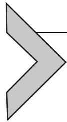

CHAPTER THREE

Pancreas morphogenesis: Branching in and then out

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Abstract

The pancreas of adult mammals displays a branched structure which transports digestive enzymes produced in the distal acini through a tree-like network of ducts into the duodenum. In contrast to several other branched organs, its branching patterns are not stereotypic. Moreover, the branches do not grow from dichotomic splitting of an initial stem but rather from the formation of microlumen in a mass of cells. These lumen progressively assemble into a hyperconnected network that refines into a tree by the time of birth. We review the cell remodeling events and the molecular mechanisms governing pancreas branching, as well as the role of the surrounding tissues in this process. Furthermore, we draw parallels with other branched organs such as the salivary and mammary gland.

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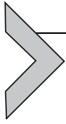


1. Introduction

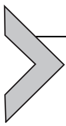
The adult pancreas is a mixed gland consisting of two functionally and morphologically distinct compartments. The exocrine pancreas (>95% of the pancreatic mass) is composed of acinar cells secreting digestive enzymes and ductal cells that secrete bicarbonate and mucus (Rahier, Wallon, & Henquin, 1981). These cells organize into monolayers to form tubes that are connected, forming a tree-like structure. Acinar cells are at the terminal ends of branches while ductal cells assemble into an arborized structure comprising narrow terminal ducts, the intralobular and interlobular ducts, draining the acini by collecting the pancreatic juice and delivering it to the duodenum (Reichert & Rustgi, 2011). The endocrine pancreas (<5% of the pancreatic mass) regulates glucose homeostasis by secreting five hormones (insulin, glucagon, somatostatin, ghrelin and polypeptide) into the bloodstream. The endocrine cells coalesce into islets of Langerhans, which are intermingled with blood vessels and neurons and scattered between the main ductal branches.

The pancreas primordium is initiated from the posterior foregut endoderm and grows into a multilayered stratified epithelium where multiple intraepithelial lumen emerge de novo. Through a series of poorly understood processes, microlumen fuse to form a continuous and highly connected luminal plexus, before branching can be seen. Progenitor cells soon reorganize into a branched epithelium while they commit to various specialized fates. The luminal network expands and remodels progressively toward a hierarchical tree where the epithelial cells are organized into monolayers lining the lumen, a process closely associated with pancreatic cell differentiation.

Pancreas branching morphogenesis presents common traits with other branched organs but also some features proper to this gland. Pancreas tubulogenesis shares similarities with submandibulary and mammary glands in which a group of initially unpolarized epithelial cells undergo polarization to form microlumens (Huebner & Ewald, 2014; Patel & Hoffman, 2014). This is distinct from processes occurring in other organs, such as the lungs, where tubes are formed by remodeling an already polarized epithelium to surround a luminal space (Swarr & Morrisey, 2015). Budding is the initial dominant branching mode of pancreatic epithelial cell but as development proceeds, clefting has been observed in the periphery of the organ. Both modes of branching coexist in many organs, although the relative fraction

of these two mechanisms differs depending on the organ and the timing in organogenesis (Andrew & Ewald, 2010; Wang, Sekiguchi, Daley, & Yamada, 2017). The formation of a highly connected plexus prior to epithelial branching and, at later stages, the combination of plexus remodeling in the center of the organ with branching at the periphery constitute features proper to the pancreas. The branching pattern of the pancreas is stochastic by opposition to the initial lung or kidney branches which are highly stereotypical. This stochasticity is associated with heterogeneity in the shape and number of branches across individuals, though some similarities in the gross pattern of branches have been reported (Villasenor, Chong, Henkemeyer, & Cleaver, 2010).

In this review, the morphological changes associated with branching of the pancreas are presented at each developmental stage. We emphasize the molecular events controlling lumen formation, tubulogenesis and epithelial branching leaving aside the description of the transcriptional regulators and signaling pathways controlling pancreatic cell differentiation, a topic covered in many comprehensive reviews (Larsen & Grapin-Botton, 2017; Pan & Wright, 2011). In a second section, we highlight the close spatiotemporal association between pancreas morphogenesis and cell fate. Finally, the interconnection between pancreatic epithelium branching, the surrounding mesenchyme, and the branched endothelial and neuronal networks are described. We limit our review to the mammalian pancreas, mostly from experiments conducted in mice. However, there is also a branched pancreas in birds, reptiles and teleost fishes and large variations in this organ in other fish species.



2. Morphogenesis

2.1 Pancreatic primordium stratification (E8.5–E10.5)

The formation of the pancreatic ductal network is rooted in the initial epithelial structure of the pancreatic anlagen. The dorsal and ventral anlagen emerge at E8.5 in the posterior foregut endoderm from a single layer of cuboidal epithelial cells expressing PDX1 (pancreatic and duodenal homeobox 1). A few hours later at E8.75, the PDX1+ cells of the dorsal primordium become columnar, creating a small protuberance in the epithelial layer, referred to as dorsal bud (Jorgensen et al., 2007; Villasenor et al., 2010). Over the next day, this single layer becomes stratified, generating multiple layers of cells between the duodenal lumen and the outer basement membrane (Fig. 1A) (Villasenor et al., 2010). The ventral pancreatic

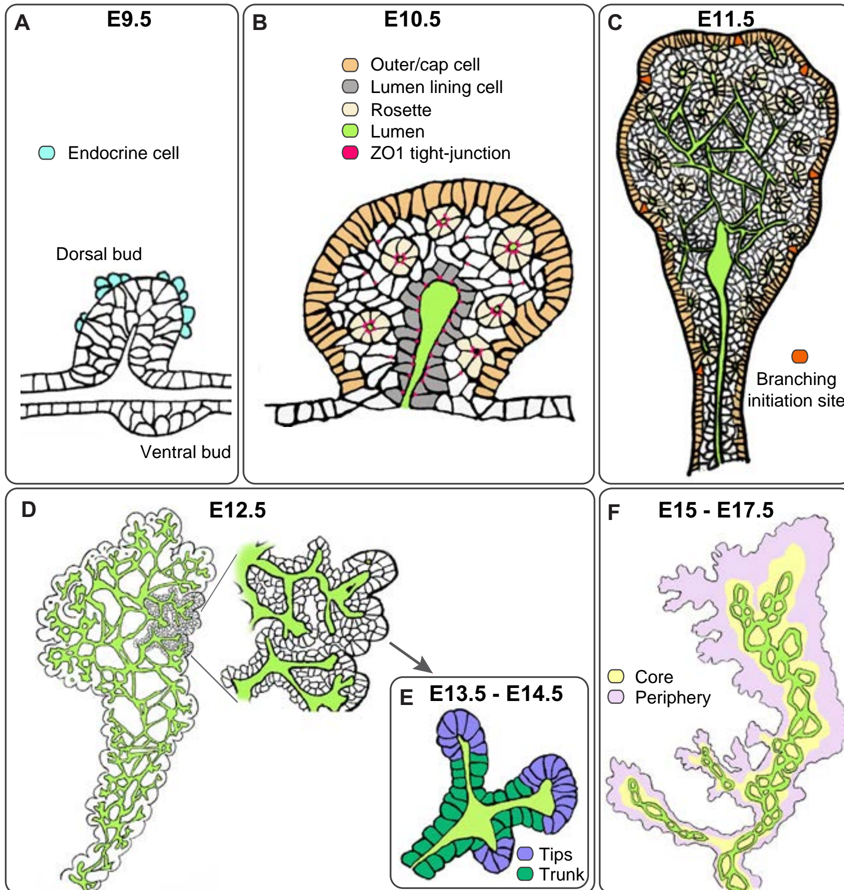


Fig. 1 Overview model of mouse pancreas lumenogenesis and branching. (A) Pancreatic primordium stratification. (B) Microlumen formation. (C) Early epithelium remodeling and luminal plexus generation. (D) First external signs of branching and luminal plexus expansion. (E) Remodeling into a monolayered epithelium and tip/trunk segregation. (F) Plexus remodeling. Regions at the center of the pancreas, termed core (yellow), contain a luminal plexus (green) in the process of remodeling, while regions at the periphery (purple) display ramifying branches. The yellow area decreases as development proceeds and disappears around E18.5 when the plexus resolves in a tree-like structure.

