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Review

From endoderm to pancreas: a multistep journey

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Abstract. The formation of the vertebrate pancreas is a complex process that typifies the basic steps of embryonic development. It involves the establishment of competence, specification, signaling from neighboring tissues, morphogenesis, and the elaboration of tissue-specific genetic networks. A full analysis of this multistep process will help us to understand classic principles of embryonic development. Furthermore, this will provide the blueprint for experimental programming of pancreas formation from embryonic stem cells in the context of diabetes cell-therapy. Although in the past decade many studies have contributed to a solid foundation for understanding pancreatogenesis, important gaps persist in our knowledge of early pancreas formation. This review will summarize the current understanding of the early mechanisms coming into play to pattern the "pre-pancreatic" region within the endoderm and, gradually, specify the pancreatic tissue.

Keywords. Pancreas, embryonic development, endoderm, diabetes, stem cells.

Introduction

The pancreas is composed of two distinct tissues with separate endocrine and exocrine functions. The exocrine portion, including acinar and duct cells, produces digestive enzymes that promote nutrient absorption in the gut. Mature endocrine cells are grouped into spheroidal aggregates, referred to as islets of Langherans, and secrete hormones that control glucose homeostasis. The majority of the mature pancreatic tissue consists of a continuum of exocrine cells within which the islets (only 1–2% of the volume of the organ) are dispersed [1, 2].

The islets of Langherans contain four principal endocrine cell types defined by the hormones that they secrete. These include insulin-producing beta-cells, glucagon-producing alpha-cells, somatostatin-producing delta-cells and pancreatic polypeptide-producing PP-cells [1, 3]. Minor islet cell types are the D1 cells that produce the vasoactive intestinal peptide, enterochromaffin cells that synthesize secretin [1, 3] and the ghrelin-producing epsilon-cells that have only recently been described [4]. The beta-cells constitute about 70% of the total islet cells in humans and are key metabolic regulators whose loss or dysfunction leads to incurable diseases such as diabetes mellitus [1, 3, 5].

Historically, electron microscopic analysis has opened the way toward recognition of individual cell types in developing as well as adult pancreatic tissue [6, 7] (Fig. 1). More recent development of immunocytochemistry and *in situ* hybridization methods have replaced electron microscopy, allowing the detection of specific terminal products of each pancreatic cell type and/or various genes that are expressed in the developing pancreas.

Embryologically, the pancreas forms from two distinct thickenings on the anterior endodermal primitive gut tube (also known as foregut in mammals). One thickening arises dorsally, posterior to the anlage of

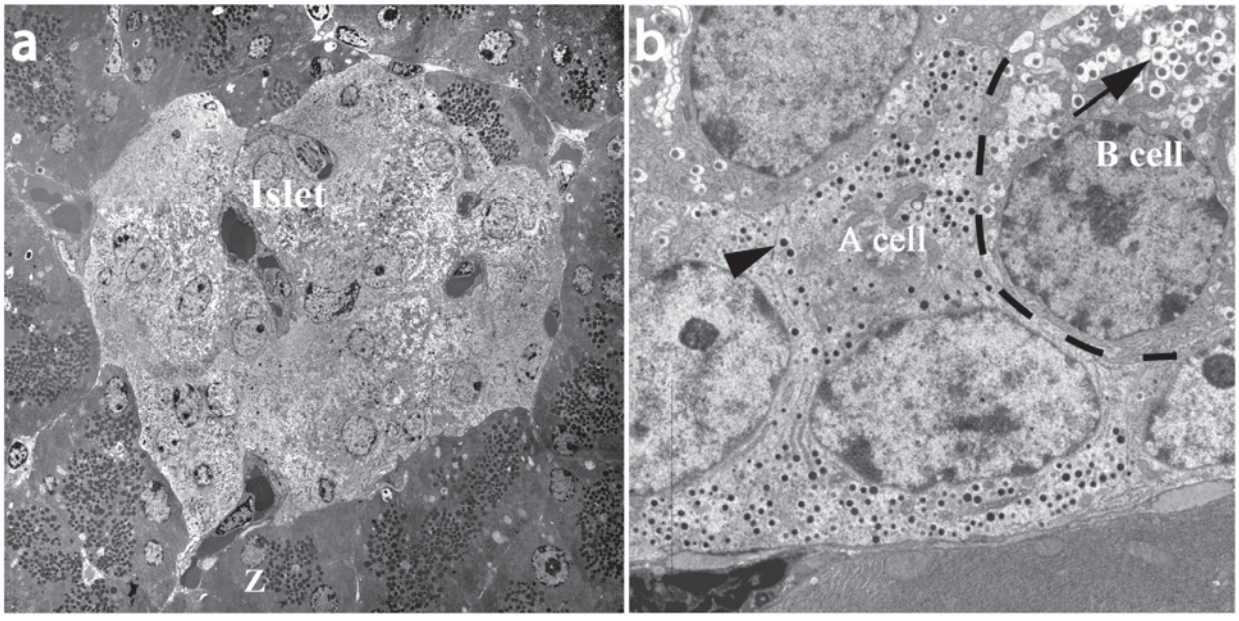

Figure 1. Architecture and histological features of pancreatic tissue. (*a*) Transmission electron micrograph of a human pancreatic islet surrounded by acinar cells. The acinar cells can be recognized by the presence of zymogen secretory granules in the apical cytoplasm (Z) and a well-developed Golgi-complex rER cisternae. Islet cells appear as clusters of pale (less electron-dense) cells displaying secretory granules of different size and density. (*b*) In the center of the islet, a beta-cell (delineated by a dotted line), which contains typical granules with a dense polyhedral core and a pale matrix (arrow), is visible. The polyhedral core is believed to be crystallized insulin. At the periphery of the islet, alpha-cells containing characteristic granules with a dense spherical core surrounded by a clear area are visible (arrowheads).

the stomach, and is referred to as the dorsal pancreatic anlange, while one or two anlagen, depending on the species, are formed ventrally adjacent to the hepatic endoderm [2, 7–10]. Subsequently, these anlagen evaginate into the surrounding mesenchyme as solid epithelial buds, which will undergo proliferation, branching morphogenesis, differentiation and fusion to generate a discrete and fully mature organ prior to birth [2, 8, 10].

