Epithelial dynamics of pancreatic branching morphogenesis

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SUMMARY

The mammalian pancreas is a highly branched gland, essential for both digestion and glucose homeostasis. Pancreatic branching, however, is poorly understood, both at the ultrastructural and cellular levels. In this article, we characterize the morphogenesis of pancreatic branches, from gross anatomy to the dynamics of their epithelial organization. We identify trends in pancreatic branch morphology and introduce a novel mechanism for branch formation, which involves transient epithelial stratification and partial loss of cell polarity, changes in cell shape and cell rearrangements, de novo tubulogenesis and epithelial tubule remodeling. In contrast to the classical epithelial budding and tube extension observed in other organs, a pancreatic branch takes shape as a multi-lumen tubular plexus coordinately extends and remodels into a ramifying, single-lumen ductal system. Moreover, our studies identify a role for EphB signaling in epithelial remodeling during pancreatic branching. Overall, these results illustrate distinct, step-wise cellular mechanisms by which pancreatic epithelium shapes itself to create a functional branching organ.

KEY WORDS: Epithelium, Stratification, Polarity, Branching, Microlumen, Tubulogenesis, Plexus, Remodeling, Eph, Ephrin, Mouse

INTRODUCTION

The mature pancreas consists of a highly branched, tubular epithelial tree-like network, which makes up the bulk of the pancreas and is primarily exocrine in function. Ductal branches are capped at the tips by acinar cell clusters and are connected to a trunk-like central duct, which shuttles digestive enzymes into the duodenum (Pictet and Rutter, 1972). Pancreatic branches ramify, as they originate within the acini as small ducts lined by centroacinar cells, followed by intercalated, intralobular and finally interlobular ducts – all of increasing caliber (Bonal and Herrera, 2008). In recent decades, understanding of the ontogeny of the pancreatic tree has grown tremendously, driven in large part by studies of endocrine differentiation, as a basis for cell replacement therapies to treat diabetes (Murtaugh and Melton, 2003; Oliver-Krasinski and Stoffers, 2008; Slack, 1995).

The entire pancreatic tree arises from an endodermally derived protodifferentiated epithelium (Cleaver and MacDonald, 2009). Multipotent progenitors (MPCs) located at branch tips give rise to the vast majority of pancreatic cells, including acinar, endocrine and ductal lineages (Gu et al., 2003; Zhou et al., 2007). As pancreatic epithelium differentiates during late gestation, acinar cells mature at branch tips, while ductal and endocrine progenitors emerge in branch stalks. Endocrine cells escape from their epithelial neighbors via delamination and coalesce into islets. The remaining tubular epithelium gives rise to the ductal branches that make up the pancreatic tree. The exocrine (acinar/ductal) and endocrine compartments thus share their origins within the pre-pancreatic epithelium and grow coordinately, albeit via dramatically different mechanisms, together forming a dual-function integrated organ.

Although a number of studies have provided excellent anatomical models of the early pancreatic bud development (Jorgensen et al., 2007; Pictet and Rutter, 1972), no in depth studies exist of its early cellular organization or later branching patterns. Shortly after evagination, pancreatic buds present as dense, convoluted epithelial structures. Initiation of branch formation has been hypothesized to occur as a result of random buckling of the endodermal epithelium (Gittes, 2009; Slack, 1995). However, the ontogeny of developing pancreatic branches remains poorly understood.

A few reports suggested that pancreatic branch, and thus lumen formation, occurs via ‘microlumen fusion’ within the progenitor epithelium (Hogan, 1999; Jensen, 2004). Recently, an elegant study reported this unusual process and demonstrated that it requires proper epithelial cell polarity (Kesavan et al., 2009). However, the mechanisms by which the pancreatic epithelium transforms into a highly branched organ remain to be determined. Understanding epithelial morphogenesis and branching in the developing pancreas has been limited by a lack of descriptive characterization, both at the macroscopic and cellular levels. As a result, no genetic models have been identified that affect these processes, in part due to the lack of an established baseline for comparison.