primordium is structurally visible slightly later (around E9) (Jorgensen et al., 2007). Despite this delay, morphogenesis of the dorsal and ventral pancreas is believed to be similar although most studies have focused on the dorsal bud. By E9.5, the dorsal pancreatic bud forms a globular mass of cells (around 900 cells) that comprises proliferative pancreatic progenitors, endocrine progenitors and few differentiated endocrine cells expressing glucagon

at the periphery of the epithelium (Jorgensen et al., 2007; Larsen & Grapin-Botton, 2017) (Fig. 1A). By E10–E10.5 the dorsal bud is composed of six cell layers in average, surrounding an elongated primary central lumen that connects the pancreatic stratified epithelium to the primitive gut tube (Larsen et al., 2017; Villasenor et al., 2010) (Fig. 1B). At these stages, the pancreata of different individuals display a nearly identical morphology and three compartments can be described based on the shape and the polarity of the cells (Villasenor et al., 2010). The peripheral cells, the basal membranes of which contact the mesenchyme, are termed "cap" or outer layer cells (Fig. 1B). They are in contact with the basal lamina, which consists, among others, of laminin and collagen, and are anchored to it via integrins. Their nuclei are localized at their basal side but they do not express classical apical markers such as aPKC, PAR3 or Ezrin (Villasenor et al., 2010). Their elongated shape is reminiscent of columnar pseudostratified epithelia with the majority of the cells presenting a large basal surface (Shih, Panlasigui, Cirulli, & Sander, 2016). On the contrary, the layer of cells directly in contact with the inner central lumen show apical polarization with accumulation of aPKC, PAR3, Ezrin and ZO1 on the lumen side, but no sign of basal polarization. In between, inner/body cells constitute disorganized layers that do not display any particular shape or sign of polarity until E10.5 (Villasenor et al., 2010) (Fig. 1B). Time lapse-microscopy on in vitro-cultured dorsal epithelium revealed that the outer layer cells differ from the body cells not only by their morphology, but also by their behavior (of note, lumen lining-cells were not included in this study as the inner central lumen is lost in explants) (Shih et al., 2016). The cap cells show rapid cell shape changes, dynamic cell intercalation and positional rearrangements within their outer layer while body cells, although also motile, maintain their shape and position. Cap and body cells display limited intermingling and their daughters remain largely in their respective layers after mitosis. Local cues from the basal membrane are believed to instruct the segregation of the two compartments as disruption of integrin-mediated cell interaction with the basement membrane renders caps cells more similar to body cells (see Section 4.2) (Shih et al., 2016). Interestingly, the pancreatic bud displays similarities to that of early mammary and salivary glands, which develop from primordia in which relatively unpolarized cells are initially densely packed (Huebner & Ewald, 2014; Patel & Hoffman, 2014). Moreover, these organs are also transiently organized into inner/body and outer/cap cells based on the cell shape as well as the observed cell mobility in the case of the salivary gland (Hsu et al., 2013; Larsen, Wei, & Yamada, 2006).

2.2 Microlumen formation (E10.5–E11.5)

At E10.5, the surface of the epithelium is largely smooth and does not present any sign of branching. The cells start reorganizing and apico-basal polarity becomes established. The first sign of apical polarity both in the cap and body cells, is the detection of the tight-junction maker ZO1 (Villasenor et al., 2010) (Fig. 1B). This protein accumulates in individual cells and forms one or two foci at cell-cell interfaces. By E10.75, the apical marker Mucin1 is also detected in the dorsal epithelium, mainly in isolated dots at the interface between several cells (Hick et al., 2009; Villasenor et al., 2010). Concomitant with this acquisition of polarity by body cells (E10.0–10.75), individual cells undergo asynchronous apical constriction, each forming a tight collar of ZO1. It is proposed that localized ZO1 in single cells is followed by cell shape changes in neighboring cells, resulting in the formation of a rosette of cells which form an initial microlumen on the apical side (Hick et al., 2009; Kesavan et al., 2009; Villasenor et al., 2010) (Fig. 1B). Among its multiple functions, PDX1 coordinates apical constriction of the bottle-shaped rosette cells via downregulation of E-cadherin and phospho Myosin light chain. Loss of PDX1 leads to the formation of microlumen with bigger diameters that will eventually fuse into a single cystic lumen (Marty-Santos & Cleaver, 2016). At E10.5, around 270 microlumen, mostly under 20 μm diameter, are found (Marty-Santos & Cleaver, 2016). As these microlumen are not connected with the central lumen but arise in a position where there was no space previously, the term de novo lumen formation is used (Sigurbjornsdottir, Mathew, & Leptin, 2014).

In unpolarized epithelia, three different mechanisms of de novo lumen formation have been described: cell hollowing, cord hollowing and cavitation (Sigurbjornsdottir et al., 2014). As apoptosis is not observed during pancreatic microlumen formation, the cavitation process can be excluded (Hick et al., 2009; Marty-Santos & Cleaver, 2016). Cord and cell hollowing both involve apical membrane material delivery via vesicle trafficking. In the first case, these vesicles are delivered at the contact between two cells where apical polarity is established, while in the second case the vesicles fuse within the cytoplasm, forming a large intracellular lumen that expands and only subsequently fuses to the plasma membrane once two cells have made contact and established junctions (Sigurbjornsdottir et al., 2014). Large intracellular lumen have not been detected so far in the pancreatic cells under normal conditions but multiple mucin-containing vesicles can be observed in scattered cells of the early epithelium (Kesavan et al., 2009). It is reasonable

to assume that these vesicles are targeted to the prospective apical surfaces, but it is not clear if apical cell junctions form before, after or concomitantly to apical protein vesicle trafficking. Indeed, there are discrepancies in the timing of apparition of ZO1 and Mucin markers in different studies (Hick et al., 2009; Kesavan et al., 2009; Villasenor et al., 2010). In other systems, the site at which the lumen will be generated is usually defined through interactions with the microenvironment via either ECM or direct cell-cell contact. However, disruption of cell–ECM interactions by pancreatic deletion of β1-integrin does not perturb microlumen formation (Shih et al., 2016) (see Section 4.2). Cytoskeletal regulators such as small Rho GTPases (CDC42, RAC1 and RHOA) are generally activated downstream of these interactions and contribute to establishing cell polarity (Sigurbjornsdottir et al., 2014). In the pancreas of CDC42- deficient mice, vesicles with the apical membrane proteins PAR6, Crumbs3 and Mucin1 are retained in auto-cellular lumen in contact with the cell surface via the tight junction (Kesavan et al., 2009). CDC42 is not required for apical membrane protein delivery to the surface or for tight junction formation but is essential for the subsequent expansion in a common luminal space with neighboring cells (Kesavan et al., 2009). In contrast, an increased luminal surface is observed at E10.5 following ablation of RAC1 in epithelial cells, suggesting that RAC1, together with PI(3)K, represses lumen formation. It is unclear if these proteins control apical membrane biogenesis, microlumen growth and/or fusion or maturation of these lumens in a connected plexus (Löf-Öhlin et al., 2017). Pancreatic deletion of RHOA does not perturb lumen formation (Azizoglu, Braitsch, Marciano, & Cleaver, 2017) although its downstream Rho-associated kinase (ROCK) effector and actomyosin contractility control the branching process. Interestingly, de novo lumen formation has also been reported in the stratified epithelium of the early mammary and salivary glands. However, mammary and salivary gland lumens are formed, at least in part, by apoptotic clearance of body cells. In addition, in the mammary and salivary gland, lumens form after the organ has already branched, whereas in the pancreas a plexus of lumen appears before branching (Huebner & Ewald, 2014; Macias & Hinck, 2012; Wells & Patel, 2010).

2.3 Early epithelium remodeling and luminal plexus generation (E11.5–E12.5)

At E11.5, while the gut tube begins to undergo a rotation movement that brings the dorsal and ventral pancreatic buds into proximity, the dorsal bud

elongates into a tear drop-shaped structure (Fig. 1C). The bud surface stays relatively smooth and no clear external signs of branching are visible yet, although internally, the epithelial cells continue to reorganize around ramifying lumens (Fig. 1C). Cap cells (around 120) remain discernable from body cells and sporadically present a constricted basal side and a wider apical surface (Hick et al., 2009; Shih et al., 2016; Villasenor et al., 2010) (Fig. 1C). Time-lapse analysis suggests that this cell shape change demarcates sites of future epithelial invaginations and subsequent branching (Shih et al., 2016). In the body cell compartment (around 250 cells), the number of rosettes increases and they incorporate more polarized cells (Villasenor et al., 2010) (Fig. 1C). Following depletion of β1 Integrin in the epithelium, both cap and body cells adopt round shapes and the first signs of branching are abolished, suggesting that integrin-mediated cues from the surrounding extracellular matrix may contribute to remodeling of the actin cytoskeleton resulting in branching (see Section 4.2) (Shih et al., 2016).

