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REVIEW

Cellular and molecular mechanisms coordinating pancreas development

Aimée Bastidas-Ponce[1,2,3,4,*], Katharina Scheibner[1,2,3,4,*], Heiko Lickert[1,2,3,4] and Mostafa Bakhti[1,2,3,‡]

ABSTRACT

The pancreas is an endoderm-derived glandular organ that participates in the regulation of systemic glucose metabolism and food digestion through the function of its endocrine and exocrine compartments, respectively. While intensive research has explored the signaling pathways and transcriptional programs that govern pancreas development, much remains to be discovered regarding the cellular processes that orchestrate pancreas morphogenesis. Here, we discuss the developmental mechanisms and principles that are known to underlie pancreas development, from induction and lineage formation to morphogenesis and organogenesis. Elucidating such principles will help to identify novel candidate disease genes and unravel the pathogenesis of pancreas-related diseases, such as diabetes, pancreatitis and cancer.

KEY WORDS: Pancreas development, Morphogenesis, Organogenesis, Lineage formation, Exocrine cells, Endocrine cells, Islets of Langerhans, β-cells, Diabetes

Introduction

The pancreas consists of an exocrine compartment that produces digestive enzymes, and an endocrine compartment that generates pancreatic hormones. The exocrine compartment comprises acinar cells that secrete nutrient-digestive zymogens, and a ductal epithelium that neutralizes these enzymes and transports them into the duodenum. By contrast, endocrine cells mediate the regulatory function of the pancreas in glucose homeostasis by producing several peptide hormones that are secreted into the bloodstream. These cells cluster in the islets of Langerhans and include α-, β-, δ-, PP- and ε-cells that synthesize glucagon, insulin, somatostatin, pancreatic polypeptide and ghrelin, respectively (reviewed by Pan and Wright, 2011; Shih et al., 2013). Malfunction of these hormone-producing cells can lead to abnormalities such as diabetes mellitus. Therefore, understanding how these endocrine cell types develop might help to unravel the pathogenesis of diabetes and identify molecular targets for therapeutic approaches. Furthermore, given that aberrant function of the exocrine pancreas can cause pancreatitis and pancreatic cancer, understanding the developmental mechanisms generating exocrine tissue might pinpoint molecular targets to treat these lethal diseases (reviewed by Dunne and Hezel, 2015; Murtaugh and Keefe, 2015).

In mice, the pancreas is derived from the foregut endoderm and its development takes place through a series of morphological processes to generate distinct cell types (reviewed by Wells and Melton, 1999; Zorn and Wells, 2009). The early organogenesis of this organ can be classified into two main stages. In the primary stage (or transition), signals from the notochord, endothelium and mesenchyme induce pancreatic buds through the formation and expansion of multipotent progenitor cells (MPCs) from embryonic day (E) 9.0 to E12.5 (reviewed by Gittes, 2009; Lammert et al., 2003; Larsen and Grapin-Botton, 2017). These cells then undergo massive proliferation to generate a multilayered epithelium in which microlumen structures develop. During the secondary stage, the fusion of microlumina creates a central plexus that further remodels into a continuous ramified epithelial network, segregated into tip and trunk domains (Bankaitis et al., 2015; Kesavan et al., 2009; Villasenor et al., 2010). These morphological events coincide with the formation of three main pancreatic cells (endocrine, exocrine/acinar and ductal cells), highlighting a tight association between morphogenesis and differentiation during pancreas development. Further interactions and interconnections between pancreatic cells and the surrounding mesenchyme, endothelium and neuronal projections (reviewed by Cleaver and Dor, 2012; Thorens, 2014) construct the final anatomy of the adult pancreas.

Over the past few decades, intensive efforts have aimed to pinpoint the molecular mechanisms governing pancreas development and organogenesis. Most studies have focused on the identification of signaling pathways and gene regulatory networks responsible for pancreas formation (reviewed by Arda et al., 2013; McCracken and Wells, 2012). However, much remains to be discovered, particularly with regard to the cellular processes that coordinate the morphogenesis of this complex organ. In this Review, we discuss our current knowledge of the cellular and molecular events orchestrating the specification and morphogenesis of pancreatic cells. Given that most studies of pancreas formation to date have been performed in mice, we focus on studies conducted in rodents, which have proved to be a popular and amenable model due to the ease with which they can be genetically modified. Studies of pancreas development in humans have also been performed; although limited, they have highlighted key similarities and differences between pancreas development in mice and man (summarized in Box 1; reviewed by Jennings et al., 2015).

Pancreas induction and specification

The first step in pancreas formation involves the specification of dorsal and ventral pancreatic buds from foregut endoderm. These buds are formed by proto-differentiated MPCs that express pancreatic and duodenal homeobox 1 (Pdx1) and pancreas-specific transcription factor 1a (Ptf1a) (Burlison et al., 2008). The subsequent growth and expansion of these cells relies on different signaling cues that are derived from the notochord, endothelium and surrounding mesenchyme.

[1]Institute of Diabetes and Regeneration Research, Helmholtz Zentrum München, D-85764 Neuherberg, Germany. [2]Institute of Stem Cell Research, Helmholtz Zentrum München, D-85764 Neuherberg, Germany. [3]German Center for Diabetes Research (DZD), D-85764 Neuherberg, Germany. [4]Technical University of Munich, Medical Faculty, 81675 Munich, Germany.
*These authors contributed equally to this work

‡Author for correspondence (mostafa.bakhti@helmholtz-muenchen.de)

H.L., 0000-0002-4597-8825; M.B., 0000-0002-2307-1122

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Box 1. Pancreas development in humans

Because of the limited access to human samples, our understanding of human pancreas development is rudimentary and still mainly derives from analysis of embryonic and fetal tissues. Similar to rodent models, the induction of human pancreas initiates with the evagination of the foregut endoderm, at Carnegie stage (CS) 9, to generate ventral and dorsal buds at CS13 (reviewed by Jennings et al., 2015). Unlike the situation in mice, the human pancreas does not undergo primary transition and NKX2.2 is not expressed in pancreatic progenitors (Jennings et al., 2013; reviewed by Pan and Brissova, 2014). By CS19, specified populations of tip-like and trunk-like domains are distinguishable (Jennings et al., 2013). During the fetal period, endocrine progenitors ($NGN3^+$ $SOX9^-$) peak at 8 weeks post coitus (wpc), decline at ~26-28 wpc and are not detectable by 35 wpc (Capito et al., 2013; Salisbury et al., 2014). Although NGN3 is transiently expressed in endocrine progenitors (Jennings et al., 2013), as occurs in mice, newly differentiated human endocrine cells also express NGN3 (Lyttle et al., 2008). In addition, in rare cases of *NGN3* homozygous mutation, patients develop a mild diabetic phenotype, suggesting the existence of NGN3-independent ways to form endocrine progenitors (Rubio-Cabezas et al., 2014). However, it should be noted that some of the human mutations are likely to be hypomorphic, as it was recently described that *GATA6* haploinsufficiency impairs the differentiation of human pluripotent stem cells into $PDX1^+$ $NKX6.1^+$ cells (Shi et al., 2017).

The first fetal β-cells emerge at ~8 wpc, followed by the formation of glucagon-expressing cells at 9 wpc (Hanley et al., 2010; Jennings et al., 2013; Jeon et al., 2009; Lyttle et al., 2008; Riedel et al., 2012). At 10 wpc, endocrine clustering starts, and at 12-13 wpc all endocrine cell types can be found in the developing islets (Jennings et al., 2013; Meier et al., 2010). Notably, the morphology of human islets changes during development: at 14 wpc, β-cells are found in the core and α-cells at the periphery, as has been reported in mouse and in small human islets, but by 21 wpc both cell types are intermingled within the islets (Jeon et al., 2009). This alteration in islet architecture, which has not been reported in mice, might be required for human endocrine cells to achieve their final mature functional state.

Table 1. Key transcription factors involved in the formation of pancreatic lineages

Developmental stage/ cell lineage	Transcription factor
Pancreas induction	Pdx1 (Mody4, STF-1), Ptf1a (bHLHa29, PTF1-p48), Sox9
MPCs, E9.5-E11.5	Pdx1, Ptf1a, Sox9, Foxa1 (Hnf3α), Foxa2 (Hnf3β), Hnf6 (Onecut1), Nkx6.1, Nkx6.2, Gata4, Gata6, Hnf1β (Tcf2, vHNF1), Mnx1 (Hlxb9), Hes1 (bHLHb39, Hry), Prox1, Nr5a2 (Ftf, LRH-1, UF2-H3B), Nkx2.2, Pax6, Tead1 (Tcf13, TEF-1), Glis3
Tip MPCs, E12-13.5/14.5	Pdx1, Ptf1a, Sox9, Nkx6.1, Gata4, Hnf1β, Nr5a2, c-Myc
Bipotent trunk progenitors	Pdx1, Sox9, Hnf6, Nkx6.1, Nkx6.2, Hnf1β, Hes1, Glis3
Acinar cells	Ptf1a, Gata4, c-Myc, Nr5a2, Mist1 (Bhlha15), Rbpj
Centroacinar cells	Pdx1, Ptf1a, Sox9, Nkx6.1
Mature ductal cells	Sox9, Hnf1β, Hnf6, Hes1, Pax6, Glis3, Prox1, Gata6
Endocrine progenitors	Ngn3, Pdx1, Neurod1 (NeuroD), Isl1, Pax4, Arx, Pax6, Nkx6.1, Nkx2.2, Rfx3, Rfx6, Insm1, Glis3
β-cell differentiation and maturation	Pdx1, Nkx6.1, Neurod1, Ins1/2, Pax4, Pax6, Nkx2.2, MafA, Mnx1, Glis3, Isl1, Rfx3
α-cell differentiation and maturation	Arx, Pou3f4 (Brn4, Otf9, Oct-9), Pax6, Rfx6, Foxa2, MafB
δ-cell differentiation	Pdx1, Pax4, Hhex, Isl1
PP-cell differentiation	Arx, Rfx3, Isl1

For reviews, see Arda et al. (2013), Bramswig and Kaestner (2011), Cano et al. (2014), Dassaye et al. (2016), Gittes (2009), Jensen (2004), Larsen and Grapin-Botton (2017), Pan and Wright (2011) and Rieck et al. (2012).