Here, we provide an anatomical model of pancreatic branching based on the analysis of hundreds of developmental intermediates. This model will serve as reference for future comparison and analysis of mutants, allowing identification of molecules that regulate pancreatic epithelial morphogenesis and branching. We demonstrate that the developing pancreas displays predictable trends in overall shape and in the elaboration of specific branches. In addition, we propose a new model for pancreatic branching in which a multi-lumen epithelial network remodels into single-lumen ramifying branches. This process involves a number of cellular events, including transient epithelial stratification, dynamic cell polarity shifts, asynchronous apical cell constriction and rosette organization, as well as microlumen formation and fusion. This mechanism of branch formation is novel and in stark contrast to classically described branching models (Andrew and Ewald, 2009). Significantly, we identify a genetic model in which these processes are affected. We demonstrate that EphB signaling is required for...
proper pancreas development, as pancreatic epithelium and predicted branching patterns are disrupted in the absence of EphB receptor function.

MATERIALS AND METHODS
Mice and embryo handling
Experiments were performed in accordance with protocols approved by the UT Southwestern Medical Center IACUC. E8.0-E18.5 CD1 embryos were fixed in 4% PFA/PBS overnight at 4°C, rinsed in PBS and stored at −20°C. EphB2lacZ/lacZ/EphB3−/− were maintained on a CD1 background. Littermates or CD1 staged matched embryos were used as controls.

Whole-mount in situ hybridization and RNA probes
Experiments were carried out as previously described (Villasenor et al., 2008). Probes used were: Barx1 (~800 bp); ephrin B1 (~800 bp); Hox11 (~2067 bp, BC018246); Ngn3 (539 bp) and Sox9 (2305 bp, BC023953) from Open Biosystems. Images were taken using a NeoLumar dissecting microscope (Zeiss) and a DP-70 camera (Olympus).

Pdx1-lacZ embryo generation and β-galactosidase reaction
Pdx1-lacZ heterozygous males were mated to CD1 females. Pancreata were fixed in 4% PFA/PBS for 15 minutes, rinsed in PBS and stained for β-gal, as previously described (Villasenor et al., 2008). Images were taken with a NeoLumar stereomicroscope (Zeiss) using a DP-70 camera (Olympus).

Immunofluorescence of paraffin sections
Sections were deparaffinized and treated with R-Buffer A or R-Buffer B in a 2100 Retriever (Electron Microscopy Sciences); blocked with CAS-Block (In Vitrogen); and incubated with primary antibody. Concentrations of primary antibodies were: rabbit α-αPKC (1:500 Santa Cruz); goat α-β-catenin (1:50 Santa Cruz); rabbit α-collagen IV (provided by R. Brekken); mouse α-E-cadherin (1:200 Invitrogen); rabbit α-ezrin (provided by T. Carroll); guinea pig α-Glucagon (1:600 provided by R. MacDonald); mouse α-GM130 (1:500 BD Biosciences); rabbit α-laminin (1:200 Sigma); rabbit α-Par3 (1:500 Invitrogen); rabbit α-Ptf1a (1:1000 provided by R. MacDonald); and mouse or rabbit α-ZO1 (1:50 Invitrogen). Secondary antibodies were from Jackson ImmunoResearch Labs or Invitrogen. Images were acquired on a LSM510 META Zeiss confocal microscope. Fluorescence intensity ratios of β-catenin were calculated by intensity of delineated cell membrane versus cytoplasm (12 different raw micrographs), within arbitrary areas containing ~15 cells each (both cap and body cells), from three individual buds (three wild type and three EphB mutant) with ImageJ software. Student’s t-test was used to calculate P value for all graphs.

Whole-mount immunofluorescence of pancreatic guts
Fixed pancreatic guts were permeabilized with 1% Triton X100/PBS for 1 hour, blocked with CAS-Block Reagent (Invitrogen), incubated with armenian hamster anti-nuc1 (1:200 Thermo Scientific) overnight at 4°C, followed by a Cy3 secondary antibody. Pictures were taken every 1 μm using a Zeiss Stereo Discovery, V12 with Pentafluor S. Image J software was used to reconstruct images.

3-D reconstructions
PFA-fixed pancreata were sucrose embedded in optimal cutting temperature (OCT) medium. Frozen sections (30 μm) were treated as described above for paraffin sections. Z-stacks were taken every 1 μm with an LSM510 META Zeiss confocal microscope. Three-dimensional reconstructions were made using Imaris software.

