare putative adult progenitor population expresses neither transgene. Unlike *Hnf1 $\beta$* -*CreER*<sup>T2</sup> [31], *CK*(*cytokeratin*)*19*<sup>CreERT</sup> [161] and *Muc1*<sup>RES-CreERT2</sup> [162], *Sox9*-*CreER*<sup>T2</sup> labels a ductal population during the immediate postnatal period which gives rise to endocrine cells [19]. Thus, it is plausible that this line is more likely to target a putative adult progenitor population than other ductally expressed CreERs.

To test whether  $\beta$ -cell neogenesis is induced from *Sox9*<sup>+</sup> cells following ligation injury, Kopp *et al.* performed PDL or sham surgery on seven-week-old *Sox9*-*CreER*<sup>T2</sup>; *Rosa26R*<sup>YFP</sup> mice four weeks after they were pulse-labeled *via* a high-

dose tamoxifen regimen, resulting in two-thirds of *Sox9*<sup>+</sup> cells being YFP-labeled [19]. Despite a 40-fold upregulation of *Ngn3* expression in the ligated tail, which was consistent with the detection of *Ngn3*<sup>+</sup> hormone<sup>+</sup> cells within *Sox9*<sup>+</sup> lineage-labeled ducts of ligated pancreata, labeling was seen in virtually no (fewer than 1 in 1,000) insulin<sup>+</sup> cells at either one or eight weeks post-PDL [19]. Accordingly, no increase in duct-adjacent small  $\beta$ -cell clusters was evident seven days after surgery [19]. Mirroring these findings, PDL of *Sox9*<sup>RES-CreERT2</sup>; *Rosa26R*<sup>lacZ</sup> mice pulsed with a high-dose tamoxifen regimen at 4 or 8 weeks of age similarly failed to elicit any appreciable  $\beta$ -cell labeling [17]. Independent of the possible failure of both *Sox9*-*CreER*<sup>T2</sup> and *Sox9*<sup>RES-CreERT</sup> to mark a scarce *Sox9*<sup>+</sup> progenitor population, it appears that PDL-induced injury does not induce neogenesis of *bona fide*  $\beta$ -cells from the *Sox9*<sup>+</sup> duct compartment. Indeed, given the replication of this outcome in transgenic lines, in which CreER is expressed in other ductal subpopulations [31, 32], it is becoming improbable that neogenesis of mature insulin<sup>+</sup> cells from ducts occurs to any appreciable extent in the PDL model. This, however, does not preclude PDL injury stimulating at least a partial program of endocrine differentiation in injured adult duct cells, as suggested by activation of ductal *Ngn3* expression post-PDL. Presumably, however, the duct-ligated adult pancreas lacks the vital cues required for the continued differentiation of endocrine progenitors into hormone<sup>+</sup> endocrine cells. This is consistent with *Ngn3*<sup>+</sup> cells in ligated adult pancreata being able to give rise to insulin<sup>+</sup> cells in embryonic pancreas explants [155]. Contingent upon feasibility, identifying the molecular cues required for terminal  $\beta$ -cell differentiation from ductal *Ngn3*<sup>+</sup> cells in the PDL model will facilitate the development of novel therapies for stimulating  $\beta$ -cell regeneration *in situ* in the diabetic pancreas.

Notably, confirming the inability of *Sox9*<sup>+</sup> cells to yield  $\beta$ -cells following PDL-induced injury, alternative pancreatic injury models including partial pancreatectomy, cerulein-induced pancreatitis or STZ-induced  $\beta$ -cell ablation all similarly failed to label  $\beta$ -cells in *Sox9*<sup>RES-CreERT2</sup>; *Rosa26R*<sup>lacZ</sup> mice [17]. Together, these observations unanimously argue that *Sox9*<sup>+</sup> cells in the adult mouse pancreas do not serve as facultative  $\beta$ -cell progenitors following injury. In apparent contrast, regeneration of  $\beta$ -cells following their specific ablation at the end of embryogenesis, is compromised in *sox9b* mutant zebrafish larvae [79]. While this result would ostensibly implicate a requirement for *sox9b*