Transcription factors involved in pancreas induction and multipotent progenitor formation

In mice, one of the first signs of pancreas development is the induction of *Pdx1* expression within the primitive gut tube at E8.5 (Burlison et al., 2008; Guz et al., 1995). Shortly after, at E9.0, pancreatic epithelium buds from the foregut endoderm. Next, *Ptf1a* expression is initiated in the pancreatic endoderm domain at E9.5 (Kawaguchi et al., 2002). The importance of these transcription factors (TFs) is reflected by pancreatic agenesis in mice with homozygous mutation for *Pdx1* or *Ptf1a*. However, initial buds are formed in these animals, indicating that we have still not identified the most upstream regulators of pancreatic bud initiation (Ahlgren et al., 1996; Jonsson et al., 1994; Marty-Santos and Cleaver, 2016; Offield et al., 1996). The absence of *Pdx1* also results in defects in gastro-duodenal development, as *Pdx1* is expressed broadly in the foregut endoderm at early stages of development and is not restricted to pancreatic buds. On the other hand, homozygous *Ptf1a* loss of function leads to ventral pancreatic agenesis and a conversion from pancreatic to duodenal fate, although a dorsal rudiment is formed (Kawaguchi et al., 2002). The expression of *Pdx1* and *Ptf1a* during these early stages of pancreas development is regulated by several TFs (Table 1), such as Sox9, Hnf1β and Foxa1/2 (Gao et al., 2008; Haumaitre et al., 2005; Lynn et al., 2007). However, how these factors activate *Pdx1* and *Ptf1a* expression remains unclear. Presumably, the cooperative functions of these TFs and other downstream mediators of common signaling cascades are required for *Pdx1* and *Ptf1a* expression. Indeed, binding sites for Sox9 and Hnf1β on putative *Ptf1a* promoter regions have been identified (Haumaitre et al., 2005; Lynn et al., 2007). Sox9 also controls the expression of *Hnf1b*, *Hnf6* (*Onecut1*) and *Foxa2*, indicating a crucial role for this TF in establishing a gene regulatory network required for MPC formation and maintenance (Lynn et al., 2007). Furthermore, together with Pdx1, Sox9 regulates lineage progression towards the pancreatic fate (Shih et al., 2015a).

From E9.5 to E12.5, pancreatic cells co-express *Ptf1a* and *Pdx1* (Burlison et al., 2008). These proto-differentiated MPCs generate all types of pancreatic cells and their number defines the final size of the adult pancreas (Stanger et al., 2007). One of the key factors regulating MPC numbers during early pancreas development is Notch signaling. Indeed, mutations in Notch signaling components such as delta-like 1 (*Dll1*), *Rbpj* and *Hes1* reduce MPC expansion or accelerate premature endocrine differentiation and ultimately decrease organ size (Ahnfelt-Ronne et al., 2012; Fujikura et al., 2006; Hart et al., 2003; Jensen et al., 2000). Pdx1 also plays a crucial role in the expansion and maintenance of MPCs, as mice null for this protein exhibit pancreatic agenesis (Ahlgren et al., 1996; Hale et al., 2005; Offield et al., 1996). MPCs also express other TFs, including *Hnf1b*, *Mnx1* and *Sox9*, that enable them to retain their multipotency (Table 1).

Signaling pathways regulating early pancreas development

The induction and growth of the early pancreas rely on a number of signaling pathways (Table 2). Many of these signals are derived from the notochord, aortic endothelium and the surrounding mesenchyme. For example, prior to pancreatic budding, the prospective dorsal endoderm is in close proximity to the notochord (Wessells and Cohen, 1967), which releases permissive morphogenic signals, including activin-βB and FGF2, to induce

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Table 2. The main signaling pathways coordinating pancreas development

Developmental stage	Inductive factor	Inhibitory factor
Dorsal bud induction	RA, Activin, FGF, VEGF	Shh
Ventral bud induction		FGF, BMP, Notch
MPC maintenance and proliferation	FGF10, Notch, Ihh, Wnt/β-catenin, Hippo	
MPC differentiation	Gdf11	Notch
Branching morphogenesis	EGF-ErbB, FGF10, Ephrin B2/3, ECM-integrin signaling	Hh
Endocrine progenitors		FGF, Notch
Endocrine specification	TGFβ, EGF, Wnt/β-catenin, Wnt/PCP, VEGF, S1p	Notch

BMP, bone morphogenetic protein; ECM, extracellular matrix; EGF, epidermal growth factor; FGF, fibroblast growth factor; PCP, planar cell polarity; RA, retinoic acid; VEGF, vascular endothelial growth factor. For reviews see Gittes (2009), McCracken and Wells (2012), Pan and Wright (2011) and Serup (2012).

pancreas formation (Kim et al., 2000; Kumar et al., 2003; Martín et al., 2005). These signals mainly repress sonic hedgehog (Shh) signaling in the dorsal pancreatic epithelium to activate pancreatic gene expression and hence allow proper pancreas induction (Hebrok et al., 1998; Xuan and Sussel, 2016). Signaling through activin receptor type A and B also inhibits Shh activity and, in line with this, mutations in activin receptors increase Shh expression and impair pancreas formation (Hebrok et al., 1998; Kim et al., 2000).

Development of the early pancreas also relies on active crosstalk between the pancreatic epithelium and the surrounding mesenchyme (Golosow and Grobstein, 1962). The mesenchyme produces factors such as FGF10, BMPs and follistatin that induce pancreas development (Bhushan et al., 2001; Miralles et al., 1998; Norgaard et al., 2003). Signaling via FGF10 and its receptor, FGFR2b, induces pancreatic progenitor proliferation and growth of the pancreatic buds by maintaining and enhancing *Pdx1* and *Ptf1a* expression (Bhushan et al., 2001). In addition, during bud formation Sox9, FGFR2 and FGF10 establish a feed-forward loop in which FGF10 maintains epithelial *Sox9* expression and Sox9 regulates the expression of *Fgfr2* to allow FGF10 signaling; disruption of this loop results in loss of cell identity and a switch towards a liver cell fate (Seymour et al., 2012).

Another prominent and well-studied extrinsic signaling pathway regulating MPC maintenance is the Notch/Delta signaling pathway (Hald et al., 2003; Murtaugh et al., 2003). The expression of Notch ligands, such as *Dll1*, starts at E9.0 and continues throughout the primary transition in the pancreatic epithelium (Apelqvist et al., 1999), inducing *Hes1* expression in pancreatic buds to maintain and increase the progenitor pool. This is achieved not only by increasing the proliferation of MPCs but also by preserving the progenitor state and preventing differentiation towards an endocrine cell fate (Ahnfelt-Ronne et al., 2012; Lee et al., 2001). This function of Notch is important in assuring an appropriate number of pancreatic cells; *Hes1* deletion in the early stages of pancreas development results in the differentiation of progenitors into endocrine cells and induces pancreatic hypoplasia (Horn et al., 2012; Jensen et al., 2000).

Specification of the dorsal and ventral pancreas

The pancreas develops from two primordia – the dorsal and ventral pancreatic buds – that later fuse to form the adult pancreas. These structures are complex, stratified epithelia, consisting of non-polarized MPCs. The dorsal bud forms at E9.0 from the dorsal foregut endoderm, whereas the ventral bud appears ∼12 h later (Fig. 1A,B) from the caudal aspect of the hepatic/biliary evagination (Villasenor et al., 2010; reviewed by Gittes, 2009). Because of their distinct surrounding tissues, different signals induce dorsal and ventral pancreatic buds. For instance, signals secreted from the lateral plate mesoderm [e.g. retinoic acid (RA)] and notochord (e.g. FGF2 and activin) are required for dorsal bud induction. By contrast, FGF signaling from the cardiac mesoderm inhibits ventral pancreatic bud specification (Deutsch et al., 2001; Hebrok et al., 1998; Kim et al., 2000; Kumar et al., 2003; Martín et al., 2005). The subsequent development and morphogenesis of these buds also differ. Around E8.5-9.0, the dorsal gut tube is separated from the notochord (due to fusion of the paired dorsal aortae in the midline), placing the dorsal pancreatic endoderm in immediate proximity to the aorta. The endothelium of the aorta provides inductive signals to the dorsal endoderm, inducing the formation of a pancreatic bud expressing *Pdx1* and *Ptf1a* (Lammert et al., 2001). By contrast, the ventral pancreatic endoderm is in close contact with the prospective hepatic bud, lateral plate mesoderm, the septum transversum mesenchyme and cardiac mesoderm (Kumar et al., 2003; reviewed by Kumar and Melton, 2003). In fact, the ventral pancreas, liver and extrahepatic biliary system all develop from the posterior segment of the ventral foregut endoderm and therefore might be derived from a common progenitor cell (Deutsch et al., 2001; Spence et al., 2009). The signaling pathways inducing ventral pancreas formation are unclear. BMP and FGF have been shown to segregate the hepatic and pancreatic lineages by inducing liver formation at the expense of a ventral pancreatic fate (Chung et al., 2008; Deutsch et al., 2001; Spence et al., 2009; Tremblay and Zaret, 2005). Furthermore, unlike the dorsal pancreas, the development of the ventral pancreas is not dependent on cues from the endothelium (Yoshitomi, 2004). However, the function of Pdx1 and Ptf1a together with Hnf1β is essential for the formation of the ventral pancreas (Haumaitre et al., 2005; Kawaguchi et al., 2002; Offield et al., 1996).