RESULTS
Anatomy of the embryonic pancreas
To understand the ontogeny of pancreatic branches, we examined and compared between 20-50 embryonic Pdx1-lacZ pancreata (Offield et al., 1996) at each stage of embryonic development (Fig. 1A-I; 10 shown per stage in Fig. S1 in the supplementary material). We identified common features of the embryonic dorsal pancreas, defined conserved landmarks not previously noted and created a representative anatomical model that summarizes distinct and reproducible trends in pancreatic epithelial morphogenesis (Fig. 1J).

Splenic and gastric lobes of the dorsal pancreas (E12.5-birth)
As previously described (Bock et al., 1997), the murine dorsal pancreas is composed of a large splenic lobe and a smaller gastric lobe (Fig. 1J). The splenic lobe extends along the stomach from the duodenum (proximal) to the spleen (distal), making up the bulk of the dorsal pancreas. The gastric lobe extends along the posterior stomach (Fig. 1D-H,J).

The anvil-shaped tail (E12.5-birth)
This comprises the distal and largest region of the splenic pancreas (Fig. 1C-H,J). Terted ‘tail’, in analogy with the distal region of the human pancreas (Cleaver and Melton, 2004), it takes on a characteristic ‘anvil-like’ shape by E14.5. The tail is grossly pyramidal and consists of compound branches.

The ridge (E11.5-birth)
This region forms the apex of the pyramid-shaped tail and runs along the proximodistal axis of the pancreas (Fig. 1). It separates an arbitrarily designated ‘left’ from ‘right’ side of the splenic lobe, when dorsal pancreas is viewed laterally (with stomach behind) (Fig. 1J,K). Ridge is initially recognizable around E11.5 (Fig. 1J), and is prominent from E14.5 (Fig. 1M) into postnatal stages (Fig. 1L).

The heel (E13.5-15.5)
The heel is a transient feature located on the ‘right’ side of the ‘anvil-shaped’ tail. It is a thick, rounded cap of mesenchyme overlying the branching epithelium of the tail, located near the spleen (Fig. 1J).

The identification of ‘ridge’ and ‘heel’ structures was based both on morphological features and on localized expression of mesenchymal genes (Fig. 1N-P). In situ hybridization for the mRNAs of Hox11 (Tlx1 – Mouse Genome Informatics), Barx1 and ephrin B1 (Ephbl – Mouse Genome Informatics) at E14.0 reveals mesodermal subdomains within the pancreatic tail. Barx1 marks the ‘left’ side of the pancreas (Fig. 1N), Hox11 marks the ‘heel’ only (Fig. 1O), and ephrin B1 marks the ‘right’ side of the pancreas (Fig. 1P).

Lateral branches (E13.5-birth)
The pancreas displays ‘lateral’ type branching (Metzger et al., 2008; Puri and Hrebko, 2007). The splenic lobe displays numerous parallel lateral branches on both sides of the ridge, along its proximodistal axis. Long lateral branches extend towards the posterior region of the stomach, on the ‘left’ side (Fig. 1E-H,J), while shorter ones are found on the ‘right’, extending towards the stomach fundus (Fig. 1D-H,J).

Ventral pancreas morphology also displayed recognizable trends of growth during development, including similar patterns and onset of branching (see Fig. S2 in the supplementary material). However, this study focused on the dorsal pancreas.

Trends in pancreatic branching patterns
To recognize branching patterns and trends, pancreata were categorized based on their most easily distinguishable features, such as tail morphology and left lateral branches. The relative frequency of each category was calculated and representative examples assembled (see Fig. S3 in the supplementary material).
We began our analysis at the earliest stage of pancreas development. As described by others (Jorgensen et al., 2007; Offield et al., 1996; Slack, 1995) E9.5 buds appeared in whole mount as ‘fin-like’ epithelial evaginations on both the dorsal and ventral sides of gut tube (see Fig. S3A in the supplementary material) and by E10.5, the dorsal bud appeared ‘fist-like’ (see Fig. S3B in the supplementary material). At these early stages, all pancreata displayed nearly identical gross morphology (see Fig. S1A,B in the supplementary material).

By E11.5, the dorsal bud elongated into a teardrop-shaped structure (see Fig. S3C in the supplementary material) and two forms of the distal tail became distinguishable: blunt or bifurcated. At this stage, branches were not distinguishable as the bud surface was relatively smooth. However, by E12.5, the bud surface became completely covered by stubby, MPC-containing ‘tips’ (Fig. 2A). Throughout this report, we will term these structures ‘tips’ for simplicity.