Although the epithelium remains globular at E11.5, small-branched lumen have emerged inside and appear to form a network of fusing lumen (Fig. 1C). Thin canals of shared apical membrane join the small lumen delimited by the rosettes, while new microlumen continue to be generated (Fig. 1C). This nascent luminal plexus eventually connects to the central lumen (Villasenor et al., 2010). The cell polarity is now firmly established as classical markers of apical polarity such as PAR3/6, Crumbs3, and aPKC are detected along the apical surface of the lumen-lining cells, while Collagen IV and Laminin mark their basal side (Kesavan et al., 2009; Villasenor et al., 2010). Concomitant to this acquisition of polarity along the apico-basal axis, the planar polarity components VANGL1/2 and Frizzled3 are detected around the apical junctions (Flasse et al., 2020). Our understanding of the lumen expansion process in the pancreas is rudimentary. In different model systems, coordinated vesicular transport to the apical domain along the cytoskeletal track allow lumen opening and growth (Sigurbjornsdottir et al., 2014). Along with this process, more cells can be recruited, increasing the luminal space and adjacent microlumen can fuse together. The presence of rosettes involving increasing numbers of polarized cells ("higher order rosette") (Petzold, Naumann, & Spagnoli, 2013), is supportive of more cells being included over time though this may occur either by epithelial rearrangement or cell division. The fusion of adjacent microlumen is suggested by the observation of thin canals, delineated by apical membranes, between microlumen (Villasenor et al., 2010). It is speculated that pancreatic lumens emerge, as proposed during the formation of the

zebrafish gut lumen, by the rearrangement of cell junctions and expansion of apical membrane domains (Horne-Badovinac et al., 2001; Petzold et al., 2013; Villasenor et al., 2010). In agreement with this hypothesis, aPKC, the role of which is crucial in zebrafish gut microlumen fusion, may also be involved in the lumen coalescence into a continuous tubular network in the pancreas. Indeed, E11.5 pancreatic explants treated with an aPKC inhibitor fail to from a continuous network in the following days of culture, the mucin-positive epithelial cell forming mostly unconnected rosettes (Kesavan et al., 2009). More insight into lumen formation was obtained by time-lapse imaging on E11.5 explants using a reporter line labelling the apical membrane (Crumbs3-GFP) (Azizoglu et al., 2017). This revealed extensive vesicular trafficking directed toward the nascent apical membrane in the cap cells, suggesting biogenesis of a new apical membrane while the central epithelium exhibited little noticeable vesicular trafficking. The existing central lumens underwent dynamic changes, extending and connecting with each other. This suggests that from this stage onward, de novo luminogenesis predominates at the periphery of the organ, while the central lumen morphogenesis occurs primarily through lumen extension. In agreement with this model, in the absence of Afadin, an adaptor protein bridging Nestin to the actin cytoskeleton at the adherens junction, lumens remained discontinuous at the tips while the central lumens remodeled relatively normally (Azizoglu et al., 2017). Detailed analysis of Afadin-depleted pancreas showed a retention of apical markers (ZO1, Mucin, PAR3, aPKC, Podocalyxin) either at the lateral membrane or in cytoplasmic vesicles, a phenotype reminiscent of CDC42 mutants though milder as it occurs only in the periphery of the epithelium. Afadin likely acts as a scaffold to localize RAB8 and CDC42 to the pre-apical domain to enable exocytosis (Bryant et al., 2010).

The connection between individual lumen is expected to rely on extensive reorganization of cell-cell contacts. The Rho-GTPase-activating protein STARD13 is involved in this process, eventually enabling the cells to reorganize into monolayers delimiting pancreatic branches (Petzold et al., 2013). Upon its inactivation, apico-basal polarity (PKCζ, Mucin, Laminin localization) is properly established but the actomyosin network usually enriched at the apical junction of bottle-shaped cells forming the rosette is abnormally distributed resulting in fewer and disorganized rosettes. STARD13 can potentially activate all small GTPases involved in pancreas morphogenesis, but the authors suggest that the STARD13 mutant phenotypes are not attributable to constitutively active CDC42, which is involved

in microlumen generation (Petzold et al., 2013). In this mutant, the pancreas fails to branch but the phenotype is also complicated by a decreased replicative activity of the multipotent progenitors resulting in pancreas hypoplasia. Similarly, Ephrin B2/B3 receptor deletion also disturbs early epithelial rosette rearrangements, without affecting the establishment of polarity, and displays branching defects as well as pancreatic hypoplasia (Villasenor et al., 2010). The luminal network is established but fails to mature properly. Decreased junctional E-cadherin and β-catenin levels suggest a fundamental change in the structural cohesion of the mutant epithelium that could account for the early epithelial disorganization. Subsequent branching and lumen remodeling are affected as the length of the branches is reduced and the lumen subsequently fail to remodel into their normal hierarchical arrangement. These defects are associated with a decrease of exocrine and endocrine cell mass attributed to a reduction of multipotent progenitor numbers at early stages (Villasenor et al., 2010). This study highlights the importance of EphrinB signaling in early pancreatic remodeling. As Eph B2 and B3 ligands are expressed in the mesenchyme (ephrin B1) and in the pancreatic arteries (ephrin B2), respectively, these tissues are implicated in early epithelial rearrangement (van Eyll et al., 2006; Villasenor et al., 2010). Taken together, while the current knowledge suggests the importance of apical secretion and cell division in lumen expansion, the cell rearrangements that enable the widening or lengthening of lumen or the connection of independent lumen remains far from understood. Whether luminal plexus formation prior to branching is more widely relevant would require more 3D investigations of the luminal network structure in other branched organs.

2.4 First external signs of branching and luminal plexus expansion (E12.5–E14.5)

By E12.5, the two pancreatic buds have fused to form one interconnected organ and the dorsal pancreas has lost its grossly symmetrical shape. The epithelium surface presents clear invaginations separating protruding rudiment of branches usually denominated as "tips" (Fig. 1D). Concomitant with this early branching morphogenesis, compartmentalization of different types of pancreatic progenitors emerge. While the early pancreas mostly consists of multipotent progenitors giving rise to the three major pancreatic lineages (Gu, Dubauskaite, & Melton, 2002; Zhou et al., 2007), clonal analysis revealed a gradual process whereby some unipotent acinar progenitors start to emerge at E11.5 (Larsen et al., 2017; Sznurkowska et al., 2018). At E12.5, an

interesting switch occurs in the localization of the proliferating cells: while the body cells proliferate more than the cap cells at earlier time points, the tips become more proliferative by E13.5 (Larsen et al., 2017; Marty-Santos & Cleaver, 2016). The tip compartment fuels the epithelium expansion with acinar-committed cells whereas the body cell compartment remodels toward a monolayer epithelium (by E13.5–E14.5) (Fig. 1E). Morphologically, pancreatic 'branches' appear as wide protrusions and at this stage (E13.5), the pattern of nascent branches exhibits the highest level of variation between individuals (Villasenor et al., 2010). Two different modes of branching have been described: splitting of the branch tip (tip bifurcation or clefting) and budding from an existing duct (lateral/side branching). The pancreas uses a combination of both. Indeed, live imaging of pancreatic explants shows that in the initial phase of branching, 85% of the new branches appear via lateral branching and 15% by terminal bifurcation or "clefting" (Puri & Hebrok, 2007). Consistently, new protrusions of similar shape and size appear laterally in vivo (Villasenor et al., 2010). At the cellular level, new branch formation can be driven by patterned cell proliferation, collective cell migration, coordinated cell deformation and/or cell rearrangement (Wang et al., 2017). While the tips are proliferative, local patterns of proliferation have not been reported in the pancreas. Similar to the uteric bud and the lung, the pancreatic branch tips are pseudostratified and/or single layered at E13.5 (Villasenor et al., 2010). A process of de-stratification, involving major cell rearrangements, must therefore accompany luminogenesis and branching. Live imaging on E11.5 explants suggests that collective cell migration participates in the budding process (Nyeng et al., 2019). However, albeit it is clear that individual progenitor cells are motile, a coordinated movement of the cells toward the budding site has not been demonstrated. Measurement of cell displacement and its directionality should clarify this point.

Actomyosin contractility plays an important role in driving the changes in organ shape associated with branching in several organs (Wang et al., 2017). The phosphorylated form of myosin regulatory light chain (pMLC), a substrate of Rho-associated kinase (ROCK) and effector/read-out of actomyosin-based contraction, is polarized at apical cell junctions of all epithelial cells in a pattern similar to the actin network (Bankaitis, Bechard, Gu, Magnuson, & Wright, 2018; Flasse et al., 2020). Although this expression pattern in suggestive of a role of the ROCK pathway and the downstream actomyosin contractility in pancreas remodeling, E11.5 explants treated with a ROCK inhibitor do not show major defects in lumen morphology or branching (Azizoglu et al., 2017).