The dorsal pancreatic bud eventually gives rise to the head, neck, body and tail regions of the adult pancreas, whereas the ventral bud generates the posterior part of the head region (Uchida et al., 1999). Importantly, these regions appear to display structural, and hence potentially functional, differences. For example, a higher islet density is found in the tail part compared with the body and head area (Wittingen and Frey, 1974). Furthermore, endocrine composition, vascularization, innervation and the expression of several xenobiotic-metabolizing enzymes differ between islets in the head and tail domains (Brissova et al., 2005; Standop et al., 2002; Stefan et al., 1982). A more recent study has also shown higher percentages of mature β-cells in the tail compared with the head part (Bader et al., 2016). Although the importance of these variations is currently unclear, they might result from the contribution of different pancreatic bud progenitors. Given that type 2 diabetes patients exhibit preferential loss of larger islets located in the head region, and that distinct types of pancreatic ductal adenocarcinoma can arise from head versus tail regions (Ling et al., 2013; Wang et al., 2013), understanding these developmental differences might be important for unravelling the pathogenesis of diabetes and cancer.

Early pancreas morphogenesis

After the pancreatic buds have formed, their morphogenesis generates a highly branched, tubular epithelial tree-like network. This highly coordinated process involves epithelial stratification, cell polarization, microlumen formation and fusion, eventually giving rise to a luminal plexus. Later, the plexus is remodeled into a complex tubular network.

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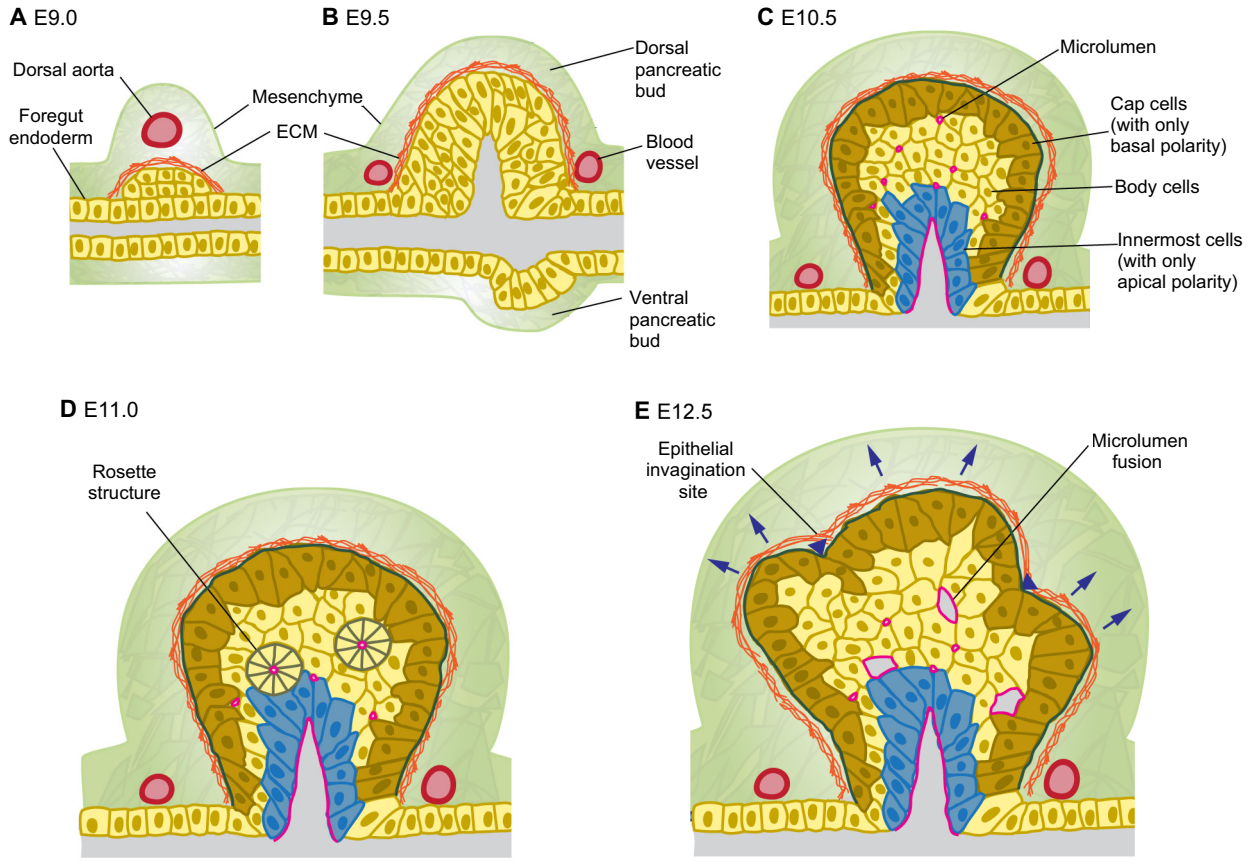

Fig. 1. The early stages of pancreas development. (A) Evagination of the foregut endoderm drives the formation of the dorsal pancreatic bud. (B) While the dorsal pancreatic bud expands due to multipotent progenitor cell (MPC) proliferation, the ventral pancreatic bud emerges from the ventral foregut endoderm. (C) Segregation of the pancreatic buds gives rise to innermost cells that exhibit only apical polarity, inner 'body' cells that are non-polarized, and outer 'cap' cells that exhibit only basal polarity. For simplicity, only the dorsal bud is shown. (D) The establishment of cell polarity in individual body cells produces microlumen and rosette structures. (E) Microlumen fusion and tubulogenesis coincide with the initiation of branching morphogenesis, which involves epithelial invagination (blue arrowhead) and outgrowth (blue arrows).

Epithelial stratification

Prior to pancreatic branching, dorsal and ventral pancreatic MPCs form a multilayered epithelium (E9.0 to E11.5). This stratified epithelium consists of two domains: an outer layer of 'cap' cells and an inner 'body' of cells (Shih et al., 2015b; Villasenor et al., 2010). The highly pleomorphic and motile cap cells are semi-polarized and express only basal markers, whereas the inner body cells are mainly non-polarized stratified cells but with a layer of innermost cells that exhibit only apical polarity (Shih et al., 2015b; Villasenor et al., 2010) (Fig. 1C). The segregation of these domains is regulated by cues from the basement membrane through the remodeling of actomyosin contractility. Indeed, disruption of extracellular matrix (ECM)-integrin signaling retains cap cells in a 'body cell state' by stabilizing E-cadherin-mediated cell adhesion (Shih et al., 2015b). However, because frequent cell movement between cap and body cells is observed, it is not clear whether each domain later differentiates into a distinct pancreatic lineage. In addition, the stratification process is accompanied by expansion of the MPC pool, suggesting that transient stratification of the epithelium might be crucial for establishing a progenitor pool that assures adequate numbers of mature pancreatic cells (Stanger et al., 2007; Villasenor et al., 2010).

Polarity establishment and microlumen formation

Individual cells within the inner body of pancreatic buds soon reacquire polarity and rearrange to form microlumina at ∼E10.5-11.5. During this process (Fig. 1D,E), the asynchronous apical constriction of individual polarized cells generates rosette structures with a central lumen that later coalesce to form tubules (Kesavan et al., 2009; Villasenor et al., 2010). Whether lumen formation in the pancreas follows the same principles as in other epithelial organs is unknown, although several common regulators have been identified. For example, an elegant study uncovered a role for the Rho GTPase cell division cycle 42 (Cdc42) as an integral player in establishing polarity during microlumen formation in pancreatic buds (Kesavan et al., 2009). Similarly, it has been shown that in the absence of the Rho GTPase-activating protein (GAP) Stard13, pancreatic epithelial cells fail to form microlumina due to impaired cytoskeleton organization and downregulated extracellular signal-regulated kinase (ERK) signaling (Petzold et al., 2013). By contrast, deletion of *Rac1* does not impact lumen formation in the pancreas (Heid et al., 2011), indicating specific roles for distinct Rho GTPases during this process. Factors that lie downstream of Pdx1 also regulate lumen formation and pancreas morphogenesis; besides blocking lineage differentiation, the deletion of this TF impairs the expansion and fusion of microlumina to form a large lumen, as well as tubulogenesis, possibly through alterations in E-cadherin-mediated cell adhesion and myosin activity (Marty-Santos and Cleaver, 2016). In addition, impaired endocrine lineage formation is observed upon defective tubulogenesis in a *Cdc42* conditional knockout pancreas (Kesavan et al., 2009), further highlighting the intimate link between pancreatic morphogenesis and differentiation.

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Plexus remodeling and branching morphogenesis

After microlumina are formed, they expand and fuse to generate continuous luminal networks, eventually forming an epithelial plexus that is remodeled into a highly branched, ramified ductal epithelium, producing all types of mature pancreatic cells (Kesavan et al., 2009; Villasenor et al., 2010). During this process, the pancreatic epithelium consists of a core region in which plexus expansion (E12.5-15.5) and plexus-to-duct transformation (E16.5-18.5) occur, and a peripheral region in which the epithelium is remodeled into branches (Fig. 2). The plexus serves as a niche, maintaining and harboring endocrine progenitors during epithelial growth and morphogenesis. Pancreatic progenitor expansion seems to be driven by a local feedback circuit in which cells expressing neurogenin 3 (Ngn3, or Neurog3) suppress endocrine progenitor differentiation in a Notch-dependent manner and thus increase the progenitor pool (Magenheim et al., 2011a). Remarkably, endocrine progenitor differentiation and expansion decline during the plexus-to-duct transformation (Bankaitis et al., 2015).