Between E12.5-13.5, bona fide branch formation initiation could be discerned. By E13.5, pancreatic ‘branches’ appeared as wide protrusions, studded with ‘tips’. Of note, at this stage, the pattern of nascent branches presented the highest level of variation, compared with all other stages (Fig. 2B’ and see Fig. S3 in the supplementary material). We speculate that this high level of observed variation results from the proliferative burst known to occur during the secondary transition, a timeframe when the pancreas is rapidly changing.

From E13.5 to E17.5, the overall morphology of individual pancreata progressively converged in appearance. Although variability remained at the level of single branches, pancreata were readily categorized based on their left lateral branches: type I, no/short left lateral branches; type II, one or two branches midway along the pancreatic axis; type III, many or long lateral branches (see Fig. S3E-I in the supplementary material); and a few pancreas at E13.5 and E14.5 displayed unclassifiable pancreatic morphology (~0-10%, data not shown). By E18.5, pancreas morphology fell into two principal categories: ‘short’ left-branches (Fig. S3J in the supplementary material) and ‘long’ left-branches (see Fig. S3J in the supplementary material). Morphological features (described above) were clearly identifiable in perinatal pancreas (Fig. 1K,L).

Thus, whereas pancreatic branching had been thought to be random, we show that, at least grossly, there is predictable patterning of branches.

**Branch formation: epithelial/ductal plexus remodeling**

To understand initiation of branch formation, we examined the first externally apparent ‘tips’, around E12.5 (Fig. 2A,A’). Given that ‘tips’ consist of MPCs (Zhou et al., 2007), the presumption is that they generate extending tubular branches. We thus expected branch formation to result from perpendicular elongation of individual epithelial ‘tips’ (model I, Fig. 2E). Surprisingly, however, we did...
not observe ‘tips’ of intermediate and variable lengths, suggesting there was no progressive ‘tube-like’ extension of ‘tips’. These short epithelial structures instead remained of fixed and uniform in size throughout pancreatogenesis, but increased in number over time (Fig. 2A’-D’; data not shown).

Indeed, at E13.5, left lateral branches appeared to form from wide lateral protrusions of epithelium that narrowed over time (Fig. 2B-D). This morphology suggested that branches formed via growth and remodeling of the epithelium, rather than by outgrowth of ‘tips’. Our observations thus favor an alternative model for branch growth in which the contribution of ‘tip’ MPCs to branch growth is via longitudinal growth, where ‘tips’ repeatedly divide to generate new ‘tips’, as well as intervening epithelium, resulting in overall extension of the underlying branch epithelium (model II, Fig. 2F).

Because the dynamics of pancreatic branch growth were unexpected, we investigated the emergence of branches from a different perspective: examining branch lumens using mucin 1 (Muc-1) staining (Pierreux et al., 2006) (Fig. 3). Surprisingly, we found that prior to appreciable branch appearance, the pancreatic epithelium had already established a complex internal network of lumens, or luminal ‘plexus’ (Fig. 3A,E). This plexus then remodels to a hierarchical system of tubes (Fig. 3E-G). Interestingly, pre-branch epithelial protrusions contained multi-lumen plaxi. As these protrusions refined into branches, their multi-lumen networks progressively remodeled into single lumens (Fig. 3D). Pancreatic branch lumens therefore developed before any external evidence of branching and the multi-lumen tubular plexus coordinately extended and remodeled into a ramifying single-lumen tree (Fig. 3A-C). To our knowledge, this is the first evidence for this type of epithelial tube and branch formation.

The pancreatic epithelium: transient stratification and de-stratification

To investigate the cellular basis of this unusual branching morphogenesis, we assessed the dynamics of pancreatic epithelial organization during development (Fig. 4). Using E-cadherin immunofluorescence, we determined that the pancreas arises from a single-layered epithelium that experienced a rapid but transient stratification, and later resolved back to a single-layered state as tubes and branches were generated.