However, E10.5 buds grown in Matrigel in the presence of a different ROCK inhibitor fail to branch properly (Flasse et al., 2020). This discrepancy may be due to the timing of ROCK inactivation (before or after the onset of branching), to the presence of mesenchyme or to the potency and side effects of these inhibitors. Activation of RhoA, upstream of ROCK, also has controversial effects as E11.5 explants treated with CNO3 are less branched (Shih et al., 2016) while treatment of E10.5 bud cultured in Matrigel with the same activator promotes branching (Flasse et al., 2020). Further investigations will be required to determine how the RhoA-ROCK pathway is involved in pancreas branching and if it promotes branching, as seen in the in salivary gland, the uteric bud or the lung (Wang et al., 2017). Actomyosin contractility, which functions downstream of the ROCK pathway, but also of other pathways, is required for pancreas branching. Indeed, inhibition of MyosinII-dependent processes with Blebbistatin or inhibition of actin polymerization with CytochalasinD disturb branching morphogenesis and lumen continuity (Azizoglu et al., 2017; Shih et al., 2016). Explants treated with Cytochalasin D display more severe phenotypes, likely due to myosin-independent functions of actin (Azizoglu et al., 2017). Interestingly, actomyosin perturbation renders the shape of cap cells more similar to that of body cells (Shih et al., 2016). This suggests that differential contractility of outer versus inner cells may be involved in branching, as proposed in the salivary gland (Hsu et al., 2013). Though these observations highlight the importance of cell rearrangements in branch formation, they should be taken with caution as they are mainly based on observations made in vitro in explants where the organ flattens. Moreover, the fraction of budding to clefting branching modes changes during organogenesis (see below).

Nascent epithelial branches protruding into the surrounding mesenchyme contain multi-lumen plexi. Looking at the branching process form the lumen point of view, the luminal network of the E12.5 pancreas is entirely connected in a plexus where no hierarchy in the connections can be identified based on lumen diameter (Villasenor et al., 2010) (Fig. 1D). This network was manually skeletonized, from whole mount stainings with the apical marker mucin, which enabled to derive its properties (Dahl-Jensen et al., 2018). At E12.5, the network is over-connected, exhibiting redundancy of paths from one point to another (network cost) and toward the duodenum outlet. A simple in silico model in which new microlumen appear close to the established pancreas network and immediately connect to the nearest lumen of the network, recapitulates the network traits of the E12.5 pancreas. Simulations best fit the biological data when new lumen only connect to slightly more than one previously generated luminal

structure. This lumen-forming rule is different from the more hierarchical and dichotomic lung branching program (Metzger, Klein, Martin, & Krasnow, 2008) or from the random walk proposed in the mammary gland (Hannezo et al., 2017). A network of redundantly interconnected lumen has not been reported in these branched organs and would deserve more scrutiny.

From E13.5, the overall morphology of individual pancreata will progressively converge in appearance, their gross anatomy revealing classes of patterns based on the length of the lateral branches (Villasenor et al., 2010). Though the branching process is not stereotyped, as described in the lung (Metzger et al., 2008), these classes reflect features in the gross anatomy shared by multiple individuals. Likewise, the digitization of the pancreatic lumen network revealed that its length and connectivity are stereotypic at each developmental stage and change as the pancreas matures (Dahl-Jensen et al., 2018). For instance, ducts form polygonal structures or loops whose number differs at E12.5 and E14.5 stages (Fig. 2). The reduction in the number of loops between these two stages suggests that alongside the connection of new lumen at the periphery, pruning occurs throughout the organ, eliminating redundant connections. In agreement with the evolution of the network toward a more hierarchical structure, the cost of the network starts to decrease at E14.5 revealing less redundancy of paths (Dahl-Jensen et al., 2018). Moreover, some hierarchy in the lumen diameter starts to be visible, with thinner lumen in the periphery of the organ (Bankaitis, Bechard, & Wright, 2015; Villasenor et al., 2010) (Fig. 2).

2.5 Segregation of tip and trunk domains (E14.5)

By E14.5, the epithelium is clearly segregated into distinct "tip" and "trunk" domains based on the fate of the cells constituting these two compartments but also on morphological features. Acinar cells arise from the extending tips of the branches and continue to undergo active proliferation, which contributes to increasing the number of acinar tips (Pan et al., 2013; Sznurkowska et al., 2018; Zhou et al., 2007) (Fig. 1E). The "trunk" part of the branches forms the primitive pancreatic ducts composed of bipotent duct/endocrine progenitors (Kopinke et al., 2011; Solar et al., 2009). The endocrine progeny delaminates and clusters into the nascent islets of Langerhans while the bipotent progenitors divide (see also Section 3.1) (Bankaitis et al., 2015; Gouzi, Kim, Katsumoto, Johansson, & Grapin-Botton, 2011). Differentiation at the branch tips is often associated with maturation of branched organs. In the pancreas, as in

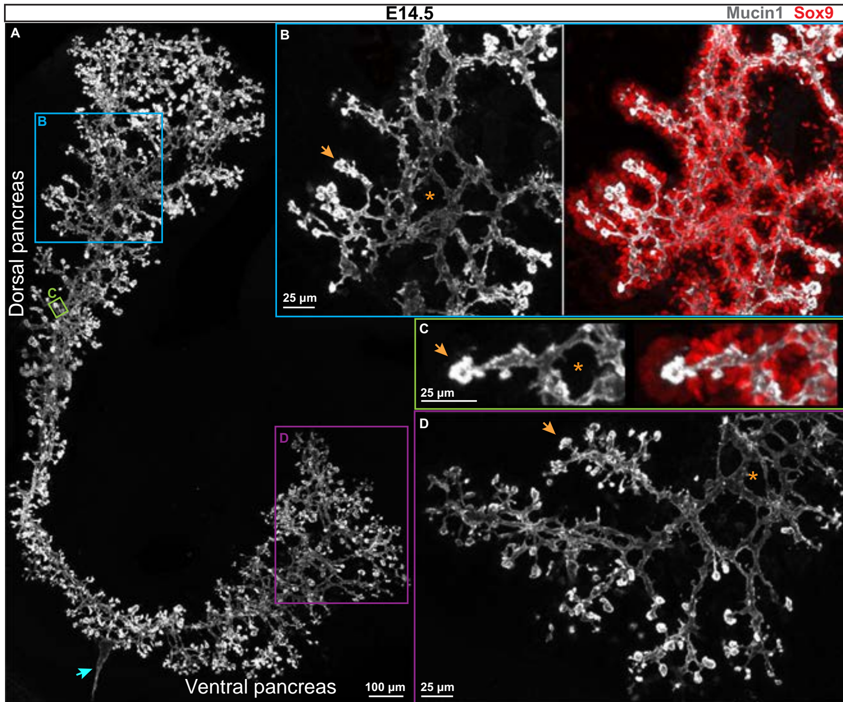


Fig. 2 Luminal network at E14.5. (A) 3D projection of a whole-mount staining of apical marker Mucin1 depicting the architecture of the pancreatic ductal tree. Rectangles delimit the area shown in (B) (blue), in (C) (green) and in (D) (pink). A blue arrow points to the duodenum connecting-duct. (B, C, D), Projection of a thinner Z stack in the area delimited in A showing "the loops" formed by the ducts that form the plexus (orange "*," one example of loop) and the peripheral plexi (orange arrow) that remodel into narrower tubular lumen. In B and C, SOX9 staining shows the progenitor nuclei arrangement around the lumen, the tips cells expressing lower levels of SOX9 compared to the trunk cells.