How branching morphogenesis is coordinated during pancreas development is not fully understood. In salivary glands, the branching process occurs via the formation of shallow clefts (reviewed by Harunaga et al., 2011), but it appears that newly formed branches in the pancreas arise mainly on the lateral sides of the growing epithelium (Puri and Hebrok, 2007; Shih et al., 2015b), although it should be noted that the short length of branches in the pancreas makes it difficult to discern lateral branches from clefts. In this context, branching ensues through the appearance of small invaginations in the epithelium followed by collective expansion and outgrowth of the domain between two invagination spots (Fig. 1E). By contrast, a separate study has proposed that epithelial branches are produced by longitudinal growth of remodeled epithelium, whereby 'tip' domains divide to generate new 'tips' (Villasenor et al., 2010). Based on this model, expansion of MPCs located at the 'tip' structures leads to growth of the epithelium by longitudinal extension. Further quantitative live-cell imaging studies, in particular over a longer time period, are thus needed to better understand the mechanisms underlying pancreas branching.

The molecular factors that regulate pancreatic branching are also unclear. A key cellular process involved in epithelial rearrangement is the remodeling of cell-cell adhesion, and this is partially regulated by EphB signaling during pancreatic microlumen formation and ductal morphogenesis (Villasenor et al., 2010). The deletion of *Stard13* or *p120-catenin* (*Ctnnd1*) also causes aberrant tubulogenesis and branching due to the stabilization of epithelial adherens junctions (Hendley et al., 2015), highlighting key roles for these factors in the developing pancreas. TFs such as Hnf1β and Nr5a2 also coordinate branching morphogenesis of the pancreatic epithelium through expansion of the MPC pool (De Vas et al., 2015; Hale et al., 2014). In addition, the mesenchyme and its secreted factors, such as FGF10, promote progenitor proliferation and thereby influence branching morphogenesis (Bhushan et al., 2001). The proliferative effect of FGF10 in this context is mediated by Notch signaling, which acts to reduce the expression of a key cyclin-dependent kinase inhibitor, P57 (Cdkn1c) (Georgia et al., 2006). Therefore, early FGF10-induced MPC proliferation might positively impact the branching process, although it should be noted that this factor is expressed in a restricted area in the mesenchyme at E11.5, when the branching starts, and is no longer expressed thereafter (Bhushan et al., 2001).

Tip and trunk patterning

From E11.5 onwards, the mouse pancreatic epithelium consists of MPCs that progressively segregate into tip or trunk domains and are allocated to acinar or bipotent endocrine/duct progenitor cell fates, respectively (Fig. 2A). Whereas cells in the tip domain express *Ptf1a* and *Nr5a2*, trunk cells express *Sox9*, *Nkx6.1*, *Hnf1b* and *Pdx1* (Solar et al., 2009; Zhou et al., 2007) (Table 1). The tip and trunk domains are enclosed by a basement membrane consisting mainly of laminin-1, collagen IV and fibronectin that separates the epithelium from the surrounding mesenchyme (Hisaoka et al., 1993). The reciprocally suppressive functions of Nkx6.1 and Ptf1a control how progenitor cells are committed and segregated into tip or trunk domains: the expression of Nkx6.1 induces trunk formation by repressing tip fate, while the activity of Ptf1a favors tip domain generation by blocking the trunk program (Schaffer et al., 2010).

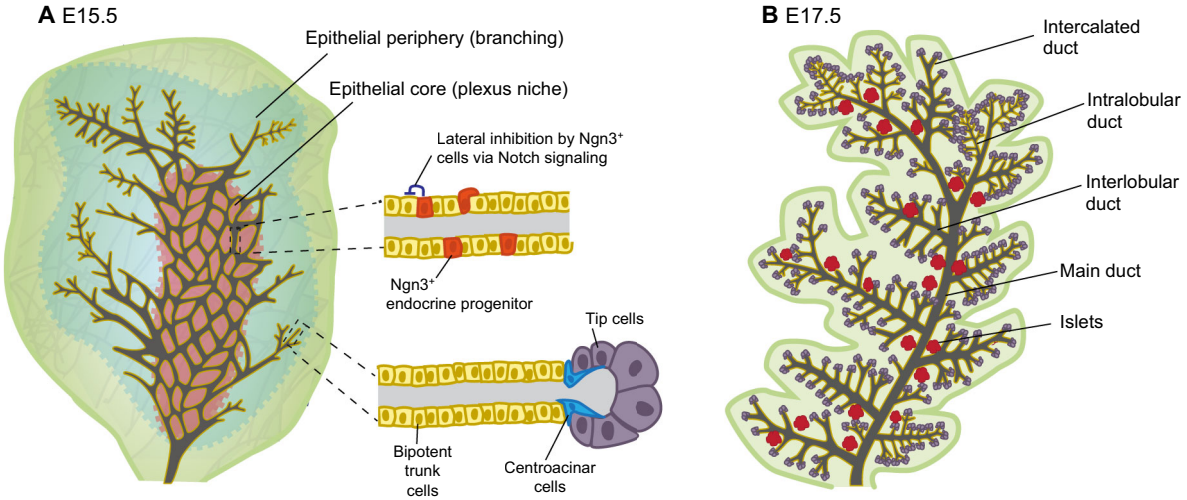

Fig. 2. Branching morphogenesis during pancreas development. (A) At E15.5, the pancreatic epithelium consists of a central plexus (red) that serves as a niche, maintaining and harboring endocrine progenitors. Within this niche, *Ngn3*-expressing progenitors prohibit the differentiation of neighboring cells through lateral inhibition in a Notch-dependent manner and thereby allow epithelial expansion. The central plexus is surrounded by an epithelial periphery (blue) in which the epithelium remodels into epithelial branches, which contain bipotent trunk cells, centroacinar cells and tip cells. (B) Remodeling of the plexus gives rise (by ~E17.5) to the formation of the main duct, which branches into interlobular ducts that are connected to acini via intralobular and intercalated ducts. At this stage, the remodeled plexus also contains developing islets.

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Signaling pathways, such as the Notch pathway, also regulate tip-trunk patterning. For instance, Notch activity in progenitors induces trunk formation via the activation of *Nkx6.1* and blocks tip fate through the repression of *Ptf1a* (Afelik et al., 2012; Esni, 2004). The function of the E3 ubiquitin ligase mind bomb 1 (Mib1) in activating Notch ligand is also essential for tip-trunk patterning. The endoderm-specific deletion of this protein results in loss of bipotent ductal progenitors concomitant with an increase in Ptf1a^{+} tip cells, highlighting a crucial role for Notch signaling in trunk cell formation (Horn et al., 2012). In addition, the mesenchyme and endothelium have opposite effects on tip-trunk patterning. Whereas mesenchymal factors and ECM components increase acinar/tip formation, the interconnection between epithelium and endothelial cells favors trunk development (Magenheim et al., 2011a; reviewed by Cleveland et al., 2012). Furthermore, by promoting expression of high levels of the angiogenesis factor *Vegfa*, the trunk domain increases the density of adjacent blood vessels, which in turn prevents differentiation into tip cells (Magenheim et al., 2011b; Pierreux et al., 2010), although the endothelium-derived signals that coordinate this process remain unidentified. Together, these findings illustrate that neighboring cell interactions as well as cell-cell and cell-matrix interactions contribute to patterning and differentiation of the pancreatic epithelium.

The formation of exocrine pancreatic cell types

The exocrine compartment of the pancreas, which makes up more than 95% of the organ, comprises acinar and ductal cells. Its development commences during epithelial remodeling and branching morphogenesis, at ∼E11.5-12.5 (reviewed by Marty-Santos and Cleaver, 2015). The presence of the mesenchyme is essential for exocrine differentiation due to its production and secretion of pro-exocrine factors such as the TGFβ antagonist follistatin, which induces exocrine differentiation but represses endocrine cell formation (Miralles et al., 1998). Canonical Wnt signaling also regulates exocrine cell number, as indicated by the impaired exocrine expansion observed when Wnt pathway components are conditionally knocked out (Baumgartner et al., 2014; Wells et al., 2007). Finally, ECM components such as laminin-1 exhibit pro-exocrine (ducts and acini) activity through α6-containing integrin receptor in 3D embryonic pancreatic explant cultures (Crisera et al., 2000).