in  $\beta$ -cell regeneration in zebrafish, it is important to consider the challenges encountered in attempting to identify comparable stages in the mouse and zebrafish pancreatic programs. Thus, while conceivable that  $\beta$ -cell regeneration occurs *via* distinct mechanisms in the mature rodent *versus* zebrafish pancreas, it is more plausible that  $\beta$ -cell neogenesis during the early post-embryogenesis stage in zebrafish corresponds with the mouse secondary transition. Thus, in zebrafish, *sox9b* appears to be required for pancreatic ductal cells to generate secondary transition endocrine cells. Given that zebrafish *sox9b* mutants are capable of surviving to adulthood [79, 112], it is tempting to speculate whether  $\beta$ -cell regeneration following ablation will be similarly compromised in mature adult *sox9b* mutants.

#### 8.4 Adult pancreatic *Sox9*<sup>+</sup> cells can be induced to form insulin<sup>+</sup> cells *ex vivo*

Whereas *Sox9*<sup>+</sup> cells in the intact adult mouse pancreas can only be stimulated to undergo partial differentiation toward an endocrine fate, they can seemingly be induced to form endocrine cells when cultured *ex vivo*. Where *in vivo* pulse-chase studies have failed, a recent *ex vivo* hematopoietic colony assay study [163] identified a putative adult pancreatic progenitor cell expressing *Sox9-eGFP* [85] as well as the ductal marker CD133 [164]. Constituting ~1% of total adult pancreatic cells, such so-called “pancreatic colony-forming units” (PCFUs) form ductal-like colonies in Matrigel-containing semisolid culture media which are capable of >100,000-fold expansion following treatment with the Wnt agonist R-spondin 1 [163]. Moreover, when transferred to a Matrigel-free laminin-based semisolid medium, some cells give rise to endocrine/acinar colonies containing glucose-responsive  $\beta$ -cell-like cells [163]. This result apparently confirms the notion that mature pancreatic *Sox9*<sup>+</sup> cells *can* be induced to form  $\beta$ -cells when provided with the requisite signaling milieu. It is also particularly striking when it is considered that the same BAC clone was used to generate both *Sox9-eGFP* and *Sox9-CreER<sup>flz</sup>* mice [19, 85]. Thus, the same *Sox9*<sup>+</sup> pancreatic population should theoretically be targeted in studies using either mouse, notwithstanding integration site-specific effects. Given the relatively young age (2-4 months) of the mice from which such PCFUs are isolated, only a few weeks after *Sox9*<sup>+</sup> cells remain duct/endocrine-bipotent [19], it will be interesting to examine whether equally multipotent PCFUs can be isolated from more mature animals. Furthermore, it is interest-

ing to speculate whether the dissociation process itself elicits a regenerative stimulus provoking a facultative progenitor response to injury. Since the *Sox9-eGFP*<sup>+</sup> CD133<sup>+</sup> ductal population comprises between 2-6% of total pancreatic cells while only ~1% of such cells exhibit colony-forming ability, further efforts should be expended on characterizing the adult ductal population to gain insight into this heterogeneity. Undoubtedly, this *ex vivo* study will redouble current efforts aimed at identifying adult pancreatic progenitor cells corresponding to PCFUs *in situ*.