the salivary gland, the epithelial cells at the branch tips are wedge-shaped columnar cells surrounding a small lumen to form the acinus (Aure, Konieczny, & Ovitt, 2015) while the ductal cells stay more cuboidal (Fig. 3A). The tips cells are not only distinguished by their shape or transcriptional identity but also by their adhesive properties. P120 catenin is known to stabilize epithelial cell adherens junctions through its interaction with the juxtamembrane domain of *E*-cadherin molecules (Ishiyama et al., 2010). Although P120 is expressed in all epithelial cells (Hendley et al., 2015), the tip cells display lower expression levels of this protein at the membrane than the trunk cells at E14.5 (Nyeng et al., 2019). This segregation is preceded by a random

distribution of low and high P120 expressing cells at E11.5. Following mosaic inactivation of P120 at E11.5, the P120 KO cells are enriched in the tips at E14.5 and live imaging on explants revealed their migration toward the periphery where they acquire an acinar fate. Similar mosaic inactivation of P120 after tip/trunk segregation also showed a bias toward acinar fate for the P120 low cells suggesting that differential P120 levels are important for acinar cell birth. Moreover, P120 KO cells exhibited lower levels of E-cadherin at the membrane and mosaic inactivation of E-cadherin also led to sorting of the E-cad-depleted cells to the peripheral tips (Nyeng et al., 2019). In their model, based on the prediction that a mixture of cells with differential adhesive or cortical tension properties would spontaneously sort (Brodland, 2002; Steinberg, 1963), the authors suggest that E-cadherin is the main effector of P120 catenin in the segregation process. However, E-cadherin is probably not the only effector as in other cell types, P120 also inhibits RHOA and activates RAC1 and CDC42, which may participate in the cell rearrangement process (Anastasiadis et al., 2000; Noren, Liu, Burridge, & Kreft, 2000). This study shows the importance of adhesion molecules in the process of tip/trunk segregation and the subsequent allocation of cell fate. It is important to note that only a differential level of expression of these adherent junction proteins results in a sorting process whereas inactivation of P120 or E-cadherin in the whole epithelium has a more profound effect. Indeed, in a prior study, Hendley et al. showed that P120 deletion in the whole epithelium generated large ductal cysts in the center of the epithelium and reduced branching from E12.5 (Hendley et al., 2015). The total amount of endocrine cells was not affected but acinar differentiation was impeded throughout development (Hendley et al., 2015). An overall reduction of adherens junction components, including E-cadherin, was observed in P120 KO cells although the protein was still detected at the membrane (Hendley et al., 2015). Pancreas-specific deletion of E-cadherin has a similar outcome, with an aberrant epithelial duct-like epithelium detected soon after birth (Kaneta et al., 2020). Interestingly, a comparable phenotype is observed in the submandibular gland following P120 deletion: acinar differentiation is blocked, resulting in a gland essentially composed of distended ducts with reduced E-cadherin levels (Davis & Reynolds, 2006). Down-regulation of E-cadherin also leads to aberrantly dilated lumen suggesting that it is an effector P120 in the salivary gland as it is in the pancreas (Walker et al., 2008). Taken together, cell sorting appears important to link morphogenesis and differentiation, enabling the segregation of acinar cells at the tips.

2.6 Plexus remodeling into a tree-like structure (E14.5–E18.5)

From E14.5, regions at the center of the pancreas, also termed core, contain a luminal plexus in the process of remodeling, while regions at the periphery display ramifying branches (Fig. 1F; Fig. 3). The central plexus eventually resolves into a ramifying tree at perinatal stages (Bankaitis et al., 2015; Dahl-Jensen et al., 2018) (Fig. 3).

After E14.5, a pervasive epithelial branching process is evident in the outskirt of the organ. Proacinar tips exhibit a variety of cleft morphologies and appear to grow and multiply progressively to generate small lobes with interconnected ductal branches linking multiple tips (Figs. 2 and 3). Concomitant with the increase in organ size, the ductal branches of the periphery multiply and lengthen, apparently by a cleaving and outgrowth process (Bankaitis et al., 2015), although no live imaging corroborates the presence of clefting at these stages. By combining clonal tracing and whole-mount staining with proliferation kinetics, a recent study suggests that expansion and branching of the ductal network is driven by self-renewing precursors localized at the ductal termini (Sznurkowska et al., 2018). This study proposes a quantitative model where equipotent ductal termini, hosting both multipotent progenitors and fate restricted acinar and ductal progenitors, driving a process of stochastic ductal branching and elongation, which terminates when termini move into proximity of neighboring ducts (Sznurkowska et al., 2018). As pro-acinar progenitors are spatially segregated from ductal progenitors after E14.5 (Kopinke et al., 2011; Solar et al., 2009), one thus has to imagine a process where cleavage of the acinar structures is coordinated with the formation of a new connecting duct. In the salivary gland, the ureteric bud and the kidney, clefting is accompanied by microscopic perforations in the basement membrane toward the tip and accumulation of basement membrane components at the cleft bottom (Harunaga, Doyle, & Yamada, 2014). It will therefore be interesting to determine if such ECM remodeling also takes place during peripheral branching in this second branching phase. Notably, establishing if the role of BTBD7 (BTB/POZ domain-containing protein 7) in clefting is conserved in the pancreas, will be informative. Loss of BTBD7 disrupts branching morphogenesis in the kidney, the lung and the salivary gland. In the later, focal induction of BTBD7 is induced at the clef site by fibronectin deposition. BTBD7 together with Snail downregulate E-cadherin, which results in a breakdown of cell-cell adhesion and promotes cleft progression (Daley et al., 2017; Onodera et al., 2010).

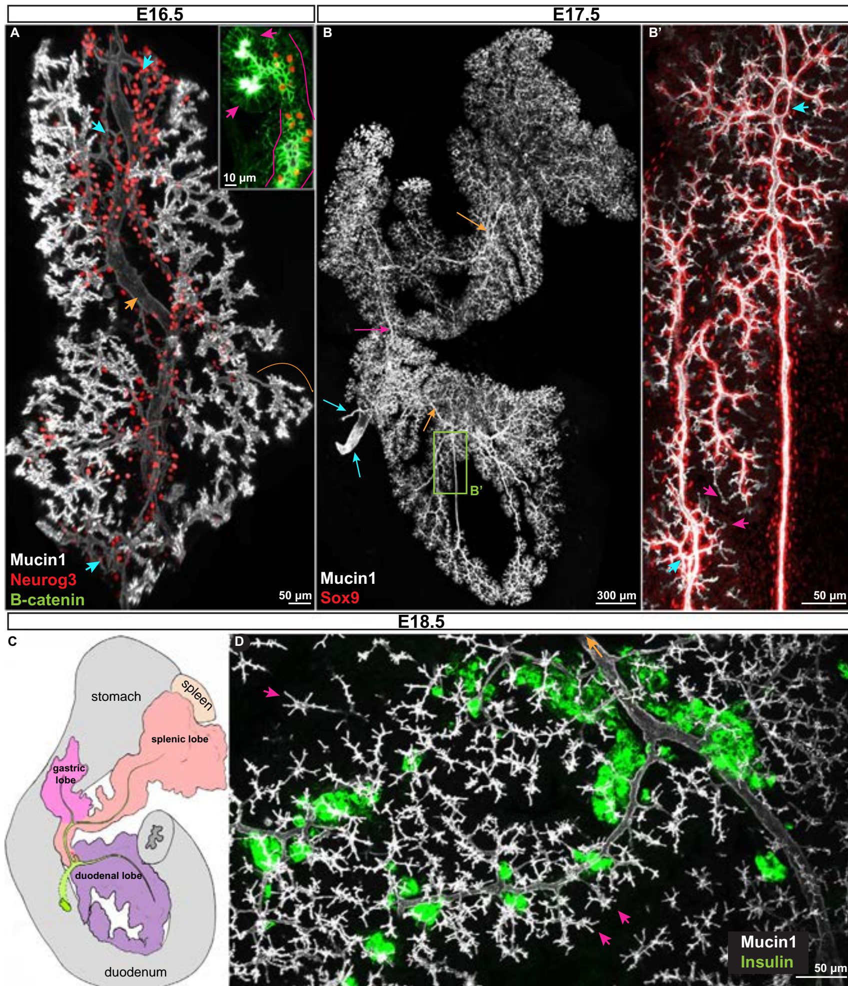


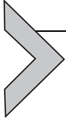
Fig. 3 Plexus remodeling into tree-like structure. A, B, D, 3D projections of a whole-mount staining of the apical marker Mucin1. (A) Shows a "core" area at E16.5 containing plexus region (blue arrow) and a central duct (orange arrow), as well as peripheral branches linking multiple tips (orange bracket). Inset showing the shape of the cells surrounding a duct (pink lines) that connects to two acini (pink arrows). While acinar cells are apically constricted, ductal cells do not present a "bottle neck" shape. Red staining marks endocrine progenitor nuclei (Neurog3 + cells) that are enriched in the plexus area. (B) Whole pancreas luminal network at E17.5. The orange arrow points to principal intralobular ducts, the pink arrow points to principal inter-lobular ducts and the blue arrow points to duodenum connecting-ducts (in this case a small accessory duct is visible). (B′) Zoom in the area delimited in green in (B). SOX9 staining shows the organization of a monolayer of ductal cells around the lumen. For note, red cells not connected to the lumen are mesenchymal cells, some of which also express SOX9. The blue arrows point to some "loops" that remain at this late stage. The pink arrows point to terminal ducts that connect the acini. These ducts are considerably narrower than in A. (C) Schematic of the pancreas around birth. The red arrow is in the common bile duct and points toward the liver. (D) Main intralobular duct (orange arrow) ramifying in smaller ducts. The arrow point toward the connection with the duodenum. Endocrine cells clustering around the mains ducts are labeled in green. Note, the star-like shape of the intercalated duct connecting the acinar cells (pink arrow).