Acinar differentiation

Adult acini consist of pyramid-shaped exocrine cells (Fig. 3) that express *Ptf1a*, *Gata4*, *Mist1* (*Bhlha15*) and *Nr5a2* (Table 1). These cells contain an abundance of rough endoplasmic reticulum and secretory granules carrying digestive enzymes, such as amylase, lipases, ribonucleases and phospholipases (reviewed by Cleveland et al., 2012), which are essential for nutrient digestion. The specification, differentiation and maintenance of acini is regulated mainly by Ptf1a; mice lacking this TF have no acinar cells (Krapp et al., 1998). Acting via a PTF1 complex, which contains Tcf3 (E47), Tcf12 (HEB), Ptf1a and Rbpj or Rbpjl, Ptf1a indirectly represses the expression of *Nkx6.1* to block trunk fate (Schaffer et al., 2010). After differentiation, acinar cells express *Rbpjl* and *Mist1* to gain their mature phenotype. A switch from Rbpj to Rbpjl within the PTF1 complex is then essential for the final step of acinar cell maturation (Masui et al., 2010). The activity of this complex in driving acinar gene expression is also enhanced by Nr5a2, which independently induces the expression of acinar-specific genes including *Ptf1a*, *Rbpjl* and those encoding digestive enzymes (Hale et al., 2014). Another regulator of acinar cell differentiation and maturation is Mist1 (Direnzo et al., 2012). This protein is not only important to allow acinar cells to exit the cell cycle,

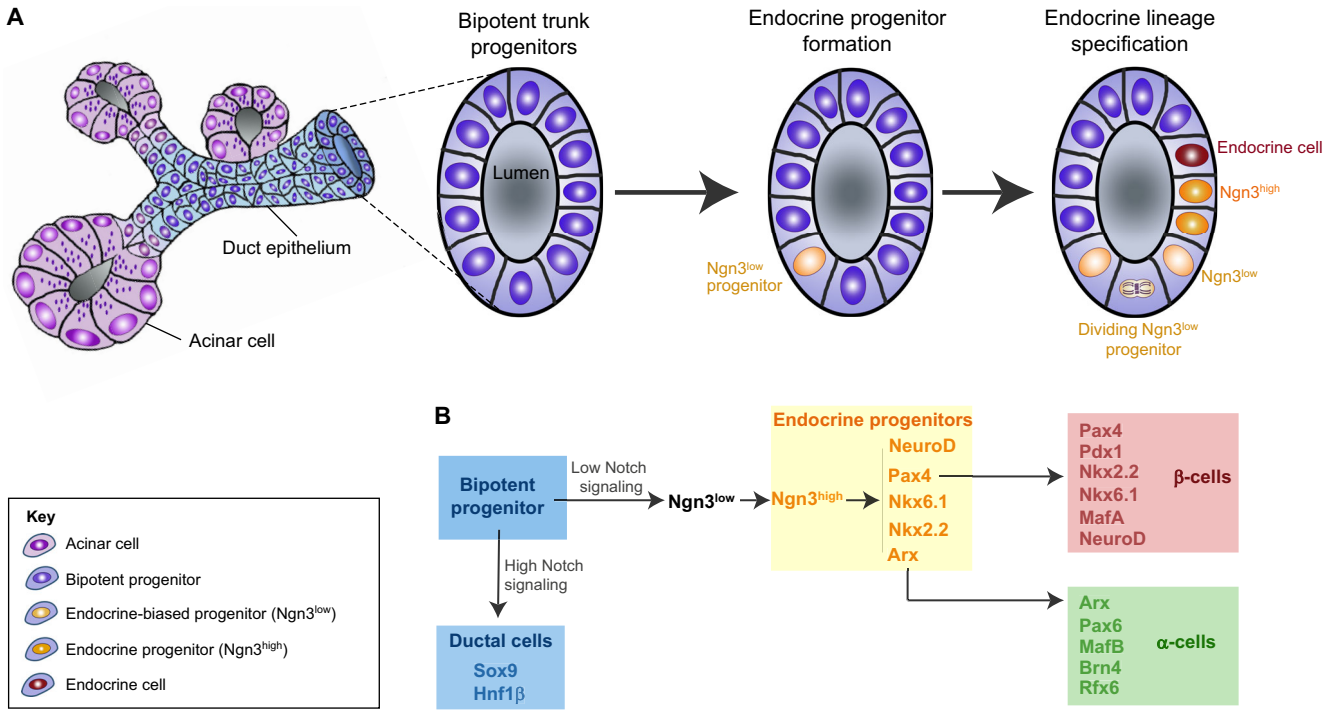

Fig. 3. Endocrine lineage formation during pancreas development. (A) Endocrine-biased progenitors (Ngn3^{low}) emerge from bipotent progenitors within the trunk epithelium between E12.5 and E16.5. These Ngn3^{low} cells give rise to post-mitotic endocrine progenitors expressing high levels of Ngn3 (Ngn3^{high}) that are committed to all endocrine cell types. (B) High or low levels of Notch signaling promote the specification of epithelial cells towards bipotent ductal cells or endocrine progenitors, respectively. The expression of different transcription factors, under the control of Ngn3, then defines the differentiation of distinct endocrine cell types.

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but is also essential for allowing them to acquire terminal differentiation and secretory function (Jia et al., 2008).

The specification of acinar cells is regulated by different signals such as Notch signaling, which competes with Ptf1a for access to Rbpj and hence blocks the expression of acinar-specific genes (Beres et al., 2006). By contrast, β-catenin inactivation in pancreatic progenitors diminishes acinar formation through reduction of *Ptf1a* expression (Murtaugh et al., 2005; Wells et al., 2007), suggesting that canonical Wnt/β-catenin signaling induces acinar cell development. Furthermore, mesenchyme and its secreted factors such as follistatin display pro-acinar activity, as the culture of embryonic pancreatic explants in the presence of mesenchyme promotes acinar cell differentiation at the expense of endocrine cells (Duvillié et al., 2006; Gittes et al., 1996; Miralles et al., 1998).

Centroacinar cell formation

Pancreatic acini are connected to ducts through centroacinar cells (CACs)/terminal duct cells (Fig. 2A) (reviewed by Beer et al., 2016) that are derived from multipotent progenitors expressing *Pdx1*, *Sox9*, *Ptf1a* and *Nkx6.1* (Kopp et al., 2011a; Schaffer et al., 2010; Solar et al., 2009). CACs exhibit high levels of aldehyde dehydrogenase 1 (ALDH1) activity and contain a low cytoplasm-to-nucleus ratio (Rovira et al., 2010). In the adult pancreas, CACs have been suggested to function as progenitor-like cells, with sustained Notch signaling being required for maintenance of their identity. Indeed, perturbing Notch activity in CACs rapidly transforms them into acinar cells (Kopinke et al., 2012). In zebrafish, CACs are enriched for progenitor markers and can generate endocrine cells upon partial pancreatectomy or β-cell ablation, which is likely to be due to the high regenerative capacity of this model organism (Delaspre et al., 2015). Whether such regenerative potential of CACs exists in mammals, and whether these cells can give rise to endocrine cells, remains controversial. While lineage-tracing experiments have failed to show the differentiation of endocrine cells from CACs in adult mice (Kopinke et al., 2011; Solar et al., 2009), *in vitro* studies have revealed a progenitor-like state of these cells (Rovira et al., 2010), suggesting potential differentiation towards both endocrine and exocrine fates in the adult pancreas.

Formation of the ductal epithelium and bipotent cells

The trunk domain of the embryonic pancreas consists of bipotent epithelial cells that express *Pdx1*, *Nkx6.1*, *Sox9* and *Hnf1b*. During development, these cells are able to differentiate into either *Sox9*/*Hnf1b*-expressing ductal exocrine cells or Ngn3/Pdx1-positive endocrine cells (Fig. 3). One of the important players in the trunk domain is Sox9, which induces the activity of Ngn3 and Hes1 (reviewed by Seymour, 2014). Whereas Ngn3 blocks Sox9 expression, Hes1 inhibits Ngn3, highlighting the tight regulation of cell fate decisions within the trunk epithelium (Lee et al., 2001; Shih et al., 2012). The main determinant favoring a ductal fate is Notch signaling, which promotes ductal cell development and inhibits acinar and endocrine cell differentiation (Hald et al., 2003; Murtaugh and Melton, 2003). Therefore, to maintain ductal cell identity, trunk cells require continuous Notch signaling. It has been suggested that upon commitment, Ngn3-expressing cells produce Notch ligands to activate the pathway in adjacent cells and prevent further endocrine differentiation (Qu et al., 2013; reviewed by Afelik and Jensen, 2013). This so-called 'lateral inhibition' is required to maintain the balance between endocrine and epithelial cells.

Mature ductal epithelium formation

The main duct of the adult pancreas is connected to the bile duct and duodenum (Fig. 2B). The main duct branches into the pancreatic lobes via the interlobular ducts that are connected to acinar cells through intralobular and intercalated ducts (reviewed by Reichert and Rustgi, 2011) (Fig. 2B). The cells within these various ducts express specific markers, including carbonic anhydrase II, mucin 1 and CD133 (PROM1). Besides transporting pancreatic zymogens, ductal cells also secrete bicarbonate for neutralizing gastric acid in the duodenal lumen (reviewed by Reichert and Rustgi, 2011). In comparison to embryonic stages, *Pdx1* and *Nkx6.1* are not expressed in mature ductal cells, although these cells still express *Sox9*, *Hes1*, *Hnf1b*, *Hnf6*, *Glis3* and *Prox1*. Of these, Hnf6 is required for maturation of the ductal epithelium (Pierreux et al., 2006). Whether other TFs also regulate ductal cell maturation remains to be shown. Furthermore, while endocrine cells can emerge from progenitors located within the ductal epithelium during development (see below), whether the mature duct is still able to produce endocrine cells remains a matter of debate (see Box 2).

The formation of pancreatic endocrine cells

During the secondary transition stage, between E12.5 and E15.5, endocrine progenitors are derived from the bipotent trunk epithelium (Solar et al., 2009). These progenitors transiently express Ngn3 and give rise to all endocrine cell types (Gradwohl et al., 2000; Gu et al., 2002). As we discuss below, coordination between extrinsic signals and intrinsic gene regulatory networks orchestrates the formation of mature hormone-producing cells.