The pancreas is the site of virtually incurable diseases, such as diabetes and pancreatic cancer. A variety of benign and malignant tumors may arise from both pancreatic tissues. However, the most frequent tumors are those from exocrine pancreas, in particular the carcinoma of the pancreas, which represents a leading cause of cancer death worldwide [1]. Diabetes mellitus type I is a widespread disease affecting the endocrine pancreatic compartment, specifically the beta-cells (see also above) [1, 5]. Despite the availability of insulin as treatment to temporarily restore glucostasis, diabetes is still incurable. One promising therapeutical approach for the cure of diabetes type I is the replacement of lost insulin-producing beta-cells by embryonic stem (ES) cells that have been differentiated *in vitro*. These future cell-based therapies entirely depend on a better understanding of the early stages of pancreas development. Indeed, the knowledge of each single step that an endodermal cell has to undergo to give rise to a functional differentiated pancreatic cell in the embryo may teach us how to generate its equivalent "*in vitro*".

In this review, I focus on recent advances in the understanding of how pancreatic fate is initially established within the endoderm, emphasizing the contribution of comparative embryological approaches to the elucidation of these very early stages of pancreatic development. For more detailed and comprehensive views of later aspects of pancreatic development, additional reviews should be consulted [2, 8, 11–13].

Molecular mechanisms underlying early stages of pancreatic development

A complex cascade of events, including a series of tissue interactions and activation of a network of regulatory genes, underlies the execution of each pancreatic morphogenetic step and leads to the formation of a mature functional organ (Figs. 2 and 3). The first step in this cascade of events is the establishment of an endodermal region that is competent to respond to subsequent pancreatic inductive signals. During gastrulation, the endoderm is composed of multipotent cells organized as a flat sheet on the outside of the mouse embryo or as a ball of cells at