Prior to budding, between E7.5-8.25, the pre-pancreatic endoderm is composed of a single layer of cuboidal epithelial cells (Fig. 4A). At these stages, the pre-pancreatic epithelium exhibits no morphological signs of organogenesis. As the embryo turns, however, we found that dorsal bud epithelial cells transformed from cuboidal to columnar, forming a ‘placode’ or epithelial thickening (Fig. 4B). Although this placode was initially a single cell layer thick, it stratified over the next 2 days, generating multiple layers of cells between the duodenal lumen and the outer basement membrane (Fig. 4B-D,I,J). As development proceeded
through ‘protodifferentiated’ stages (E8.75-11.5), the number of cell layers increased to approximately six to eight thick (Fig. 4K), surrounding a primary central lumen (PCL) (see Fig. S4 in the supplementary material).

Of note, we consider all cells within this multilayered bud to be ‘epithelial’ in nature, as they express E-cadherin and do not express mesenchymal markers, such as vimentin (data not shown). High-resolution 3D reconstructions and z-stack movies revealed that, within the most highly stratified region of the E10.5 bud, inner cells contacted neither the PCL nor the peripheral basement membrane, either directly or via thin extensions (Fig. 4J and data not shown).

As the pancreas progressed through the secondary transition, the multi-layered pancreatic epithelium began to resolve and to progressively decrease in thickness (de-stratified) as branches formed (Fig. 4E-H). This resolution was the result of two major cellular changes: (1) Epithelial cells rearranged to form single cell layered epithelial tubes; and (2) The ductal network remodeled from a multi-dimensional plexus to a hierarchical ramifying tree. Interestingly, these changes were concomitant with the differentiation of protodifferentiated cells into the different pancreatic cell lineages: endocrine cells, acini and ducts.

Pancreatic epithelial polarity dynamics
To further understand how the pancreatic epithelium generated organized tubes, we examined the dynamics of cell polarity throughout stratification and de-stratification (Fig. 5). Although the pancreatic bud originated from a typical polarized epithelium (data not shown), during stratification some of these epithelial cells partially or completely lost their polarity. This was a transient phenomenon, as these cells recovered typical polarity when the epithelium resolved back to a single layer and branches formed.

Once fully stratified, at E10.5, pancreatic epithelial cells organized in a manner similar to the mammary gland (Lu and Werb, 2008; Mailleux et al., 2008; Sternlicht, 2006), with at least three recognizable domains: an outer layer of ‘cap’ cells, interim ‘body’ cells and innermost ‘lumen-lining’ cells (Fig. 5H). ‘Cap’ cells maintained their basal polarity, as they directly contacted the basement membrane (laminin and collagen on basal surface, basal nuclear localization) (Fig. 5A,B), but displayed no apical polarity, as they lost expression of the apical PAR complex (APKC, Par3) (Fig. 5C-D) or ezrin (Fig. 5E). ‘Body’ cells also displayed no apical membrane domain, as assessed by the absence of aPKC; however, they displayed little basal polarity, as assessed by laminin or collagen IV deposition (Fig. 5A,B; data not shown). The lumen-lining cells, in contrast to cap cells, showed only apical polarization, as they exhibited direct contact with luminal space and significant accumulation of all apical markers, including aPKC, Par3, ezrin and ZO-1 (Fig. 5A-E, and data not shown), but no basal polarization.

Loss of polarity in the stratified pancreatic epithelium was transient and step-wise. The first sign of reacquired polarity in both cap and body cells was apical localization of ZO-1, as early as E10.5 (Fig. 5F; Fig. S5 in the supplementary material). At this time, however, no other apicolateral markers were detected in body cells. Of note, we frequently observed single body cells that harbored ZO-1 on multiple sides (see Fig. S5B,F in the supplementary material). In addition, cap cells became elongated over time, exhibiting basal nuclear localization (Fig. 5F) and apicolateral localization of the Golgi (GM130) (Fig. 5G).

The progressive re-emergence of complete apical and basal polarity was temporally associated with the secondary transition. Polarized, monolayered epithelial cells reemerged during this time
(Fig. 5A’-E’,H), while gradually fewer stratified body cells remained (Fig. 5A’-E’). At about E15.5, almost all cells within the branched epithelium displayed full apicobasal polarity and were integrated into a single-layered branched epithelium (data not shown).