#### 8.5 *Sox9*<sup>+</sup> cells in adult liver are competent to initiate a pancreatic program

Intriguingly, while *in vivo*, *Sox9*<sup>+</sup> duct cells in mature adult pancreas apparently cannot be coaxed into giving rise to insulin<sup>+</sup> cells, those in the liver can. In a recent study by Slack and colleagues STZ-induced diabetes in NOD-SCID mice was rescued by adenoviral administration of a transcription factor cocktail comprising Pdx1, Ngn3, and the  $\beta$ -cell maturation factor MafA [165, 166]. This was associated with a 25-fold increase in hepatic insulin content consistent with the presence of glucose-responsive insulin-immunopositive CK19<sup>+</sup> ectopic duct-like structures in the livers of infected mice. While insulin<sup>+</sup>, such cells appear to be poly-hormonal, additionally expressing glucagon and somatostatin, consistent with the expression of the pan-endocrine markers Isl1 and Rfx6 [165, 167, 168]. Using *Sox9-CreER<sup>flz</sup>*-mediated lineage tracing, such insulin<sup>+</sup> cells were demonstrated to arise from *Sox9*<sup>+</sup> hepatic cells, proposed by the authors to constitute:

1. Small bile ducts,
2. Hepatoblast-like progenitors assumed to reside in the adjoining periportal zone, or
3. Peribiliary glands associated with larger bile ducts.

Supporting the second hypothesis, the Chan group convincingly showed that helper-dependent adenoviral delivery of Ngn3 and the growth factor betacellulin in STZ-treated diabetic mice induced the appearance of periportal “neo-islets” comprising the four principal endocrine cell types, including functional insulin-immunopositive  $\beta$ -cell-like cells capable of restoring euglycemia in infected mice [169].

Several strands of evidence including marker expression, location, morphology, and lineage trac-

ing supported the cell of origin being hepatic oval cells, bipotent hepatic progenitors able to give rise to hepatocytes or biliary cells [170]. Thus, it is plausible that in both studies, insulin<sup>+</sup> cells in the adult liver arise from the same progenitor population in which *Sox9* expression affords endocrine competence. Supporting this contention, in both healthy adult mice and those subjected to an oval cell-inducing injury (treatment with the chemical DCC), *Sox9* marks a clonal liver progenitor population residing within biliary ducts that is capable of *in vitro* bilineage (hepatocyte and biliary duct cell) colony formation [171]. This differentiation potential of *Sox9*<sup>+</sup> cells in adult liver is conserved *in vivo*: genetic pulse-chase labeling using either *Sox9*<sup>JRES-CreERT2</sup> [17] or *Sox9-CreER<sup>T2</sup>* [171] mice reveals that *Sox9*<sup>+</sup> cells contribute to both hepatocyte and bile duct compartments in the uninjured adult liver during normal tissue homeostasis. Hepatic injury through treatment with either DCC [171] or a methionine- and choline-deficient/ethionine-supplemented diet [17] to induce an oval cell response, dramatically augmented the contribution to both lineages from *Sox9*<sup>+</sup> progenitors, as did acute liver insult through carbon tetrachloride administration or bile duct ligation [17].

These studies thus show that *Sox9* marks a bipotential adult hepatic progenitor which gives rise to both hepatocytes and biliary duct cells even in the absence of a regenerative stimulus. Therefore, while *Sox9*<sup>+</sup> ductal cells in the adult pancreas do not appear to serve an obvious progenitor role, at least during normal organ homeostasis, they evidently do so in the mature liver. The ability to reprogram adult hepatic *Sox9*<sup>+</sup> progenitors into insulin<sup>+</sup> cells when provided with the appropriate cues suggests that transcriptional programs are at least partially conserved between *Sox9*<sup>+</sup> progenitors in adult liver and those in the embryonic pancreas. By extension, it will be fascinating to test whether their expression of *Sox9* provides hepatic progenitors the competence to give rise to insulin<sup>+</sup> cells. This might be examined by testing whether the adenoviruses mentioned above are still able to induce hepatic insulin expression following conditional hepatic *Sox9* ablation.