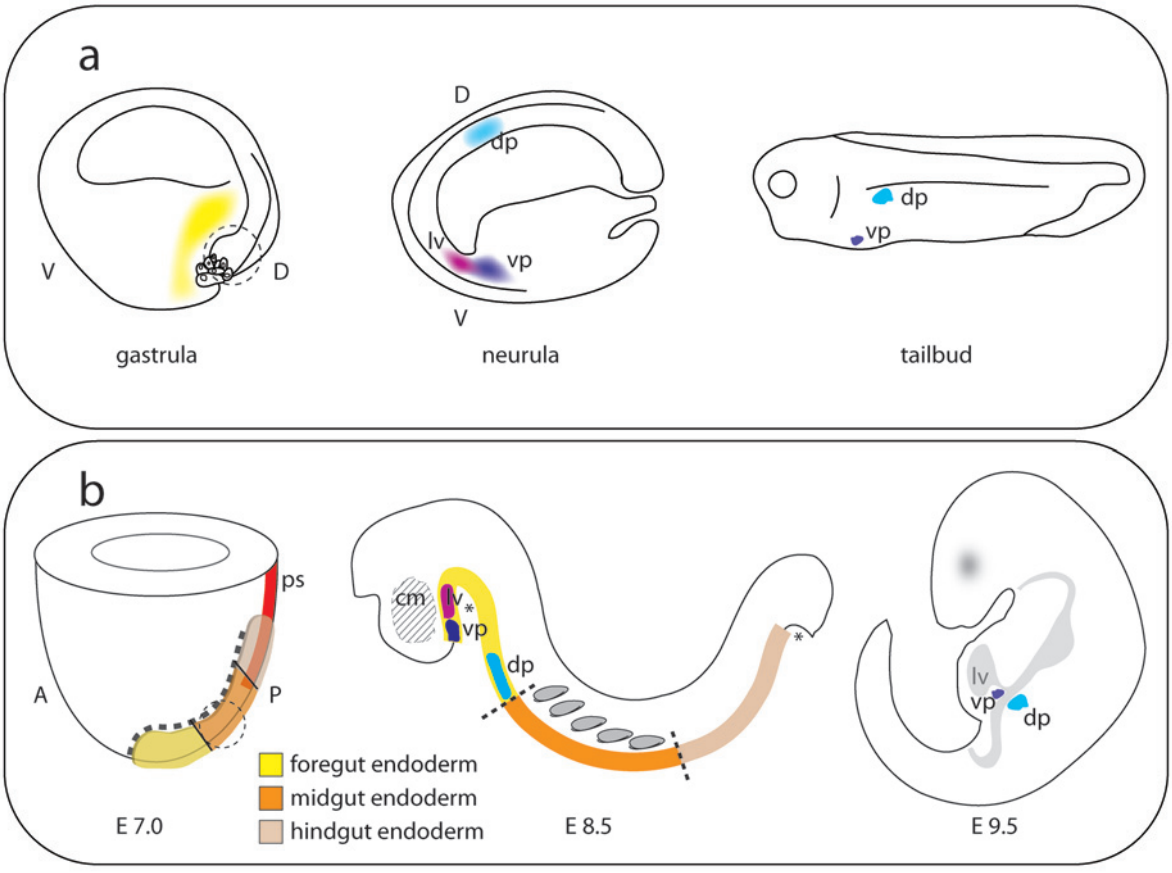

Figure 2. Regionalization of the endoderm from gastrula stage onward. Schematic representation of the development of anterior endoderm and its derivatives in Xenopus (*a*) and mouse (*b*) embryos. At gastrula stage, endoderm that will form the pancreas is in yellow, referred to as foregut endoderm in the mouse (*b*). The organizer in (*a*) and the prospective node in (*b*) are indicated as dotted circles. In neurula stage Xenopus embryo (*a*), ventral anterior endoderm containing progenitors of ventral pancreas (vp) and liver (lv) and dorsal middle endoderm that will give rise to dorsal pancreas (dp) are illustrated [14]. At tailbud stage (*a*) and at E9.5 in the mouse (*b*), dorsal and ventral pancreatic buds are formed. In Xenopus, the two buds migrate toward each other and, following gut rotation, fuse by stage 41 [10]. In the mouse embryo, the elongation and opposing movements toward the midline of the anterior and caudal intestinal pockets (asterisks), which are formed respectively at the anterior and posterior tips of the embryo at the beginning of somitogenesis, promote rostral-to-caudal closure of the primitive gut. In addition, the lateral walls of the embryonic gut fold ventrally, leading to the final closure of the primitive gut at E9–9.5 [15, 19]. Subsequent gut rotation from E12.5 onward brings the ventral pancreatic bud into contact with the dorsal bud and they fuse. The following abbreviations are used: V, ventral; D, dorsal; A, anterior; P, posterior; vp, ventral pancreas; dp, dorsal pancreas; lv, liver; cm, cardiac mesoderm; ps, primitive streak.

the vegetal pole of the Xenopus embryo (Fig. 2) [14–18]. At the completion of gastrulation, endodermal cells undergo morphogenetic movements to generate a primitive gut tube. Evaginations arising from different portions of the tube will give rise to various differentiated organs, such as the thyroid, pancreas, liver and lung [15, 19]. While much is known about how these organs undergo differentiation, growth and morphogenesis, we know little about how different domains of the endoderm become specialized to generate distinct organ primordia at appropriate locations along the gut tube.

The initial commitment of different portions of the endoderm toward specific cell fates according to their position along the anterior-posterior (A/P) and dorsal-ventral (D/V) axes is referred to as regional specification. This patterning may still be reversible [20]. The signals that control regionalization are inductive signals, and the ability to respond to them is referred to as competence.

Endoderm regionalization: fate map analysis. Increasing evidence suggests that regionalization of the endoderm in vertebrates occurs at relatively early developmental stages, beginning with a "pre-pattern" within the endoderm as it first emerges during gastrulation. In the mouse embryo, the definitive endoderm might acquire positional identity even as it exits the primitive streak, such that cells recruited earlier will form the foregut and those recruited later will contribute to the posterior gut [19, 21, 22] (Fig. 2). In frog and zebrafish, gene expression and fate

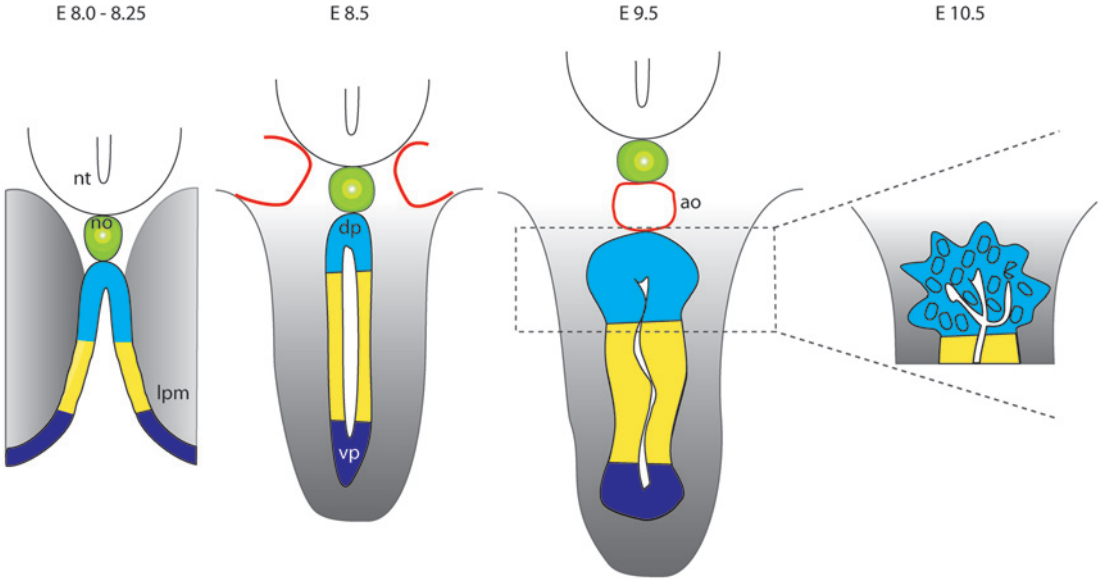

Figure 3. Tissue interactions underlying early stages of pancreas formation. Schematic cross-sections of developing mouse embryos at the level of the pancreatic endoderm. Ventral pre-pancreatic rudiment (vp; in dark blue) is surrounded by lateral plate mesoderm (lpm; in dark gray). Dorsal pre-pancreatic rudiment (dp; in turquoise) establishes sequential tissue interactions: first, with the notochord (no; in green) at the time of tissue specification; second, with dorsal aorta (ao; in red) at the time of budding; and then with surrounding mesenchyme (in light gray), which sustains proliferation, branching and cell differentiation of the pancreatic tissue.

mapping analysis has unveiled the existence of a significant pre-patterning along both A/P and D/V axes within the endoderm long before organogenesis [14, 23, 24]. For instance, in Xenopus embryos, dorsal endoderm explants, once dissected from early gastrula stage embryos and cultured alone, express pancreatic markers, while ventral explants do not [10, 25]. This suggests that as early as gastrula stage, the endoderm is broadly regionalized, and pre-pancreatic endoderm forms in the quarter of the embryo around the dorsal blastopore lip (Fig. 2a).