In the core, only few, unclefted, branch tips are visible during plexus remodeling (Bankaitis et al., 2015). At 16.5, while the plexus extends with organ growth, transformation of the plexus into an arborized ductal system is observed (Fig. 3A). This process occurs asynchronously across the organ and is manifested by an increased lumen diameter and the appearance of a central duct lined by flattened cells. At E17.5, interlobular ducts are formed and connect the thinner intralobular ducts (Fig. 3B). By E18.5, only rare and scattered plexus regions remain visible (Bankaitis et al., 2015). At the molecular level, the core plexus remodeling process is poorly understood. RHOA-mediated actomyosin contractility together with Afadin seem essential for this process. When both these proteins are inactivated in the pancreatic epithelium, the plexus is discontinuous and fails to resolve into a hierarchical tubular tree by E18.5 (Azizoglu et al., 2017).

The transition of the pancreatic ductal network from an interlinked mesh to a tree-like structure is also reflected in changing network properties. This transition optimizes the distance from the acinar ends to the duodenum while reducing redundancy in the ductal network (Dahl-Jensen et al., 2018). At E18.5, the network contains almost no redundant connections, only few central polygonal loops are retained (Bankaitis et al., 2015; Dahl-Jensen et al., 2018). From E18.5, the tubular lumens clearly organize in a tree-like structure with a large tube connecting the pancreas to the duodenum and branches of progressively decreasing diameter toward the periphery (Fig. 3C and D). In silico modeling shows that the transition from the E14.5 plexus to this hierarchical structure can be reproduced by pruning the network based on a flux of fluid running through the pancreatic network to the duodenum (Dahl-Jensen et al., 2018). The flux-base model leads to the widening of ducts with greatest flow, i.e. main duct toward the exit, while the redundant ducts are eliminated starting from the periphery (Dahl-Jensen et al., 2018). This is similar to what is reported for the maturing vasculature (Honda & Yoshizato, 1997), in which the flux drives remodeling and vessel diameter. Detection of epithelial fluid secretion as early as E12.5 and the absence of network optimization in organoids, which lack an outlet, support this model though the flow itself remains to be seen and measured (Dahl-Jensen et al., 2018). How this pruning occurs also remains to be determined but since there is little apoptosis during pancreas development it is likely that the cells of pruned connections are recycled (Dahl-Jensen et al., 2018). This may also provide an efficient way to widen or elongate ducts, while remodeling the plexus. Branch elongation occurs via several processes in other organs, such as elongation of cells and intercalation in

the Drosophila trachea (Caussinus, Colombelli, & Affolter, 2008), convergence extension in the kidney tubules (Derish et al., 2020; Karner et al., 2009; Lienkamp et al., 2012) or oriented cell divisions in the lung (Tang, Marshall, McMahon, Metzger, & Martin, 2011) and the kidney (Fischer et al., 2006; Saburi et al., 2008). All these cellular mechanisms could potentially contribute to tube elongation in the pancreas. Orientation of cell division and convergence extension processes are both regulated by the PCP pathway. As we know that the PCP core component, VANGL, is planar polarized in the pancreatic duct, it will be interesting to determine if the length of the branches and/or their orientations is perturbed in VANGL loss of function model (Flasse et al., 2020).

Anatomically, the dorsal pancreas presents two main lobes at perinatal stages: the splenic lobe that extends along the stomach from the duodenum to the spleen forming a typical "anvil-like" shape, and the gastric lobe that expands along the posterior stomach (Figs. 1F and 3C). The splenic lobe projects long lateral branches toward the posterior region of the stomach, their length reaching occasionally (18%) the gastric lobe extremity. On the opposite side, shorter branches extend toward the stomach fundus. This grossly predictable pattern of branches is conserved in the mature organ (Villasenor et al., 2010). The ventral pancreas is considered as a third lobe corresponding in human to the pancreatic head while gastric and splenic lobes are homologous to both the body and the tail in human (Dolensek, Rupnik, & Stozer, 2015). Ducts are typically classified by size and position within the ductal epithelial tree, with the most terminal/intercalated ducts draining into a main intralobular duct, followed by an interlobular duct (Reichert & Rustgi, 2011) (Fig. 3C). The arrangement of the outlets leading to delivery of the pancreatic secretion into the duodenum can vary. The splenic and the gastric intralobular ducts always merge first in a main interlobular duct before opening either directly into the duodenum (10% of the time) or in the common bile ducts (90% of the time) (Fig. 3C). On the other hand, the intralobular duodenal duct always connects to the common bile duct (Watanabe, Abe, Anbo, & Katoh, 1995). Importantly when considering flow, the choledochoduodenal muscle (sphincter of Oddi in human), controlling the opening of the hepatopancreatic duct can be seen as early as E15.5 (55 days in human) though it is not known when it becomes functional (Higashiyama & Kanai, 2020). Histologically, the most terminal ductal epithelial cells (intercalated duct) exhibit a flattened, almost squamous-like shape while intralobular ducts are lined by cuboidal cells and interlobular ducts by a columnar epithelia (Reichert & Rustgi, 2011).



3. Coupling morphogenesis to pancreas differentiation

3.1 Endocrine differentiation

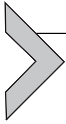
Close spatiotemporal association between pancreatic lumen formation and cell fate determination brings up the question of whether lumen morphogenesis influences pancreatic cell fate decisions. A growing number of studies link the development of epithelial architecture to endocrine fate, leading to the concept of an endocrine cell niche. Pancreatic endocrine cells derive from a Notch-responsive SOX9+ progenitor pool via activation of the endocrine-lineage determinant Neurogenin 3 (NGN3, Neurog3). NGN3 is necessary and sufficient for endocrine-cell birth (reviewed in Larsen & Grapin-Botton, 2017; Rieck, Bankaitis, & Wright, 2012). Following tip-trunk domain segregation, a massive wave of endocrine differentiation occurs, NGN3+ cells being generated at a constant yield from E14.5 to E17.5 (Bankaitis et al., 2015). Cells with high-level NGN3 egress from the epithelial trunk domain and cluster adjacent to the forming nascent islets of Langerhans (Gouzi et al., 2011; Sharon et al., 2019) (Fig. 3A and D). Interestingly, Bankaitis et al. reported that the magnitude of endocrine differentiation (endocrine yield) is highest in the plexus and significantly lower or absent in the distal branches and in the matured duct (Fig. 3A). The replacement of the plexus by a tree-like ductal epithelium is associated with the reduction in the production of new endocrine cells after E17.5 (Bankaitis et al., 2015). Supporting the notion that the plexus acts as an epithelial niche for endocrine cell generation, perdurance of the core plexus following double inactivation of Afadin and RHOA leads to a dramatic increase in endocrine mass by the time of birth, while other cells types (acinar and ductal cells) are unaffected (Azizoglu et al., 2017). Notably, the total number of SOX9 bipotential progenitors remains the same but their prolonged exposure to the core region relative to controls increase endocrine cell differentiation. The time spent by progenitors in the plexus state seems therefore to impact cell fate. In addition, the plexus niche may control the subtype of endocrine cells expressing different hormones (Nyeng et al., 2019). Which signaling pathways are required to maintain a niche favorable to endocrine differentiation is not fully understood but a spatial variation in the level of Notch pathway activity may contribute (Bankaitis et al., 2015). Interestingly, NGN3 loss results in a dysmorphic plexus that is precociously transformed into more mature epithelial duct and branched states highlighting a feedback mechanism from the delaminating NGN3+ cells to

maintain the plexus state (Bankaitis et al., 2015; Magenheim et al., 2011). As NGN3 expression increases in endocrine progenitors, their apical surface narrows, they move to the basal side and eventually lose their apical surface, though they remain connected to the epithelium (Bankaitis et al., 2018). ROCK/non-muscle MyosinII-dependent processes mediate this cell egression sequence and the progression through NGN3 Low and NGN3 High states, connecting epithelial morphogenesis programs and intrinsic cell fate gene regulatory networks (Bankaitis et al., 2018). Moreover, increased NGN3 levels control Snail2 protein expression post-transcriptionally to repress E-cadherin, which is sufficient to promote delamination from the epithelium (Gouzi et al., 2011). Additionally, the inhibition of aPKC via RAC, triggered by activation of the epithelial growth factor receptor (EGFR) signaling in endocrine progenitors, contributes to the reduction of the apical domain size. This narrowing of the apical domain leads to Notch signaling inhibition and NGN3 upregulation to promote endocrinogenesis (Löf-Öhlin et al., 2017). Finally, the Planar Cell Polarity pathway (PCP) also couples epithelial morphogenesis to endocrine cell fate. Inactivation of CELSR2/3 in pancreas progenitors causes a large reduction of β cell birth by preventing NGN3 upregulation in the endocrine progenitors (Cortijo, Gouzi, Tissir, & Grapin-Botton, 2012).