Microlumen formation and tubulogenesis
To characterize luminal network formation, we examined Muc1 staining of the pancreatic bud (Fig. 3). The PCL, an initially prominent central cavity within the bud (Fig. 4D), quickly thinned as the bud elongated and de novo lumens emerged. The late E10.75 bud therefore consisted primarily of a forming plexus (Fig. 6A). At this stage, multiple small isolated lumens were evident, as well as networks of fusing lumens (Fig. 6B). Serial z-stack reconstructions showed that, over time, isolated lumens integrated into the tubular network. Connection of microlumens to each other appeared to occur via ‘polarized canals’, or tracts of polarized epithelial cells that presaged future lumens (Fig. 6C).

Analysis of adjacent serial sections revealed that body cells underwent dramatic cell shape changes and rearrangements during lumen formation. Most body cells at E10.5 initially lacked obvious polarity. However, by E10.75 they quickly reacquired polarity, as assessed by re-emergence of ZO-1 immunoreactivity (see Fig. S5 in the supplementary material). Single body cells often displayed multiple foci, or aggregates, of ZO-1 expression at this stage (Fig. 6F,G).

In some single cells, expression of ZO-1 was distinguishable in an O-ring pattern, forming an apical ‘collar’ (Fig. 6D,E). When following single cells across serial sections, we determined that O-rings represented the constricted apical side of a bottle-shaped body cell, within the stratified epithelium (see Fig. S6 in the supplementary material). These cells appeared to have undergone asynchronous apical constriction within the multilayered epithelium, as they appeared in no specific order and in random locations.

As development progressed, we noted the increasing propensity of bottle-shaped body cells to form distinct ‘rosettes’ with other similarly shaped body cells (Fig. 6F-I). These rosettes of newly polarized bottle cells oriented their ZO-1 expressing apical ends at the center. Rosettes appeared to ‘open up’ isolated microlumens between them (Fig. 6I). Such isolated epithelial ‘pockets’ were readily detected in 3D reconstructions and appeared to progressively connect to each other during development, forming continuous lumen networks (Fig. 6C,J).

Taken together, these data demonstrate that branch formation is preceded by epithelial stratification, cell shape changes and polarity shifts, microlumen formation and fusion, followed by tubular plexus remodeling into the tree-like system of pancreatic branches.
EphB2lacZ phenotype was variable, with 54% of the total number of pancreatic development. The penetrance and severity of the mutant mesenchyme and epithelium is probably required for proper the supplementary material), indicating crosstalk between EphB signaling is required for proper pancreatic branching and development

EphB2 and EphB3 receptors are expressed in the pancreatic epithelium (see Fig. S7 in the supplementary material) (van Eyll et al., 2006) and the compound EphB receptor mutant mice EphB2ΔNucZlacZ/EphB3−/− display defects in epithelial organization in the thymus and intestine (Batlle et al., 2002; Garcia-Ceca et al., 2009; Miao and Wang, 2009; Munoz et al., 2009). However, to date, pancreas structure has not been evaluated in these mutants. Using our criteria for normal pancreatic development, we assessed whether EphB signaling was required for pancreatic epithelial development and branching.

To visualize pancreas epithelium and gross morphology in EphB2ΔNucZlacZ/EphB3−/− mice, we assayed Ngn3 and Sox9 expression (Villasenor et al., 2008). Mutant pancreata displayed defects in overall morphology and branching, including a reduction in size and thickness of both splenic and gastric lobes, and loss of the characteristic ‘anvil-shaped’ tail (Fig. 7A,B,G). The lengths of many left lateral branches in the distal region of the dorsal pancreas were severely reduced in mutants and the ‘ridge’ was greatly flattened (Fig. 7C,D). In addition to branching abrogation, the regionalization of mesenchymal genes was also lost (see Fig. S8 in the supplementary material), indicating crosstalk between mesenchyme and epithelium is probably required for proper pancreatic development. The penetrance and severity of the mutant phenotype was variable, with 54% of the total number of EphB2ΔNucZlacZ/EphB3−/− displaying disrupted morphology and 30% exhibiting severe phenotypes (similar to mutants in Fig. 7).

Disrupted branching morphology was present at later stages in mutants and was accompanied by decreased exocrine mass (data not shown). E16.5, P14 and adult mutant pancreata were smaller, displaying shorter branches and reduced tails (Fig. 7E,F; data not shown). In addition, although adults were viable and fertile, they had lower body weights, displayed smaller less branched pancrea and exhibited defects in glucose homeostasis (data not shown).