Endocrine progenitor cell formation

The master regulator of endocrine cell formation is Ngn3 (Gu et al., 2002). This protein is transiently expressed in $Sox9^{+}$ bipotent progenitors and induces several endocrine-specific genes, such as *NeuroD* (*Neurod1*), *Insm1*, *Irx1/2*, *Rfx6*, *Pax4* and *Nkx2.2* (Petri et al., 2006). The first $Ngn3^{+}$ cells appear at ∼E9.0, with their numbers rising during the secondary transition stage and then declining at E17.5 (Villasenor et al., 2008). Mice lacking *Ngn3* have no endocrine cells in the pancreas and die shortly after birth (Gradwohl et al., 2000). By contrast, the ectopic expression of *Ngn3*, or its downstream target *NeuroD* under the control of the *Pdx1* promoter, results in the formation of hormone-producing cells (Schwitzgebel et al., 2000). Notch signaling is the main negative regulator of *Ngn3* expression and function (Apelqvist et al., 1999; Jensen et al., 2000; Lee et al., 2001). High levels of Notch signaling induce Hes1 activity that not only blocks the expression and activation of Ngn3, but also enhances its degradation (Qu et al., 2013). On the other hand, low levels of Notch signaling induce *Sox9* expression, which further activates Ngn3 and drives endocrine differentiation (Shih et al., 2012). This highlights a dose-dependent function of Notch signaling in endocrine cell formation.

The increase in Ngn3 levels (i.e. in $Ngn3^{high}$ cells) triggers endocrine commitment, cell cycle exit and delamination from the epithelium (Wang et al., 2010). However, not much is known about the cells expressing low levels of this protein (i.e. the $Ngn3^{low}$ cells). Recently, using a new transgenic reporter mouse model, a population of mitotic $Sox9^{+}$ cells that are transcriptionally active for *Ngn3* has been identified within the plexus state epithelium (Bechard et al., 2016). These endocrine-biased progenitors are different from pro-ductal progenitors, and they undergo symmetric cell divisions to either maintain the self-renewing progenitor pool or produce two differentiated endocrine cells (Fig. 3). Whether lineage commitment towards a specific endocrine cell type already occurs in these progenitors needs further exploration.

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Box 2. Cell plasticity during pancreas development and regeneration

The developing pancreas contains populations of multipotent progenitors that, during the course of normal development, can give rise to multiple pancreatic cell types. However, it is not yet clear if this potential exists in the adult pancreas. For example, whether the mature pancreatic duct is still able to produce endocrine cells in adults remains controversial. Such plasticity could be due to either the existence of a progenitor pool within the ductal epithelium or the ability of quiescent ductal cells to regain a progenitor state. However, under homeostatic conditions, no progenitor markers have yet been found in adult pancreatic ducts. In addition, several studies have obtained contradictory results regarding the formation of endocrine cells from mature ductal epithelium in different models of injury (Bonner-Weir et al., 1993, 2004; Inada et al., 2008; Kopinke and Murtaugh, 2010; Kopinke et al., 2011; Kopp et al., 2011a,b; Li et al., 2010; Solar et al., 2009; Xu et al., 2008). This discrepancy might be due to the different lineage-tracing approaches used to label rare progenitor cells within the ductal epithelium. In adult mice, forced conversion of α-cells to β-cells induces glucagon deficiency, which provokes α-cell neogenesis from the ductal epithelium via an Ngn3-expressing state (Al-Hasani et al., 2013; Collombat et al., 2009). The formation of β-like cells from terminally differentiated acinar cells upon transient cytokine treatment in diabetic mice has also been reported (Baeyens et al., 2013). Moreover, several studies have succeeded in differentiating adult ductal cells towards endocrine cells *in vitro* (Bonfanti et al., 2015; Bonner-Weir et al., 2000; Rovira et al., 2010; Valdez et al., 2016), although the characterization of these cells was mainly based on immunostaining analysis, which might generate false-positive results and would need further testing. While these findings support the generation of endocrine cells from the mature ductal epithelium, the extent to which this event contributes to islet regeneration is not well understood. Furthermore, whether ductal-derived islet neogenesis contributes to β-cell regeneration in humans needs further investigation.

Plasticity between endocrine fates has also been observed. Notably, α-cells can successfully be converted to β-cells in adult mice *in vivo* by changing the Pax4-Arx transcriptional network (Courtney et al., 2013). More recently, γ-aminobutyric acid (GABA) and $GABA_A$ receptor signaling have been discovered to promote the transdifferentiation of α-cells to β-cells in adult mice through downregulation and inactivation of *Arx*, respectively (Ben-Othman et al., 2017; Li et al., 2017). Moreover, the conversion of α-cells to β-cells due to loss of *ARX* and DNA methyltransferase 1 (*DNMT1*) has been reported in human type 1 diabetes islets (Chakravarthy et al., 2017). These findings suggest that forced α-cell to β-cell transdifferentiation might be an effective approach to regenerate β-cells in type 1 diabetes patients, where α-cells are still present.

How the epithelium assures the correct balance between self-renewing endocrine progenitors and differentiated cells is also not understood, although a recent study has shed some light on this issue (Kim et al., 2015). In this study, which used *in vivo* clonal analysis and 3D time-lapse imaging of pancreatic explants, three types of pancreatic bipotent progenitor cell divisions were identified: symmetric self-renewing divisions that produce two pancreatic progenitors; symmetric divisions that produce two endocrine cells; and asymmetric divisions that produce one pancreatic progenitor and one endocrine cell (Kim et al., 2015). It was proposed that the timing of Ngn3 induction during the cell cycle defines progenitor differentiation in a stochastic process. In this model, if the induction happens at a late stage of the cell cycle, cells proceed with the division and form two endocrine progenitors. However, when induced at an early stage, cells exit the cell cycle and produce progenitors. Therefore, the production of one progenitor and one endocrine cell in this context results from the stochastic induction of differentiation in one of these daughter cells after cell division. This finding might exclude the existence of classical asymmetric cell division, resulting from the unequal distribution of cell fate determinants during cell division, in pancreatic progenitors. The contribution of $Ngn3^{low}$ cells to the epithelial-endocrine balance in this model, however, remains ambiguous. Furthermore, while cells in the $Ngn3^{high}$ state are unipotent, it is not clear when they become specified towards distinct endocrine subtypes, although the timing of Ngn3 induction might play a role in this process. For example, it has been shown that Ngn3 induction at early stages of pancreas development generates mainly α-cells, whereas induction at E11.5 or E14.5 favors the formation of β/PP-cells or δ-cells, respectively (Johansson et al., 2007). This finding, however, does not exclude the possible involvement of extrinsic signals in priming specific endocrine subtypes, and requires further investigation.

Signaling pathways regulating endocrine cell differentiation

Once cells are committed to become endocrine progenitors, they differentiate into specific endocrine cell types. These differentiation steps involve the expression of different pro-endocrine and endocrine-specific TFs that are directly regulated by Ngn3, as well as extrinsic signaling cues such as those derived from the surrounding mesenchyme (Table 2). FGF10, for instance, triggers Notch signaling to negatively regulate endocrine cell differentiation (Hart et al., 2003). Interestingly, mesenchyme-derived FGF10 triggers the expansion of $Pdx1^+$ progenitor cells, which was found to increase final β-cell number in a 7-day *ex vivo* differentiation protocol (Attali et al., 2007). The canonical Wnt pathway also affects endocrine cell development: deletion of β-catenin in pancreatic progenitors reduces β-cell number, and this is due to a decrease in early MPC expansion rather than defects in β-cell differentiation (Baumgartner et al., 2014). More recently, it was shown that mesenchyme-derived Wnt signaling has a regulatory function in endocrine differentiation (Larsen et al., 2015). Specifically, the deletion of *Hox6* genes (*Hoxa6*, *Hoxb6* and *Hoxc6*) in the mesenchyme was shown to reduce *Wnt5a* expression, ultimately preventing further differentiation of $Ngn3^+$ cells into endocrine cells. Wnt5a is known to activate the planer cell polarity (PCP) pathway, which has been shown to regulate endocrine cell differentiation. Indeed, mice null for the core PCP surface receptors *Celsr2* and *Celsr3* exhibit a reduction in differentiated endocrine cells, despite normal $Ngn3^+$ progenitor induction, and this effect is mediated through the c-Jun N-terminal kinase (JNK, or MAPK8) pathway (Cortijo et al., 2012). Recently, the Wnt/PCP pathway has also been shown to be important for pancreatic β-cell maturation in mouse and human (Bader et al., 2016), although how Wnt/PCP controls endocrine cell formation and maturation still needs to be explored further. Another recently described pathway regulating endocrine lineage specification is sphinosine-1-phosphate (S1p) signaling. The activity of S1p through the G protein-coupled receptor (GPCR) S1pr2 stabilizes and attenuates yes-associated protein (YAP) and Notch signaling, respectively, which promote progenitor survival and endocrine cell specification (Serafimidis et al., 2017). Because YAP is negatively regulated by Hippo signaling during pancreas development (Gao et al., 2013; George et al., 2012), the crosstalk between the S1p and Hippo pathways during endocrine cell differentiation requires further clarification.

Gene regulatory networks underlying endocrine cell differentiation and maturation

Upon receiving autocrine and paracrine signals, endocrine progenitors proceed to differentiate via the stepwise activation of

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complex gene regulatory networks (reviewed by Arda et al., 2013; Jensen, 2004) (Table 1). During this time, several TFs that are initially co-expressed in Ngn3$^+$ cells progressively become restricted to specific endocrine cell types. The most prominent example of this is seen during the differentiation of α-cells versus β-cells. Two TFs – Arx and Pax4 – play a reciprocal counter-inhibitory role in this process. Both genes are targets of Ngn3 and are initially expressed in Ngn3$^+$ progenitors in a balanced fashion (Collombat et al., 2003). However, as differentiation proceeds, the expression balance between Arx and Pax4 tips towards one side. If cells express higher levels of *Arx* they become committed to the α-cell fate, whereas if progenitors express higher levels of *Pax4* then they differentiate into β/δ-cells (Collombat et al., 2005; Sosa-Pineda et al., 1997). Interestingly, Arx and Pax4 suppress the expression of each other (Collombat et al., 2003). As both genes are activated by Ngn3, an unknown signal or factor might coordinate the dominant expression of one over the other, although the nature of such an intrinsic or extrinsic determinant remains to be uncovered.