### 8.6 *Sox9* is a key player in the initiation of pancreatic ductal adenocarcinoma

In light of many tumors originating from adult organ stem cells [172], it was almost inevitable that a potential role for *Sox9* in pancreatic cancer pathogenesis would be examined. Recent studies

have revealed that *Sox9* does indeed serve a crucial role in the initiation of pancreatic ductal adenocarcinoma (PDAC). In the US, PDAC is the fourth leading cause of cancer-associated mortality, with a five-year survival rate of less than 5%, which is in large part due to the advanced and/or metastatic state of neoplastic lesions at the time of diagnosis [173, 174]. The development of both early detection assays and novel therapies will require an understanding of the mechanisms underpinning PDAC initiation. PDAC is a malignant neoplasm which arises from normal pancreatic tissue through a series of histologically well-defined preinvasive ductal lesions (termed pancreatic intraepithelial neoplasias or PanINs) to invasive carcinoma [175, 176]. PDAC is widely regarded as a “genetic disease” with genetic mutations accumulating in lesions during PDAC progression, the first of these being activating mutations of *KRAS* [177, 178]. Seminal studies in the field of pancreatic cancer biology revealed that expression of constitutively active *Kras*<sup>G12D</sup> in mice induces PDAC several months after PanIN induction [179], roughly at the time when acinar-to-ductal metaplasia (ADM) occurs [180, 181]. Despite PDAC progression being histologically well-characterized, the cellular origin of PDAC has remained unproven: tumor histology can quite often be deceptive in terms of ascertaining tumor origin. While centroacinar cells have been widely implicated by several studies [182, 183], the lack of transgenic lines expressing CreER exclusively in centroacinar and ductal cells until recently has precluded testing this hypothesis. Activation of oncogenic *Kras* in cells of the larger ducts has revealed a low propensity for PanIN induction from ductal cells [184, 185], while its activation in acinar cells has been shown to convert them into duct-like cells and PanINs [180, 186-189], implicating ADM as a putative cause of PanIN formation.

In an effort to dissect the mechanisms inducing PanIN and PDAC formation, Kopp and colleagues demonstrated that activation of oncogenic *Kras*<sup>G12D</sup> in acinar cells using the *Ptf1a*<sup>CreERTM</sup> line readily induces PanINs while *Sox9-CreER<sup>T2</sup>*-driven *Kras*<sup>G12D</sup> activation in ductal/centroacinar cells does not (**Figure 3D**) [22, 89]. Consistent with this, acute cerulein-induced pancreatitis, previously shown to exacerbate *Kras*<sup>G12D</sup>-mediated PanIN and PDAC formation [180, 188, 190, 191], potentiated *Kras*<sup>G12D</sup>-mediated PanIN formation from acinar, but not ductal, cells [22]. Thus, the metaplasia of acinar to ductal cells associated with PanIN induction has served as a red herring, hampering the



identification of the acinar cell as the true cell of origin of pancreatic cancer. Kopp *et al.* went on to show that aberrant induction of Sox9 expression in *Kras*<sup>G12D</sup>-expressing acinar cells precedes ADM and PanIN initiation [22]. Another recent study suggests that this event is conserved in human PDAC etiology: Sox9 and a second pancreatic ductal factor, Onecut1 [59, 60], are ectopically expressed in metaplastic acinar cells in close proximity to PDAC lesions in sections of resected human pancreas [192]. If this Sox9 induction is prevented in mice by simultaneous acinar-specific *Kras*<sup>G12D</sup> expression and *Sox9* deletion, PanIN formation is blocked, revealing Sox9 induction in *Kras*-active acinar cells to be necessary for PanIN initiation [22]. In support of this result, Prévot *et al.* showed that ADM induced by PDL-mediated pancreatitis is blunted by acinar-specific *Sox9* deletion, revealing Sox9 to be required in acinar cells for their metaplasia [192]. The converse is also true in that forced expression of *Sox9* in *Kras*<sup>G12D</sup>-expressing acinar cells accelerates both ADM and PanIN formation [22] (**Figure 3D**). The expression of ductal markers in *Sox9*-expressing acinar cells in the absence of oncogenic *Kras* suggests that this is associated with a “destabilization” of acinar identity, increasing the potential for ADM given the appropriate inflammatory cues [22, 192]. Consistent with this, forced acinar *Sox9* expression, mirroring oncogenic *Kras*, promotes ADM following cerulein-induced pancreatitis [22].