A detailed fate map analysis of the Xenopus endoderm at neurula stage has shown that anterior/dorsal endoderm gives rise to proximal gut structures, suggesting a link between the A/P and D/V axes in the endoderm, as previously described in the other germ layers [14, 26]. Specifically, the two pancreatic primordia emerge from the dorsal middle region of the endoderm (prospective dorsal pancreatic bud) and the most anterior-ventral endoderm (prospective ventral pancreatic bud) [14]. This anterior-ventral region gives rise to a relatively large number of organ rudiments, including ventral pancreas and liver [14] (Fig. 2).

Even though an accurate fate map of the pre-pancreatic endoderm in the mouse embryo is not yet available, a recent cell labeling study of the mouse foregut just prior to tissue specification [approximately mouse embryonic day (E) 8.25] has shown that descendants of the ventral foregut contribute primarily to the liver bud and, in small number, also to ventral pancreas [27]. These observations are consistent with the fact that a close developmental relationship exists between the two tissues across species. How and when cell fate restriction occurs within this endodermal region, for instance between pancreatic and hepatic fate, remain poorly understood (see also below).

Fate map analysis allows the *in vivo* identification of broad regions of the endoderm that contain, for instance, pancreatic precursors long before organogenesis starts. Ideally, specific gene expression domains should be associated with these endodermal domains, predicted by fate mapping. Unfortunately this has been hampered by the paucity of early regionally restricted molecular markers in the endoderm; indeed, few genes are known to be expressed asymmetrically in the gastrulating endoderm: cerberus, hex (hematopoietically expressed homeobox) and Foxa2 (forkhead box A2) are expressed anteriorly; and cdx2 (caudal type homeobox) and IFABP (intestinal fatty acid binding protein) are expressed posteriorly [18, 24, 28]. This permits subdivision of the endoderm into broad A/P domains but does not address D/V patterning and, more importantly, does not allow accurate tracking of the pre-pancreatic domain from the gastrula stage onward. Future identification of additional regionally expressed markers will further our understanding of how the endoderm is progressively patterned to generate the presumptive pancreatic tissue.

Early extrinsic regulators of the "pre-pancreatic pattern". Commitment of multipotent endodermal cells toward pancreatic fate is a multistep process involving a long-lasting crosstalk between the endoderm and surrounding tissues [16, 18, 29]. According to this view, beginning at the gastrula stage, signals released from the other germ layers would confer positional identity on the endoderm and establish A/P and D/V patterns of gene expression.

The molecular basis of this early endodermal patterning is clearly less understood than that of the ectoderm and mesoderm, but several observations suggest that the molecular signals regulating early events of regionalization are shared among the three germ layers [24, 26, 30, 31]. For instance, in Xenopus and mouse embryos, transforming growth factor (TGF)β signaling influences anterior specification and patterning in both mesoderm and endoderm [31–33]. Furthermore, studies in Xenopus have suggested that the endoderm may be patterned by signals from the Spemann's organizer; bone morphogenetic protein (BMP) antagonists released from the organizer specify dorsal fates within the ectoderm (neural tissue) and the mesoderm (notochord and somites) [30] and may also promote endoderm of dorsal character [34, 35]. In line with this, Sasai et al. [35] have shown that overexpression of the BMP antagonist chordin in animal caps leads to the induction of endodermal markers, such as Edd (endodermin) and the pancreatic marker Pdx1 (pancreatic-duodenal homebox 1), and this activity can be counteracted by co-injection of BMP4. In mouse gastrula embryos, cell-labeling experiments have shown that anterior cells located next to the node and further away from the posterior primitive streak will contribute to the foregut (Fig. 2b) [21, 22]. It is likely that BMP antagonists released from the node, which is homologous to the amphibian organizer, influence D/V patterning of the endoderm in the mouse. The role of endogenous BMP signaling in early endoderm regionalization and pancreas development remains to be investigated.

Slightly later, at early somite stage in the mouse, a similar antagonistic role of BMPs toward pancreatic fate has been described. Both *in vivo* and in tissue explants, BMPs impart hepatic competence to the endoderm and prevent pancreatic fate (see also below) [36]. Conversely, in zebrafish, BMP signaling has been described to promote pancreatic fate [37]. In the zebrafish swirl mutant, which is deficient in BMP2b, expression of the pancreatic marker NeuroD is reduced, whereas chordino (a BMP inhibitor)-mutant embryos show an enlargement of the pancreas, as detected by Islet-1 expression [37]. However, this study does not conclusively address the role of BMPs in patterning of the pre-pancreatic endoderm at early stages, as it is based on a very limited number of late-stage pancreatic markers.

Fibroblast growth factor (FGF) signaling has been shown to play a role in the A/P patterning of the endoderm in addition to its control of mesoderm and neural tube patterning. In mouse embryos, using *in vitro* culture of endoderm isolated at E7.5, and *in vivo* in chick embryos, FGF4 has been characterized as a posterior morphogen for the endoderm [18, 28]. High concentrations of FGF4 promote a posterior/intestinal endoderm cell fate, whereas lower concentrations of FGF4 are required for the pancreatic region to appear [28]. These data are consistent with FGF4 expression in the embryo, as it is strongly expressed between gastrulation and early somite stage in the posterior mesoderm and ectoderm and weakly in tissues adjacent to the posterior foregut, where the pancreas is specified.