3.2 Exocrine differentiation

As discussed above in the case of Ephrin and STARD3 mutants (see Section 1), disturbing pancreas morphogenesis can lead to a general hypoplasia affecting both endocrine and exocrine differentiation to the same extent but few studies couple defects in pancreas architecture specifically to acinar and ductal differentiation. In zebrafish, Yee et al. identified several mutants presenting altered ductal branching associated with impaired acinar cell differentiation while the endocrine compartment remained unaffected. Although these mutations have not all been mapped and only few mechanistic data are available, this suggests that duct morphogenesis and exocrine differentiation are coupled (Yee, Lorent, & Pack, 2005). In mice, CDC42 inactivation, which blocks epithelial tubulogenesis, resulted in an increase of acinar cells. This phenotype was attributed to the absence of tube formation and the exposure of progenitor rosettes to mesenchyme conducive of acinar differentiation (Kesavan et al., 2009). However, there is also evidence that acinar cell fate and the structure of the ductal network are established independently. Indeed, although JAG1 deletion in the endoderm led to a drastic

switch from bipotent progenitors to proacinar fate, the plexus was unaffected. Only finer morphological features in the intercalated ducts exhibited an abnormal appearance (Seymour et al., 2020).



4. Role of the mesenchyme, endothelial and neural networks in epithelial branching

4.1 Mesenchyme

In many branched organs such as the lungs, kidney, salivary glands, mammary glands, prostate and pancreas, the mesenchyme plays a crucial role in branching morphogenesis. It impacts the development of the nearby epithelium by secreting extracellular matrix and key signaling factors involved in proliferation, polarity establishment and maintenance, migration and differentiation. The mesenchyme is associated with the pancreatic endoderm before the dorsal and ventral anlagen emerge (E9.0–E9.5) and wraps the buds as they form (Golosow & Grobstein, 1962; Sakhneny, Khalifa-Malka, & Landsman, 2019). Mesenchymal cells remain associated with the epithelium, but the proportion of mesenchymal area decreases from 90%, at E11.5, to 11% and 6%, at E15.5 and E18.5 respectively (Landsman et al., 2011). Depleting NKX3.2 positive (BAPX1) mesenchymal cells at precise stages by expression of diphtheria toxin leads to an impairment of epithelial progenitor proliferation. Early mesenchyme ablation (from E9.5 to E10.5) prevents the emergence of both ventral and dorsal anlagen while later ablation (from E13.5) results in an aberrant pancreas morphology by E18.5, the epithelium remaining smooth and the ductal network condensed. Interestingly, similar morphological defects are observed following induction of mesenchyme hyperplasia by downregulation of the Hedgehog pathway in NKX3.2 positive cells (Hibsher, Epshtein, Oren, & Landsman, 2016). As in other branched organs, mesenchyme-secreted factors are involved in this mesenchyme-epithelium crosstalk. In early pancreas development, the best-described factor is fibroblast growth factor 10 (FGF10) (Bhushan et al., 2001). FGF10 is secreted transiently from E9.5 to E11.5 by mesenchymal cells and binds to its receptor FGFR2b located on epithelial cell membranes (Pulkkinen, Spencer-Dene, Dickson, & Otonkoski, 2003). The deletion of either FGF10 or FGFR2b in mouse leads to a decrease in progenitor proliferation, persistent pancreas hypoplasia and a poorly branched network (Bhushan et al., 2001; Jacquemin et al., 2006; Pulkkinen et al., 2003). On the contrary, ectopic and prolonged expression of FGF10 in the pancreatic epithelium induces cystic lumen, an overall enlarged pancreas, increased

epithelial cell proliferation, and decreased numbers of differentiated amylase and NGN3-expressing endocrine progenitors (Norgaard, Jensen, & Jensen, 2003). These observations, together with other studies (Cozzitorto & Spagnoli, 2019), show that FGF10 promotes multipotent progenitor proliferation and maintenance. It notably activates the Notch pathway, maintains PDX1 (Bhushan et al., 2001; Jacquemin et al., 2006; Norgaard et al., 2003) and SOX9 expression (Seymour et al., 2012) and induces PTF1a expression (Jacquemin et al., 2006). When the epithelium starts branching, FGF10 is no longer expressed, consequently its effect on the branching process is indirect, unlike what has been proposed in other branched organs such as the lungs or salivary gland (Jaskoll et al., 2005; Yin & Ornitz, 2020). Other mesenchymal secreted factors play a later role in branching morphogenesis; however, their implication in mesenchymal/epithelial interaction is less clear. Indeed, their expression or the expression of their receptor(s) is not restricted to mesenchymal cells and epithelial cells, respectively. This is notably the case for the canonical WNT pathway, which controls acinar and progenitor proliferation from E12.5 (Baumgartner, Cash, Hansen, Ostler, & Murtaugh, 2014; Heiser, Lau, Taketo, Herrera, & Hebrok, 2006; Heller et al., 2002) and for Activin, which was shown to impact proliferation and branching in several organs, including the pancreas (Ritvos et al., 1995). Though Activin is expressed in the mesenchyme and epithelium, Follistatin, its antagonist, appears strictly mesenchymal (Miralles, Czernichow, & Scharfmann, 1998; Ritvos et al., 1995; Zhang et al., 2004).

This is also the case for EGFR, which controls both epithelial proliferation and the early establishment of apico-basal polarity, which is expected to impact branching (Löf-Öhlin et al., 2017; Miettinen et al., 2000). Of two ligands expressed in the pancreas, EGF, which is expressed in both the epithelium and the mesenchyme, was shown to control polarity and lumenogenesis in this system (Gittes, 2009; Löf-Öhlin et al., 2017). Downstream of EGFR, RAC1 and aPKC control polarity (Löf-Öhlin et al., 2017). EGFR also controls the activity of two metalloproteinases, MMP2 and MMP9, implicated in ECM remodeling (Miettinen et al., 2000).

Some mesenchymal signals also influence branching morphogenesis in the pancreas without controlling proliferation. In early branching steps, the stromal cell derived factor 1 (SDF-1/CXCL12) is expressed in the mesenchyme and in endothelial cells and its receptor CXCR4 in the epithelium and the endothelium (Hick et al., 2009). Pharmacological inhibition of

CXCR4 in E12.5 pancreas explants or explants from CXCR4 knock-outs both reveal a multi-layered epithelium that fails to remodel into a polarized monolayer (Hick et al., 2009).

In addition to the few molecules which have been functionally investigated, proteomics and transcriptome analyses have identified many other proteins/RNAs expressed in the mesenchyme (Russ et al., 2016). Further investigations are needed to discriminate paracrine and autocrine activity of the different extracellular signals and to determine the steps of branching morphogenesis they may control.

4.2 Extracellular matrix

The basement membrane (BM) is a sheet-like extracellular matrix (ECM) forming a scaffold on which epithelial cells are attached. It is a dense network of macromolecules, composed mainly of Laminin, Fibronectin and Collagen, creating a physical barrier between any epithelium and the stroma. In branching morphogenesis, and other tissue remodeling processes, the BM plays a critical role and its composition is highly dynamic (Lu, Takai, Weaver, & Werb, 2011). On a cellular level, modification of the BM is associated with changes in epithelial cell polarity, motility, proliferation and differentiation. In the pancreas, the BM is observed as soon as the primordium stratifies (Villasenor et al., 2010). At least until E12.5, it forms a homogeneous layer at the junction between the mesenchyme and the pancreas. The distribution changes at E14.5, when the BM becomes thinner at the tips, where the branches arise, than in the trunk (Heymans, Degosserie, Spourquet, & Pierreux, 2019; Hisaoka, Haratake, & Hashimoto, 1993). Modifying this repartition, by adding Laminin in explants in culture, reduces pancreas growth and blocks acinar cell differentiation (Anita et al., 2007; Heymans et al., 2019). The thin layer of ECM at the growing tip may facilitate expansion of the branches in the surrounding tissue, in agreement with previous observations made in other branching organs (Lu et al., 2011). Interestingly, after E18.5 the distribution of Laminin is again homogenous (Heymans et al., 2019; Hisaoka et al., 1993; Jiang, Naselli, & Harrison, 2002; Villasenor et al., 2010). Both epithelial, mesenchymal and endothelial cells can secrete ECM proteins and proteases to degrade it. Currently, the knowledge on ECM protein secretion is increasing tremendously thanks to single cell RNA sequencing, and provide important information on the cells producing its different components (Krentz et al., 2018). To degrade the BM, cells can secrete different types of ECM-degrading enzymes: MMP

(metalloproteinases) and ADAMTS (A Disintegrin and Metalloproteinase with Thrombospondin motifs). Members of both families are expressed in mesenchymal cells and epithelial cells (Krentz et al., 2018). As previously discussed (Section 4.1), the activity of MMP-2 and -9 is linked to the activation of the signaling pathway downstream EGFR in epithelial cells (Miettinen et al., 2000), which means that the mesenchyme may participate in BM remodeling. However, no branching defect was observed in MMP2/9 double knock-out or upon over-expression of the wide-spectrum MMP inhibitor TIMP1 (Perez et al., 2005).