Finally, Kopp and coworkers showed that, while acinar-specific *Sox9* ablation is able to block *Kras*<sup>G12D</sup>-mediated ADM and PanIN initiation, it is insufficient to prevent ADM following cerulein-induced pancreatitis [22], which suggested that Sox9 is dispensable for ADM in this pancreatic injury model. While this is the case, Sox9 expression is obligatory for the further progression of ADM into PanINs since cerulein-treated mice subjected to acinar-specific *Kras*<sup>G12D</sup> expression and *Sox9* deletion develop virtually no PanINs [22]. Together, these studies show that *Sox9* is crucially required for the conversion of acinar cells into PanINs by conferring them with ductal characteristics [22, 192], which is consistent with the aforementioned role of Sox9 in regulating duct-specific genes [20, 79]. Kopp and colleagues showed that, while mature acinar cells are readily PanIN-competent following *Kras*<sup>G12D</sup>-induced *Sox9* activation, *Sox9*-expressing ductal and centroacinar cells remain highly refractory to PanIN initiation [22] (**Figure 3D**). The relatively low efficiency of *Sox9-CreER*<sup>T2</sup>-mediated recombination (in ~12% of Sox9<sup>+</sup> cells) does not, however, preclude the existence of scarce

(either Sox9<sup>+</sup> or Sox9) PanIN-initiating cells in the ductal tree which were not recombined in this analysis. Notwithstanding this caveat, while tumor histology has long implicated ductal/centroacinar cells as the originator of PDAC, they paradoxically represent the only pancreatic lineage so far examined that does not ostensibly undergo neoplastic transformation readily. Contingent upon these pathophysiological findings in mouse translating to human PDAC etiology, they suggest that: 1) Sox9 (and Onecut1) constitute novel biomarkers of ADM and 2) prophylactic therapeutic strategies for PDAC should focus on preventing PanIN initiation from the acinar compartment by preventing acinar *Sox9* induction. Thus, befitting the intimate relationship between Sox9 and Notch signaling in the pancreas, Sox9 now joins the Notch pathway as a therapeutic target in pancreatic cancer prevention.

## 9. Summary and future perspectives

As well as being required for maintaining progenitors in the developing neural crest [13], intestinal epithelium [96], hair bulge/follicle [97, 193], and pancreas [16], Sox9 has been subsequently shown to maintain the neural stem cells of the CNS [194] and mammary stem cells [195] and mark progenitors of the developing lung [196, 197] and retina [198] and adult liver [17, 171] and intestine [17]. Undoubtedly, this roster of organ-specific stem/progenitor cells marked and/or maintained by Sox9 will be added to by future studies. While this progenitor marker and maintenance function has seemingly defined its role in the pancreatic program, the multiple indispensable tasks performed by Sox9 in the developing and adult pancreas outlined here show Sox9 to be far more multi-functional than generally perceived. In sum, during the morphogenesis and postnatal maintenance of the pancreas:

1. **Sox9 marks and maintains multipotent pancreatic progenitors** by promoting their survival and proliferation as well as retention of their undifferentiated status. Sox9<sup>+</sup> cells retain their multipotency throughout gestation, including the secondary transition, but become progressively “locked in” to a ductal fate as birth approaches. Maintenance of the MPC pool is likely to be achieved in part through modulation of Hes1/Notch signaling. Furthermore, by maintaining MPC Fgfr2b expression and receptivity to mitogenic mes-

enchymal Fgf10, Sox9 participates in a feed-forward Fgf10/Fgfr2b/Sox9 loop to maintain MPC proliferation and pancreatic commitment. Consequently, pancreas-specific *Sox9* ablation produces pancreatic hypoplasia and a pancreas-to-liver fate switch.