Retinoic acid (RA) is also a well-known mediator of A/P patterning in the three germ layers [13, 29]. With regard to the endoderm, gain- and loss-of-function studies suggest that RA mediates early patterning of the endoderm, specifying the position of the pancreas along the A/P axis of the gut in different vertebrate species. In zebrafish and frog embryos, RA signaling seems to be required for pancreas specification during gastrulation [34, 38, 39]; this is supported by the juxtaposed expression domains of the RA-producing RALDH (retinaldehyde dehydrogenase)-positive cells and RA receptor (RAR)-positive responsive cells at the level of the prospective pancreatic region during gastrulation [34, 38, 39]. However, studies in the mouse have suggested a later role for RA [40]. Other differences concerning the RA signaling activity have been highlighted among species. For instance, in mouse and Xenopus, RA is required only for dorsal pancreas development [34, 40, 41], while in zebrafish inhibition of RA signaling has detrimental consequences for both liver and pancreas formation [38]. These discrepancies may reflect differences in the relative locations of germ layer derivatives among species. It has also been unclear whether RA synthesized in the surrounding mesoderm reaches the endoderm and acts directly in the pre-pancreatic endoderm or is relayed *via* the mesoderm as *in vitro* studies in the chick would suggest [42]. Recent transplantation experiments in fish and studies in the mouse using reporters driven by RA-responsive elements (RARE-LacZ embryos) indicate that RA acts primarily on the endoderm [39–41]. It is important to identify downstream target genes executing the RA-mediated differentiation program and to analyze potential crosstalk between RA and other signaling pathways known to control pancreatic development.

Signals required for pancreatic development may function in either a permissive or instructive fashion. Permissive factors allow cells to continue along a pre-specified differentiation program, whereas instructive signals confer positional information, establishing pancreatic identity within naive endoderm. The early signals that pattern the endoderm at gastrulation, including RA and FGF4, render endodermal cells competent to respond to later pancreatic inductive cues and, to some extent, seem to act as instructive signals [18, 34, 39]. However, further investigation is required to better define the molecular identities of early pancreatic instructing signals.

Early intrinsic regulators of the "pre-pancreatic pattern". In addition to signals coming from other tissue layers, it is possible that the formation of boundaries between endodermal regions involves intrinsic regulatory signals that act directly within the endoderm. Future work in this direction may elucidate this point.

Specification of the pancreatic tissue within the endoderm is characterized by activation of a panel of pancreatic cell-autonomous factors. The transcription factor Pdx1 [also known as Ipf1 (Insulin-promoter factor 1) in human and Xlhbox8 in Xenopus] is generally recognized as the earliest specific marker of pancreatic endoderm; however, its expression is induced just prior to organogenesis [31, 32, 43, 44]. In the mouse, Pdx1 expression can be detected in ventral and dorsal pancreatic anlagen starting at E8.5 and E9, respectively [45]. Subsequently, its expression expands to include the duodenum and posterior stomach. Genetic-lineage tracing shows that Pdx1-expressing cells represent the progenitors of all the mature pancreatic cell types, including endocrine, exocrine and duct cells [46]. At later developmental stages (after E13.5 in the mouse), Pdx1 becomes restricted to insulin-expressing beta-cells, where it dictates important functional aspects [8, 11]. Although Pdx1 has been shown to be required for pancreas formation in the mouse [43, 44] as well as in humans [47], initial bud formation and a first wave of differentiation of endocrine cells still occur in Pdx1-mutant mice [48]. Furthermore, ectopic expression of Pdx1 is not sufficient to promote pancreatic differentiation in chick or frog non-pancreatic endoderm [49, 50]. Thus, neither the timing nor localized expression of Pdx1 is sufficient to explain how the pancreas forms in this region of the endoderm.

Recent studies in the mouse and Xenopus have discovered a synergism between Pdx1 and another transcription factor, Ptf1a (pancreatic transcription factor 1a), which together define the pancreatic precursor cell status in the endoderm [49, 51]. Ptf1a is specifically expressed in the pancreatic rudiment, and its onset corresponds approximately with that of Pdx1 expression, starting around E9.5 in the mouse embryo [51]. Thus, it appears that pancreatic fate may be induced in the endoderm by alternative mechanisms independent of or parallel to Pdx1 [25, 48, 50]. However, pancreatic precursor cells require the combination of Pdx1 and Ptf1a activities to undergo subsequent terminal differentiation [11, 49, 51].

Although Pdx1 and Ptf1a are markers of the pancreatic endoderm, their expression starts relatively late, most likely when the "pre-pancreatic pattern" has already been defined within the endoderm. What lies upstream these transcription factors? At present, there is a significant gap in our knowledge of endodermal players acting in the window of time between the early stages of endoderm formation and expression of Pdx1. Molecular characterization of this period of time might shed light on the intrinsic endodermal mechanisms regulating the "pre-pancreatic pattern". It is commonly accepted that specification and differentiation of the endoderm do not occur autonomously [16, 18]. For instance, mouse embryonic endoderm explanted at E7.5 and cultured alone does not express Pdx1 [18]. However, it might be that Pdx1 is not the appropriate temporal marker to analyze, and earlier regional intracellular factors should be taken into account.

Some early endodermal factors have been identified as putative activators of Pdx1 by promoter analysis, including Foxa2 [52] and the one cut transcription factor HNF6 (also called OC-1) [45]. However, ablation of Foxa2 in the gut endoderm before the onset of pancreatic differentiation does not impair Pdx1 activation, though it has later effects on alpha-cell differentiation [53]. Similarly, in HNF6-null mice, Pdx1 induction is slightly delayed but still occurs, and more important consequences have been observed at later stages of differentiation of endocrine precursor cells [45].

Genetic screens in zebrafish and subsequent analysis of the mouse mutant have unveiled a potential function for the POU-homeobox transcription factor vHNF1 (also called HNF1β or TCF2), upstream of Pdx1, in pancreas formation [54, 55]. In vHNF1 zebrafish mutants and null mice, regional specification of the foregut endoderm is perturbed, and reduction of the Pdx1 domain of expression along with concomitant expansion of Shh (sonic hedgehog), a well characterized antagonist of pancreatic fate, is detected [54, 56]. However, vHNF1 does not properly demarcate the early presumptive pre-pancreatic endoderm, as it is also expressed in extra-embryonic endoderm and the gut, from foregut to midgut, at E8–8.5 [54].