In addition to Arx, the differentiation of α-cells relies on other TFs such as Pax6, Rfx6, Brn4 (Pou3f4), Foxa2 and MafB (reviewed by Bramswig and Kaestner, 2011). On the other hand, Pdx1 and Nkx6.1 are essential for β-cell formation. During development, the expression of *Pdx1* becomes progressively restricted to β-cells and to a subset of δ-cells. One of the direct targets of Pdx1 is the insulin gene, indicating the crucial role of this TF not only in cell fate determination, but also in β-cell function (Ahlgren et al., 1998; Hani et al., 1999). The expression of *Nkx6.1* also becomes gradually limited to insulin$^+$ cells during development, and elimination of this factor leads to a significant reduction in β-cell mass (Nelson et al., 2007; Sander et al., 2000; Schisler et al., 2008; Tessem et al., 2014). Remarkably, the expression levels of both *Pdx1* and *Nkx6.1* increase as β-cells differentiate, suggesting a dose-dependent function for these TFs on their β-cell-specific targets. Another important TF involved in endocrine cell differentiation is Nkx2.2, which plays an essential role in the differentiation of α-, β- and PP-cells (Churchill et al., 2017; Sussel et al., 1998). In Ngn3$^+$ progenitors, Nkx2.2 represses and activates *NeuroD* to generate α-cells and β-cells, respectively (Mastracci et al., 2013). This contradictory function of Nkx2.2 in promoting or suppressing *NeuroD* expression might be due to cooperative function with other TFs and co-factors in α-cells and β-cells. In differentiated endocrine cells, Nkx2.2 is only expressed in β-cells, where it suppresses *Arx* expression and blocks the α-cell program by contributing to a repressor complex, thereby maintaining β-cell identity (Papizan et al., 2011).

Compared with α-cells and β-cells, less is known about the determination of other endocrine cell types. In the case of δ-cells, Pax4 might play a role: after induction of a β/δ-cell program, cells further differentiate into either β-cells or δ-cells, and the expression or loss of *Pax4* in these cells defines β-cell or δ-cell fate, respectively (reviewed by Napolitano et al., 2015). It is not clear whether the absence of Pax4 triggers default δ-cell differentiation or if another, as yet unidentified factor drives the formation of these cells. Another player involved in δ-cell development is hematopoietically expressed homeobox (Hhex), which has been identified as a specific regulator of δ-cell differentiation (Zhang et al., 2014). The mechanisms underlying the differentiation of PP-cells and ε-cells are largely unknown. Moreover, how the spatiotemporal regulation of these coordinated gene networks defines the final endocrine cell composition within the islets, and how much this depends on ventral or dorsal pancreatic fate, requires further investigation.

After differentiation, endocrine cells undergo maturation to acquire their glucose-responsive hormone-producing phenotype. Two main TFs essential for α/β-cell maturation are MafA and MafB. After differentiation, α/β-cells express MafB, which progressively becomes limited to α-cells, promoting their maturation and identity (Artner et al., 2007; Conrad et al., 2016). In β-cells, a switch from MafB to MafA is crucial for maturation (Nishimura et al., 2006). *Mafa* is under the regulation of β-cell-specific TFs, such as NeuroD, Nkx6.1, Nkx2.2, Foxa2, Rfx6, Pax6 and Glis3 (reviewed by Arda et al., 2013), and together with Pdx1, Nkx6.1 and NeuroD regulates the transcription of insulin (Gao et al., 2014; Taylor et al., 2013; Zhang et al., 2005). In addition, Pdx1 and Nkx2.2 play an important role in β-cell maturation by inducing mature β-cell markers such as glucokinase (*Gck*) and *Glut2* (*Slc2a2*) (Bastidas-Ponce et al., 2017; Doyle and Sussel, 2007; Hani et al., 1999).

Generation of the islets of Langerhans

Starting from the secondary transition, differentiated endocrine cells leave the ductal epithelium, migrate into the surrounding mesenchyme and coalesce to form proto-islets (Figs 3 and 4). The interconnections of these structures with endothelial, mesenchymal and neuronal cells promote the formation of functional islets of Langerhans. This overall process, as we highlight below, is regulated by the spatiotemporal activities of a number of different signaling factors and the coordination of cell dynamics.

Endocrine cell delamination

The first step of islet formation involves the delamination of differentiated endocrine cells from the trunk epithelium (Fig. 4A,B). Although these cells are known to undergo single-cell delamination (reviewed by Pan and Wright, 2011; Pictet and Rutter, 1972), our knowledge regarding the molecular mechanisms underlying the delamination process is rudimentary. It has been proposed that the onset of Ngn3 expression triggers the machinery required for delamination. Indeed, upon increasing their Ngn3 levels, endocrine progenitors acquire a bottle-shaped morphology, suggesting that Ngn3 is involved in repolarization and outwards epithelial migration into the surrounding mesenchyme (Gouzi et al., 2011). The cellular mechanisms that govern delamination in this context also remain unclear, with two possible mechanisms having been proposed: asymmetric cell division and epithelial-mesenchymal transition (EMT). In several other epithelial systems, the unequal distribution of fate determinants results in asymmetric cell division, producing two different daughter cells (reviewed by Nuemueller and Knoblich, 2009), and due to the orientation of the mitotic spindle in this type of division one of the cells is excluded from the epithelial plane (reviewed by Morin and Bellaïche, 2011). It is possible that a similar process occurs during endocrine cell delamination. However, there is no evidence to support the existence of classical asymmetric cell division or changes in mitotic spindle during pancreatic endocrine cell formation and delamination.

The other possible scenario for endocrine cell delamination is EMT (Cole et al., 2009). During this process, cells lose their epithelial characteristics and acquire a mesenchymal phenotype. These changes are accomplished by loss of cell polarity, remodeling of adhesion molecules and alterations in the cytoskeleton. Several findings suggest that this mechanism might play a role in the pancreas. For example, the Snail superfamily of TFs is known to play an important role in EMT, activating mesenchymal genes while blocking an epithelial program (Lee et al., 2011). It has been shown that, shortly after endocrine progenitors become committed, Ngn3 triggers *Snail2* (*Snai2* or *Slug*) expression, resulting in a switch from E-cadherin to N-cadherin and expression of the intermediate filament vimentin, both of which are signs of EMT (Gouzi et al., 2011; Rukstalis and

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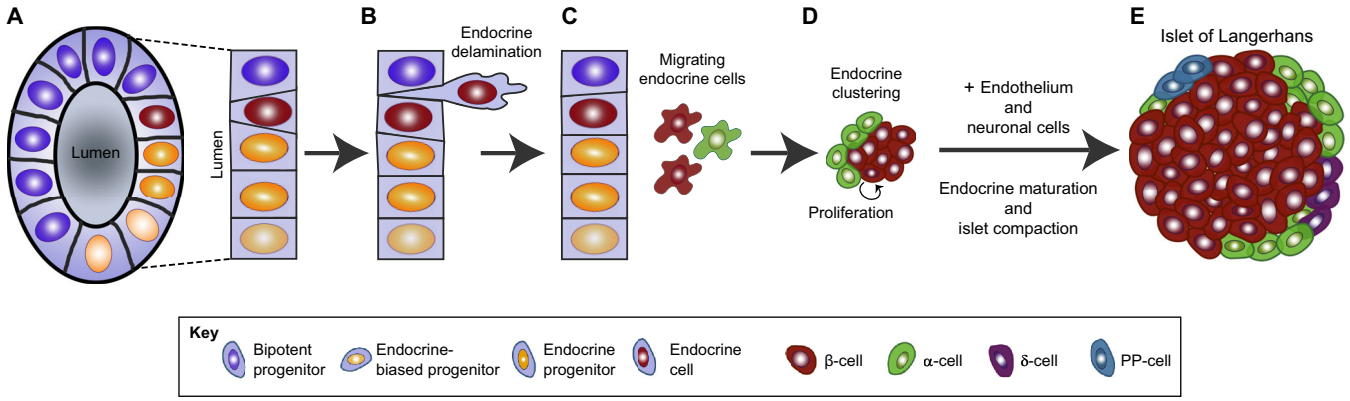

Fig. 4. Endocrine cell delamination, migration and clustering. (A,B) After differentiation within the epithelium, endocrine cells leave the epithelial plane by a delamination process that remains poorly characterized. (C) After detachment from the epithelium, endocrine cells migrate into the surrounding mesenchyme. (D) Within the mesenchyme, endocrine cells cluster and aggregate to form proto-islets. (E) The interconnection of these structures with endothelial and neuronal cells generates the functionally mature islets of Langerhans.

Habener, 2007). The downregulation of E-cadherin during endocrine delamination is partially mediated by the co-repressor Groucho-related gene 3 (Grg3, or Tle3), which is expressed in $Ngn3^{+}$ cells (Metzger et al., 2014). Of note, endocrine cells retain low levels of E-cadherin expression, suggesting the possible involvement of partial EMT during their delamination (reviewed by Morris and Machesky, 2015). Rearrangement of the actin cytoskeleton also plays a crucial role during EMT, and it was recently shown that the sustained activity of Cdc42 stabilizes filamentous actin and cell-cell junctions, impairing endocrine delamination in the pancreas (Kesavan et al., 2014). Extrinsic factors such as TGFβ and canonical Wnt signaling, which are known to regulate EMT in carcinoma cells (reviewed by Zhang et al., 2016), are also present in the developing pancreas (Afelik et al., 2015; Tulachan et al., 2007), but it is unclear if they coordinate the EMT process in endocrine cells. Finally, the expression of EphB3 in delaminating endocrine cells has been described (Villasenor et al., 2012), but whether this protein plays a role in the process needs to be clarified.