2. **Sox9 marks secondary transition duct-endocrine bipotent pancreatic progenitors and is absolutely required for their endocrine differentiation.** Like Hes1, Sox9 is a Notch target in bipotent progenitors which lay the foundations of the presumptive ductal tree. While pro-endocrine *Ngn3* is inhibited by Hes1, it is directly activated by Sox9. As a consequence of Sox9 being more sensitive than Hes1 to increased Notch activity: 1) bipotent progenitors with high Notch activity express both Hes1 and Sox9 and remain undifferentiated while 2) those with intermediate Notch activity induce only Sox9. Because of a positive autoregulatory feedback loop, once expression of *Ngn3* is initiated, an *Ngn3*<sup>hi</sup> state is rapidly attained, ensuring endocrine commitment. Cell-autonomous repression of *Sox9* by *Ngn3* is then required for endocrine differentiation. Sox9<sup>+</sup> bipotent progenitors which fail to initiate an endocrine program adopt the alternative ductal fate instead. Consistent with Sox9 being an obligatory effector of *Ngn3* induction and endocrine commitment, morphogenesis of the endocrine compartment is more susceptible to *Sox9* dosage than that of the exocrine tissue such that *Sox9*-haploinsufficiency manifests in islet-specific hypoplasia, phenocopying the islet defects seen in human CD cases.
3. **Sox9 is directly required for differentiation and maintenance of pancreatic ducts and their cilia,** in part by maintaining expression of ductal genes including *Spp1* and *Pkd2*, which maintains primary cilia. Concordantly, *Sox9* ablation either prior to the secondary transition or in adulthood results in duct dysgenesis and cysts as well as loss of primary cilia.
4. **Sox9 marks duct and centroacinar cells in the postnatal pancreas which retain some neogenic potential.** *In vivo*, the *Sox9*<sup>+</sup> pancreatic compartment is able to give rise to small numbers of endo-

crine cells in the immediate neonatal period, but not beyond. However, given the requisite culture conditions, isolated *Sox9*<sup>+</sup> adult ductal cells are able to expand and give rise to acinar and endocrine cells, including  $\beta$ -cell-like cells *ex vivo*. While pancreatic injury such as PDL is ostensibly able to initiate an endocrine program (as indicated by *Ngn3* induction) in adult pancreatic Sox9<sup>+</sup> cells, endocrine differentiation is incomplete, presumably owing to the absence of the necessary cues. Reduction of *Sox9* dosage in adult pancreas appears to invoke their acinar (but not endocrine) differentiation, revealing plasticity of mature *Sox9*<sup>+</sup> duct/centroacinar cells.

5. **Ectopic activation of Sox9 expression in acinar cells is integral for their metaplasia to ductal cells and subsequent PanIN/PDAC progression, unveiling Sox9 as a key player in the initiation of pancreatic cancer.**

These divergent roles are clearly dependent upon the interaction of Sox9 with various transcription factors and signaling pathways, foremost Fgf and Notch. It is highly probable that this milieu of interacting partners is spatiotemporally dynamic during the course of pancreatic organogenesis, changing with both developmental stage and cellular context. It is envisaged that characterizing the Sox9 interactome throughout pancreas organogenesis and in both the healthy and injured pancreas will provide great insight into the mechanisms through which Sox9 regulates pancreogenesis and adult pancreas homeostasis. This will require ChIP-Seq of various *Sox9*<sup>+</sup> pancreatic cell populations to identify Sox9 pancreatic targets on a genome-wide scale. Such analyses are of course contingent upon the future development of necessary ChIP-grade reagents. Dissecting *Sox9*-mediated governance of the pancreatic program will undoubtedly provide unprecedented insight into the transcriptional mechanisms regulating the differentiation of pancreatic endoderm into  $\beta$ -cells. Such knowledge will be highly beneficial in optimizing current protocols for differentiating hESC/iPS cells into functionally-mature insulin<sup>+</sup> cells. Elucidation of the changing pancreatic Sox9 interactome is in turn likely to lead to the identification of further roles for Sox9 in the pancreas, assuring its position as a multi-tasking master regulator of the pancreatic program during pancreas development and beyond.

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