Similarly, the early endodermal marker Sox17α, whose disruption in the mouse leads to general deficiency of definitive endoderm and absence of Pdx1 expression at E9.5, shows broad expression in the gut endoderm [57]. Thus, these transcription factors cannot be considered as specific to the pre-pancreatic intracellular program. More likely, they act in concert with other intrinsic and extrinsic regulators that are yet to be discovered.

The Gata family genes might be intracellular factors that potentially define the pre-pancreatic endoderm. Gata4, Gata5 and Gata6 represent a subfamily of zinc-finger transcriptional activators that play a crucial role in endoderm development across different species [58]. Specifically, in zebrafish and Xenopus embryos, Gata5 is abundantly expressed in endodermal cells at early gastrula stage and onwards [59, 60]. By neurula stage, its expression becomes restricted to the anterior endodermal region that will form liver and pancreas according to fate map analysis [14, 60]. Loss-of-function studies in Xenopus and genetic evidence from the zebrafish Gata5 mutant, faust, have demonstrated its requirement for the development of anterior endodermal derivatives, including the liver and the pancreas [59, 60]. Thus, Gata5 may represent the link between early endodermal formation and later endodermal region-specific genes. However, the cascade of molecular events downstream of Gata5 in the endoderm leading to Pdx1 induction has yet to be defined.

A similar role for mouse Gata5 in endoderm development has not been described [61]. One explanation for this discrepancy might be that functional redundancy exists among different members of the Gata family, and another family member may compensate for the loss of Gata5 in the mouse. Indeed, recent studies using transient transgenic mice that express a dominant-negative Gata6-Engrailed driven by the Pdx1 promoter have suggested a role for this member of the family in pancreas formation [62]. Further analysis is required to determine whether the Gata factors act coordinately at earlier stages to establish the pancreatic domain in foregut endoderm. This might reproduce the "hepatic specification paradigm", in which Foxa1 and Foxa2 have been shown to cooperate to establish competence of the foregut endoderm to receive and respond to pro-hepatic signals [63].

Finally, genetic analyses of the contribution of many endodermal transcription factors toward organogenesis have been hampered because of their expression in the extra-embryonic endoderm of the mouse. For instance, $Gata6^{-/-}$ mice fail to develop beyond gastrulation due to defects in extra-embryonic function [64]. A conditional loss-of-function allele of Gata6 has been recently generated by Stephen Duncan's laboratory [64], and this will help to move the field forward. In addition, vertebrate model systems devoid of extra-embryonic endoderm, such as Xenopus and zebrafish, remain of enormous value for dissecting the early events of regionalization of the endoderm.

Permissive pancreatic tissue interactions: the emergence of differences between dorsal and ventral bud

After gastrulation, as a result of morphogenetic movements, the endoderm forms the primitive gut tube and establishes a new set of tissue interactions. Accordingly, the pre-pancreatic endoderm is exposed to a sequence of distinct mesodermal cell populations, such as the notochord, dorsal aorta and lateral plate mesoderm, which lead to final pancreatic differentiation [8, 12, 29]. These surrounding tissues release permissive signals acting upon endoderm that has already received a "pre-pancreatic pattern" during gastrulation.

It is at this developmental time (approximately head-fold to early somite stage in vertebrates) that differences between dorsal and ventral pancreatic rudiments start to become evident (Fig. 2). For instance, specification of the two buds occurs slightly asynchronously, as detected by Pdx1 expression, which starts ventrally at E8.5 in the mouse and dorsally 12–16 h later [45]. Later in development, the two buds fuse to form the definitive pancreas [2]. However, despite the fusion and consequent cell mixing, the dorsal and ventral rudiments contribute, to some extent, to distinct portions of the fused pancreas. For instance, in humans the mature pancreas is an elongated gland composed of a head, body and tail. Morphogenetically, the ventral pancreatic bud forms the posterior part of the head, and the dorsal pancreatic bud contributes mainly to the tail and body of the organ [3]. In addition to this distinct anatomical contribution, regionalization of hormone expression between dorsal and ventral derivatives is also evident in some species. For instance, in humans the tail is the only part of the pancreas in which PP cell-containing islets can be found [65]. In Xenopus laevis and medaka, analysis of cell type-specific markers has shown that insulin expression is confined exclusively to the dorsal pancreas [10, 25, 66]. In zebrafish, undeniable evidence of existing differences between the differentiation and morphogenetic programs of the two buds came from the analysis of the heart and soul (has) mutant, in which the two pancreatic buds never fuse [9]. In has mutant embryos, the pancreatic duct and exocrine tissue are formed in the anterior/ventral bud,

whereas the dorsal/posterior bud generates the islets [9].

The differences between the morphogenetic and differentiation programs of dorsal and ventral buds can be primarily ascribed to different tissue interactions established by the two endodermal domains during the course of development (Fig. 3).

Dorsal pancreas. Dorsal pancreatic endoderm lies at the midline of the embryo and establishes direct contact with the notochord at the time of its specification. Studies in different species have described the notochord as a source of signals (including activin and FGFs) that, by repressing Shh in the underlying endoderm, permit expression of Pdx1 [56, 67]. Subsequently, fusion of the two aortic rudiments, around E9 in the mouse, displaces the notochord, and direct interaction between endothelial cells and dorsal pancreatic anlage is established. This promotes the appearance of the first differentiated endocrine cells [11, 68]. Examination of mice deficient for the receptor tyrosine kinase flk1 (also known as Kdr), which lack endothelial cells, has shown the absence of dorsal pancreas emergence and suggested a direct link between this tissue interaction and activation of Ptf1a [69]. However, the failure of Ptf1a induction is not solely responsible for the failure of dorsal pancreatic budding in $flk1^{-/-}$ mice, as $Ptf1a^{-/-}$ embryos develop an initial dorsal pancreas [51, 70]. More recent investigations have pointed out a relay network between endothelium and surrounding mesenchymal cells in promoting dorsal pancreatic morphogenesis subsequent to pancreatic specification [71]. According to this model, endothelial cells would promote dorsal pancreas development indirectly by supporting survival of dorsal mesenchymal cells.