Endocrine cell migration

After delamination, endocrine cells migrate within the mesenchyme to form proto-islets (Fig. 4C). In order to migrate, cells need to coordinate their cellular machinery in response to external stimuli. One of the events during migration is the remodeling of adhesion molecules. An example is the switch from E-cadherin to N-cadherin during cell movement (Scarpa et al., 2015). The small GTPase Rac1 is known to regulate E-cadherin-mediated cell adhesion and to subsequently coordinate cell migration, and it has been shown that Rac1-mediated endocrine cell migration is partially regulated by betacellulin (Greiner et al., 2009). EpCAM is another adhesion molecule that is upregulated in migrating endocrine cells, but is downregulated upon clustering and formation of the islets (Cirulli et al., 1998). Roles for integrins in endocrine cell motility have also been reported. For example, the expression of αvβ5 and αvβ3 integrins in human pancreatic epithelial cells and their binding to collagen IV, fibronectin and vitronectin is important for cell migration (Cirulli et al., 2000). Interestingly, the transplantation of human fetal explants into mice in the presence of the integrin-blocking RGD peptide results in failed endocrine cell migration and islet formation (Cirulli et al., 2000). Moreover, α6β4 and α3β1 integrins are expressed in pancreatic epithelial cells and regulate endocrine motility upon interaction with netrin 1, a laminin-1-like protein that directs neuronal axon guidance (Yebra et al., 2003).

The identity and source of the guidance cues orchestrating endocrine cell motility are also poorly characterized. It is possible that such signals are derived from the endothelium and/or the nervous system. Indeed, crosstalk between endothelial and neuronal cells is known to coordinate neurovascular pattering during development. This crosstalk is mediated by guidance cues such as semaphorins and VEGFs and their respective receptors, namely plexins/neuropilins and VEGFRs (reviewed by Andreone et al., 2015). Therefore, it would be interesting to investigate whether these factors also mediate endocrine cell migration during islet morphogenesis.

Endocrine cell clustering and islet assembly

The final step of islet formation involves aggregation of endocrine cells to create proto-islets (Fig. 4D). How endocrine cells find each other during this clustering process is unclear. It is possible that the assembled endocrine cells send guidance signals to the newly delaminating cells. However, the existence and identity of such signals remains obscure. By contrast, the clustering of, and communication between, endocrine cells is known to be mediated by heterotypic and homotypic interactions between adhesion molecules, such as cadherins, integrins, connexin 36 (Gjd2) gap junction, cell adhesion molecules (CAMs) and Eph-ephrins (reviewed by Jain and Lammert, 2009). In particular, cadherins have been shown to be important for β-cell aggregation during development (Dahl et al., 1996), although they are downregulated during the delamination process. CAMs are important in endocrine communication and islet formation. The deletion of *Ncam1* impairs endocrine aggregation due to the requirement of this molecule for α-cell and PP-cell adhesion (Cirulli et al., 1994; Esni et al., 1999). Interestingly, isolated single pancreatic endocrine cells spontaneously reaggregate to form pseudo-islets (Halban et al., 1987), highlighting the intrinsic properties of adhesion molecules mediating endocrine cell clustering and the self-organizing nature of endocrine cells.

Mature islets are also in close contact with endothelial cells and neurons, both of which can influence islet architecture and function. Endothelial cells, for instance, provide the basement membrane for developing islets that is important for expansion, survival and activity of endocrine cells (Nikolova et al., 2006). In addition, endothelium-derived collagen IV and laminin-1 regulate endocrine development and function through integrin β1 signaling (Diaferia et al., 2013). In turn, by producing VEGFA and angiopoietin, endocrine cells coordinate the formation of endothelial networks

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within the islets (Brissova et al., 2014). Accordingly, the deletion or overexpression of *Vegfa* in Pdx1^{+} cells results in an absence or increase in islet capillaries, respectively, suggesting tight regulation of endothelial density by endocrine cells to ensure appropriate glucose sensing and insulin release. Furthermore, pancreatic islets are innervated by sensory, parasympathetic and sympathetic neurons (reviewed by Rodriguez-Diaz and Caicedo, 2014; Thorens, 2014). This interconnection between neuronal projections and endocrine cells is initiated at E15.5, although the innervation of islets continues postnatally. Interestingly, by providing a vascular scaffold, endocrine-derived VEGF regulates islet innervation (Reinert et al., 2014), highlighting the coordination of islet neurovascular patterning by endocrine cells.

As islets develop and mature, they adopt a spherical shape in which β-cells, which constitute ∼60-80% of mature islets (Steiner et al., 2010), are located at the core and are surrounded by a mantle of α-cells and δ-cells (Fig. 4E). This abundance of pancreatic β-cells in mature islets results from the proliferation of pre-existing β-cells. After birth, β-cells undergo maturation to achieve their final hormone-producing glucose-responsive phenotype. This process occurs postnatally and is defined by the expression of the gene urocortin 3 (Blum et al., 2012). Several TFs have been shown to regulate β-cell maturation, including MafA, Nkx2.2, Pdx1 and Foxa2 (Bastidas-Ponce et al., 2017; Doyle and Sussel, 2007; Gao et al., 2014; Nishimura et al., 2006). Furthermore, the 3D organization of islets also impacts endocrine cell maturation and function. Specifically, interconnections between endocrine and non-endocrine cells within the islet microenvironment are thought to produce a 3D compacted structure that supports physiological function and allows the pancreas to respond to blood glucose levels (Bader et al., 2016; reviewed by Roscioni et al., 2016). How islets acquire this final architecture and full maturation status remains unclear, however, and is a topic of intense research.

Conclusions

Over recent decades, our understanding of the mechanisms underlying rodent pancreas development – from early organ induction to formation of the exocrine and endocrine pancreas – has vastly increased. In particular, a number of signaling pathways and gene regulatory networks that are responsible for the formation of this organ have been identified. It has also been noted, perhaps not surprisingly, that mutations in genes encoding key pancreatic regulators can lead to pathological abnormalities in humans (Box 3). Much less is known about the cellular and molecular processes driving pancreas morphogenesis and organogenesis. For instance, knowledge about the cell polarization, microlumen formation and plexus remodeling events that occur during early pancreas development is rudimentary. Furthermore, the mechanisms that control the spatiotemporal regulation of epithelial remodeling, endocrine cell delamination and migration as well as islet assembly remain elusive. Answering such questions might identify novel molecular targets for the treatment and prevention of pathological conditions such as pancreatitis, pancreatic cancer and diabetes. In addition, although our understanding of the mechanisms driving human pancreas development is growing (Jennings et al., 2013; reviewed by Jennings et al., 2015), more efforts are required to dissect these mechanisms and relate them to those already identified from mouse studies.

Moving forward, these advances and approaches are likely to have important implications for generating improved treatments, notably cell-based therapies, for diabetes. Indeed, current efforts to devise cell-replacement therapies for type 1 diabetes have focused on the *in vitro* generation of β-like cells from pluripotent stem cells (Pagliuca et al., 2014; Rezania et al., 2014; Russ et al., 2015), and obtaining a comprehensive picture of the developmental programs of endocrine cell formation will no doubt aid in the generation of functional glucose-responsive and hormone-producing cells. Understanding the principles of islet formation during development will also help to induce islet neogenesis in diabetic patients and to generate islet biomimetics for cell-replacement therapy. The recent establishment of 3D pancreatic organoid cultures (Greggio et al., 2013), CRISPR-Cas9 technology (reviewed by Sander and Joung, 2014) and single-cell-based analyses of the human pancreas (Wang et al., 2016a,b; Xin et al., 2016) have expanded the tool-box that can be employed to answer key questions regarding pancreatic cell formation, organization and function. In the future, these – together with continued studies of pancreas formation in rodents – will hopefully provide us with a comprehensive picture of the mechanisms that govern pancreas development and disease.

Box 3. Human genetic mutations associated with abnormal pancreas development

Mutations in genes involved in pancreas development can cause various pathological abnormalities in humans. For example, the complete lack of pancreas (pancreatic agenesis) is observed in patients carrying homozygous mutations in genes involved in early pancreas development. In addition, monogenic diabetes, which represents 5% of diabetes cases and is divided into permanent neonatal diabetes mellitus (PNDM; present in newborns and infants) and maturity onset diabetes of the young (MODY; occurring predominantly in children and young adults), is due to inherited or spontaneous mutations in genes essential for pancreas and β-cell development.

Key pancreatic genes that, when mutated, are implicated in human pancreatic disorders:

- *PTF1A*: pancreatic agenesis, isolated pancreatic agenesis and PNDM (Sellick et al., 2004; Weedon et al., 2013)
- *GATA4/6*: PNDM, pancreatic agenesis, hypoplasia disorders and adult-onset diabetes (De Franco et al., 2013a; Lango Allen et al., 2012; Shaw-Smith et al., 2014)
- *PDX1*: MODY4, pancreatic agenesis and PNDM (De Franco et al., 2013b; Stoffers et al., 1997)
- *HNF1b*: MODY5, pancreatic hypoplasia and agenesis (Haumaitre et al., 2006)

Other genes involved in the development of PNDM include:

- *GLIS3* (Rubio-Cabezas et al., 2011; Senée et al., 2006), *RFX6* (Smith et al., 2010), *NEUROD1* (Rubio-Cabezas et al., 2010), *NKX2.2*, *MNX1* (Flanagan et al., 2014) and *PAX6* (Solomon et al., 2009)

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Competing interests

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