To attach to this dynamic matrix, epithelial cells use Integrins, a family of 24 different transmembrane heterodimeric glycoproteins formed by 18 α and 8 β subunits. Among the β subunits, the β1 subunit is abundant in the pancreas and heterodimerizes with several α subunits to act as a receptor for Laminin, Collagen and Fibronectin. Its expression is required to enable the movement of the outer shell cells at E11.5 and affects branching (Shih et al., 2016). However, the effect of this inactivation was not analyzed at late developmental stages. Inactivation strategies targeting more efficiently the acinar cells led to a severe hypoplasia and thereby likely to a reduction of the number of branches, though specific branching defects were not analyzed (Bombardelli et al., 2010). A reduction of acinar cells was also observed upon Laminin1 down-regulation (Crisera et al., 2000; Li et al., 2004).

The extracellular matrix also serves as a growth factor and morphogen reservoir. Some growth factors, such as FGF, WNT and VEGF, mentioned to explain the impact of the mesenchyme and endothelium in pancreas branching morphogenesis, can be sequestrated or released in the BM. The mechanisms involve Syndecans (aka HSPG), transmembrane proteins interacting with the BM and extracellular sulfatases (Rosen & Lemjabbar-Alaoui, 2010). However, to our knowledge, this role has not been studied in pancreas development.

4.3 Endothelium

During the formation of the ductal network, there is a parallel development of the vascular network (Azizoglu et al., 2016). This starts during the emergence of the dorsal and ventral anlagen (E8.5), where the aorta and the vitelline veins interact with the endoderm (Lammert, Cleaver, & Melton, 2001; Yoshitomi & Zaret, 2004). In the beginning, a network of endothelial cells forms in the surrounding mesenchyme (Pierreux et al., 2010).

Once the pancreas starts branching, between E11.5 and E12.5, the vasculature is reorganized and vessels appear intercalated at the base of branches. This reorganization is currently understood as a passive process, resulting from the outgrowth of the pancreas in the vascularized mesenchyme, rather than being the result of sprouting of the blood vessels (Azizoglu et al., 2016). The vascular plexus is then remodeled and forms a hierarchical network connected to the main artery running along the main pancreatic duct. It is important to note that the blood vessels are restricted to the trunk zone and do not wrap the tips where acinar cells differentiate (Azizoglu & Cleaver, 2016).

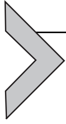
It is well established that the vasculature is essential for organ development and its role is not restricted to oxygen or nutrient supply. Indeed, paracrine signals from endothelial cells are important for organ morphogenesis and cell differentiation, starting at the onset of pancreas formation. Impaired endothelial development in mice knock-out (KO) for vascular endothelial growth factor receptor 2 (*Vegfr2/Flk1*) prevents the formation of the dorsal bud at E9 (Jacquemin et al., 2006; Yoshitomi & Zaret, 2004). Experiments performed in vitro demonstrated that this was due to the presence of endothelial cell signals affecting the endoderm rather than to signals from the bloodstream (Jacquemin et al., 2006; Yoshitomi & Zaret, 2004). Using the same mutants, Jacquemin et al. found that the aorta was also signaling indirectly by maintaining the dorsal mesenchyme, which secretes FGF10 (Jacquemin et al., 2006). After the initial bud induction, the presence of a network of blood vessels around the pancreas before branching is not necessary for the formation of the ductal plexus and for the initiation of branching since pancreas organoids, in the absence of endothelial cells, can form a ductal plexus and branch (Greggio, De Franceschi, Figueiredo-Larsen, & Grapin-Botton, 2014). However, the presence of blood vessels affects the ratio between ductal and acinar cells and thereby branching (Pierreux et al., 2010). Structurally, the endothelial and ductal networks appear to follow similar spatial patterns (Azizoglu et al., 2016; Pierreux et al., 2010) and mutual regulation has been uncovered. The reorganization of the endothelium is controlled by VEGF expressed in the pancreas by trunk cells (Azizoglu et al., 2016). In the absence of VEGF, there is a decrease of blood vessel density. Acinar cells express lower levels of VEGF possibly explaining why blood vessels associate primarily with ducts. In the absence of VEGF, there is excessive acinar differentiation and excessive growth in vitro. In contrast, when expressing VEGF broadly in the pancreas, or when VEGF is added to E12.5 pancreas explants, there is

reduced acinar differentiation, the pancreas decreases in size and the epithelium is less packed and less branched (Magenheim et al., 2011; Pierreux et al., 2010). This effect may be mediated by an induction effect of the Notch pathway, HEY1 and HEY2, by endothelial signals of unknown nature and subsequent PTF1a repression. Accordingly, Notch inhibition (in pdx1 tTA;TET VEGF E12.5 hypervascularized explants) resulted in the partial rescue of pancreas branching (Magenheim, Ilovich, et al., 2011). In addition, Laminin α1β1ϒ1, a basement membrane protein secreted in part by endothelial cells, becomes transiently more concentrated around the trunk at E14.5 and may indirectly control the ductal epithelium (Heymans et al., 2019).

4.4 Nervous system

The mammalian pancreas is densely innervated by both the sympathetic and parasympathetic nervous systems, which control exocrine and endocrine secretion (Ahren, 2000; Gilliam, Palmer, & Taborsky, 2007; Havel & Ahren, 1997; Taborsky, Ahren, & Havel, 1998). Improvement in three-dimensional imaging revealed that the neural network is enriched around the islets (Hsueh et al., 2017; Tang et al., 2018, 2018; Yang, Kawakami, & Stainier, 2018). It is also coordinated with the epithelial network from early stages of development, with lower densities around the ducts than the islets and very low densities close to acini (Burris & Hebrok, 2007; Plank et al., 2011; Reinert et al., 2014). During embryonic development, neural crest (NC) cells colonize the pancreatic epithelium as soon as E11.5 and will contribute to the enteric plexus. By E12.5, some NC start differentiating into neurons while glial cells are first observed at E13.5. From E14.5, sympathetic innervation enters the pancreas in mice (Borden, Houtz, Leach, & Kuruvilla, 2013) while in Zebrafish vagal parasympathetic innervation precedes sympathetic innervation from the celiac ganglion (Burris & Hebrok, 2007; Yang et al., 2018). From there on, the number of neuronal and glial cells dramatically increases, and most of them are closely associated with endocrine cell clusters (Munoz-Bravo et al., 2013; Nekrep, Wang, Miyatsuka, & German, 2008; Plank et al., 2011). Maturation of neuronal cells takes place during the first weeks of postnatal life. The role of NC-derived cells in pancreas morphogenesis is poorly understood. Sympathetic innervation is required for establishment of proper islet architecture and maturation during development (Borden et al., 2013). However, to our knowledge, no branching or epithelial defect have been

reported following genetic or pharmacological ablation of innervation. Though the vascular and neural networks are closely associated, their interdependence remains a controversial matter (Arntfield & van der Kooy, 2013; Cabrera-Vasquez, Navarro-Tableros, Sanchez-Soto, Gutierrez-Ospina, & Hiriart, 2009; Munoz-Bravo et al., 2013; Nekrep et al., 2008; Plank et al., 2011; Reinert et al., 2014).



5. Conclusion

The pancreas branching process we described, when compared to other branched organs reviewed in this issue, illustrates the diversity of branching mechanisms different tubular organs can adopt. Additional comparison with less studied branched organs, such as the salivary and lacrimal glands, in various species would be needed to understand the diverse strategies to form branched organs and their evolution. It will be important to further assess the existence of fluid flow, its sensors and downstream pathways and its role in cell rearrangement and duct remodeling, both in the pancreas and in other organs. More generally, the influence of tissue mechanics, including luminal flow, as well as the role of forces developed intrinsically in the epithelium and imposed by surrounding tissues on branching morphogenesis, is an area expected to develop in the coming years.

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