It is still debated whether cell interactions or bloodstream factors mediate the endothelium effects on pancreas development. Whereas tissue explant assays support the first assumption [69, 71], circulating factors, such as shingosine-1-phosphate, have been recently shown to promote budding of the pancreatic endoderm by triggering dorsal mesenchymal cell proliferation [72]. It is likely that the two components work together or in parallel.

Apparently, the role of vascular endothelium in pancreas development is not conserved across species. Zebrafish Cloche mutant embryos, which lack most endothelial cells, show normal pancreas morphogenesis as well as endocrine and exocrine differentiation in both pancreatic rudiments [9]. This is another example of possible differences between fish and amniotes and might be explained by compensatory signals deriving from other tissues.

After specification and initial bud formation, pancreatic tissue still requires interaction with the mesenchyme for subsequent proliferation, branching and cell differentiation [73–75]. For instance, mice mutants for the Islet-1 [76] or N-cadherin [77] genes do not exhibit lateral plate convergence around the dorsal pancreatic bud and show a sharp downregulation of Pdx1 expression, together with arrest of organ development after initial pancreatic budding has occurred. In addition, mesenchymal signals, such as FGF10 and FGF7, have been described as critical for proliferation and branching morphogenesis of pancreatic cells in mice and humans [73, 78].

Importantly, the mesenchyme that surrounds the dorsal pancreas also contributes to the development of mesodermal derivatives, such as the spleen [79]. Careful analysis of mice lacking the transcription factor Bapx1 has shown that the asplenia observed in these embryos is accompanied by the occurrence of metaplastic conversion of most dorsal pancreatic tissue into gut-like structures [79]; this suggests that Bapx1 dictates organ position and separation of the spleen from dorsal pancreas. Likewise, FGF10, another mesenchymal factor, has been described to demarcate the boundaries between the hepatopancreatic duct system and connected organs, such as the liver and pancreas, in zebrafish [80]. These studies underscore that a certain plasticity still exists within the cells of the developing foregut endoderm, and spatial and temporal relationships among tissues have to be tightly regulated *in vivo*.

Ventral pancreas. During morphogenesis, the ventral pancreatic endoderm is exposed to a completely different set of tissue interactions compared to its dorsal counterpart. In vertebrates, ventral pancreatic endoderm lies close to the prospective hepatic bud, and they are both exposed to lateral plate mesoderm and its derivative, the septum transversum mesenchyme, as well as to cardiac mesoderm [29, 36]. Recent work from the Zaret laboratory has proposed the existence of a bipotent hepatic-pancreatic progenitor in the ventral endoderm [81]. Supposedly, the fate of the entire ventral foregut endoderm is pancreatic by "default"; however, this can be prevented and diverted to hepatic by signals from cardiac mesoderm. Indeed, ventral endoderm isolated from 2- to 6-somite stage mouse embryos and cultured alone without cardiac mesoderm expresses Pdx1 prior to the time of pancreatic specification *in vivo* (about 7- to 9-somite stage), but does not express the hepatic marker albumin [81]. Candidate liver-inductive signals from the surrounding mesoderm are FGFs and BMPs, and they seem to act in concert [36].

Conversely, in chick endoderm-mesoderm recombinants experiments, lateral plate mesoderm appears to be a potential source of pancreatic instructive signals, including BMPs, that are capable of inducing Pdx1 in naive endoderm [42]. However, BMP is not sufficient to induce Pdx1 when added alone to endoderm culture, and its effect is mediated *via* the mesoderm, most likely by the activation of additional signals [42]. These opposite effects of BMP might be ascribed to differences in the origin and positioning of the pancreatic precursors within the endoderm among species. Alternatively, BMPs might have different effects at different stages of pancreatic development, depending on the competence of the endoderm to respond to such signals. An answer to this might come from temporally and spatially controlled inactivation of BMP signals during development.

It is tempting to speculate that the future pancreatic region in the mouse embryo possesses unknown protective mechanisms against FGF and/or BMP signaling. A mechanism by which ventral pancreas may escape inhibitory signaling has been proposed by Ken Zaret and colleagues [82]. In this elegant study, they show that the homeobox gene Hex controls proliferation of ventral foregut endoderm, allowing the prospective ventral pancreas endoderm to grow past the cardiac mesodermal signaling center. However, this mechanism alone cannot be sufficient against BMP, which is a morphogen able to act even at great distances [83].

Finally, formal proof of the existence of a single cell within the ventral endoderm having the dual potential to differentiate along the hepatic and pancreatic lineages is not yet available. Alternatively, it is possible that committed precursors of both liver and pancreas coexist in the same region. If a common precursor happens to be isolated, then it would be of great interest to identify factors that regulate the pancreatic *versus* hepatic fate switch *in vivo*. This information might be crucial for the establishment of cellular therapies based on transdifferentiation between liver and pancreas. In turn, studies in ES cells may help to discover a possible bi-potential precursor through controlled differentiation to endoderm, followed by clonal analysis of the fate of cellular progeny.

Genetic studies in the mouse have indicated that, in addition to the differences in the mesenchyme that surrounds the two endodermal regions, distinct intrinsic regulators exist within the dorsal and ventral pancreatic buds. For instance, in Pdx1- and vHNF1-knockout mice, dorsal bud formation is initiated, while the ventral bud is undetectable [43, 44, 48, 54]. Similarly, in Ptf1a$^{-/-}$ embryos, ventral cells do not bud, and their cell identity is redirected to an intestinal fate, demonstrating again the plasticity of this endodermal region [51]. On the other hand, Hlxb9 (homeobox gene B9) is required specifically for dorsal pancreas formation [84, 85]. However, as all these transcription factors are known to be expressed in both regions, it is likely that cooperative interactions with the surrounding signals or still unknown endodermal regulators are responsible for their differential impact on ventral and dorsal pancreatic endoderm.

Notably, most of the signaling factors that have been reviewed here seem to be able to influence various steps of pancreatic development and sometimes exert opposite functions. One paradigmatic example is FGF signaling in the mouse, which acts in a pro-pancreatic manner at late gastrula stage and, conversely, as an antagonist of ventral pancreatic fate later [18, 36, 81]. This provokes the question of how the same factor regulates such a range of developmental responses. One obvious answer is that the competence of the tissue to respond to the signal must evolve and change over the course of development. Alternatively, the opposite effects of FGF signaling might be due to different biological activities of the various FGF ligands that are expressed in tissues surrounding pancreatic endoderm during the course of development.

Finally, other signaling pathways have been shown to be dynamically expressed in the developing pancreas and to play crucial roles in pancreatic organogenesis and cell differentiation, including the Wnt and Notch pathways [86, 87]. In addition, a complex network of transcription factors acting on subsequent steps of differentiation of each pancreatic cell lineage has been characterized [11]. For reasons of space and because these players act at later developmental stages, they are not discussed further here. These aspects are covered in great detail in other reviews [11, 12].

In the quest for novel regulators of early pancreatic development

Despite recent progress that has been made in the field of pancreas development, it seems that there are still many open questions. In particular, much remains to be discovered about how the endoderm is patterned and subdivided into specific organ domains. In addition, the number of pancreatic instructive signals identified so far is extremely limited. Hopefully the list will grow soon, as these signals represent crucial components of any protocol to induce differentiation of stem cells into pancreas cells. Thus, it is likely that novel early pancreatic regulators remain to be identified and characterized.

As discussed above, significant conservation of the early mechanisms underlying pancreas formation

exists across species. The ease with which embryo and tissue explants can be manipulated will allow the Xenopus, zebrafish and chick models to permit higher throughput analysis of pancreas development than is generally afforded by the mouse. Efforts in this direction have already started and have, indeed, unveiled the role of novel mechanisms in regulating pancreas specification. For instance, an expression cloning strategy in Xenopus and genetic screen in zebrafish have led to the identification of two RNA-binding proteins, Vg1RBP in frog and nil-per-os in fish, as novel pancreatic factors [25, 88]. These studies have highlighted, for the first time, the contribution of post-transcriptional mechanisms in endoderm/gut development. Further investigations will elucidate the mechanisms of action of these RNA-binding proteins in endodermal patterning and regional specification and their integration with the known pancreatic gene network.

Using a large-scale differential *in situ* hybridization screen, Costa and Zorn [89] have identified a small set of genes that show spatially restricted expression in the endoderm before organogenesis in Xenopus embryos. This study clearly suggests that endoderm has significant molecular patterning as early as neurula stage and provides us with some useful markers for studying endodermal patterning.

An ethyl-nitroso-urea (ENU)-mediated mutagenesis screen specifically aimed at the identification of novel genes required for the specification of beta-cells and islet morphogenesis was performed recently in zebrafish [90]. Several interesting mutants affecting different processes, such as pancreatic endoderm specification, insulin cell specification and migration, are now available for further investigation.

Furthermore, zebrafish embryos have become an attractive model for studying beta-cell regeneration. Two recent reports have described a genetically inducible method for beta-cell ablation based on the bacterial gene nfsB, encoding the enzyme nitroreductase, and the prodrug metronidazole as a substrate [91, 92]. This represents a unique resource to conduct large-scale screens for pharmacological and genetic modifiers of beta-cell regeneration and can be extended to study earlier developmental aspects, such as tissue interactions, by changing the promoter driving nfsB.

From the embryo to ES cells: future perspectives

The lessons learned from comparative developmental studies will enrich the basic understanding of pancreas development and, hopefully, contribute to the advancement of translational efforts toward therapeutic beta-cell neogenesis in humans. Indications that this is the right path to follow came from recent studies in human ES cells: D'Amour and colleagues [93] report a five-stage protocol for efficiently differentiating human ES cells into beta-cell progenitors through a series of endodermal intermediates, resembling those occurring during pancreatic development *in vivo*. Thus, it is possible to fully recapitulate the process of pancreas and beta-cell differentiation in ES cells based on the current knowledge of normal pancreatic development. However, to attain the final goal of having a renewable source of beta-cells for therapeutical purposes, for example for people with diabetes, there are still a number of major obstacles to overcome. One major consideration is the autoimmune reaction that causes diabetes type I in the first place. In addition, differentiation protocols of human ES cells need to be optimized and become less empirical. For instance, with regard to the endodermal lineage, new methods need to be developed to distinguish definitive endoderm from extra-embryonic endoderm in human ES cell cultures. This problem can be solved in part through the use of the chemokine CXCR4, which can distinguish embryonic versus extra-embryonic tissues, but CXCR4 is not a specific marker of endoderm [94, 95]. More promising information has come from a recent microarray analysis of mouse embryonic endoderm that provided us with a "gene expression signature" of the two populations [96]. As a next step, these "endoderm signature" genes will serve as valuable markers for purification and characterization of endoderm differentiated from mouse and human ES cells.

Finally, the human ES cell model represents an unprecedented opportunity to address how much of the information obtained from the study of development in classic embryology systems is relevant in the context of human development. This will be fundamental in the context of our own pancreatic development, as little of what is currently known comes from the human point of view.

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