To discern whether the observed phenotype might be due to a single EphB receptor, we examined EphB single mutants. The EphB2ΔNucZlacZ/EphB3−/− mice are null for the EphB3 receptor and dominant negative for the EphB2 receptor. The cytoplasmic domain of EphB2 is replaced with β-galactosidase, thereby blocking forward signaling but allowing reverse signaling via Eph ligands. To determine which receptor was required for proper branching, the compound mutant EphB2ΔNucZlacZ/EphB3−/− was compared with the single mutants EphB3−/− and EphB2ΔNucZlacZ. Neither of the single mutants showed defects in pancreatic gross morphology or branching (see Fig. S9 in the supplementary material), suggesting that both EphB2 and EphB3 receptors are required coordinately for pancreatic development.

EphB2ΔlacZlacZ/EphB3−/− mutants display disrupted epithelial organization and lumen formation

To visualize lumens in wild-type versus EphB2ΔlacZlacZ/EphB3−/− mutants, 3D reconstructions of Muc1 immunostained pancreata were examined at different stages (E12.5, E14.5, P1). At E12.5, mutant lumens were initially narrower (collapsed) compared with wild type (see Fig. S10A-D in the supplementary material). By
E14.5, narrow lumens opened up, but the ductal system failed to completely remodel into its normal hierarchical arrangement (Fig. 7H,I,J; see Fig. S10E-H in the supplementary material). By P1, reduced expression of Muc1 in the large ducts of EphB mutants precluded analysis of the full extent of remodeling (see Fig. S10I,J in the supplementary material). Together, the data suggests that the luminal system in EphB mutants is delayed in its progression of tubulogenesis and remodeling.

Disorganization of the mutant epithelium was also noted when compared with wild type (Fig. 7L,M). Mutant pancreatic epithelium exhibited a marked decrease in membrane-associated β-catenin, especially in the outer cap cells (Fig. 7K-O; see Fig. S11 in the supplementary material). We note, however, that within body cells, β-catenin was often at higher levels in irregular aggregates (see Fig. S11 in the supplementary material). These data suggest that Eph signaling is required for both proper levels and cellular localization of β-catenin. In addition, as β-catenin is a component of adherens junctions (Gottardi and Gumbiner, 2001), we examined epithelial junctional complexes in EphB2lacZ/lacZ/EphB3−/− mutants. A similar decrease in junctional E-cadherin was evident (Fig. 7P,Q), suggesting a fundamental change in the structural cohesion of mutant epithelium. Accordingly, sections revealed disorganized rosettes (Fig. 7R,S) and confirmed early lumen ‘collapse’ (Fig. 7T,U).

As β-catenin influences growth of the pancreas (Dessimoz et al., 2005; Murtaugh et al., 2005; Wells et al., 2007), we examined whether MPCs or progenitor epithelium might show early reduction in EphB mutants. Although the number of CPA+ cells at E11.5 were relatively unchanged, the number of Ptf1α+ cells at 7.5dpc was significantly decreased (Fig. 7V,W; see Fig. S12 in the supplementary material) As Ptf1α+ epithelium gives rise to all pancreatic lineages (Kawaguchi...
et al., 2002), we examined endocrine numbers. Indeed, EphB2lacZ\textsuperscript{–/–} pancreata developed significantly fewer endocrine cells throughout development and into adulthood (data not shown). Interestingly, this decrease was more prominent in the distal region of the tail where most branching defects were found. Together, these results demonstrate a previously unreported role for EphB signaling in pancreatic epithelial development and branching, via proper β-catenin levels and cellular localization.

**DISCUSSION**

In this study, we provide an in depth analysis of pancreatic branching that includes both an ‘external’ analysis of the overall branch morphology and an ‘internal’ analysis of the pancreatic epithelium and its developing tubular network. It has long been thought that the pancreas displays random branching patterns (Slack, 1995); however, our observations demonstrate predictable branching trends. Furthermore, our analysis elucidates novel cellular dynamics that underlie pancreatic branching. We show dramatic transient stratification of bud epithelium, in which microlumens form as a result of cell shape changes and fuse to one another to form a luminal plexus. This complex network of tubes then undergoes remodeling to later generate pancreatic branches. We also demonstrate genetic regulation of the branching program, as compound EphB receptor signaling mutant mice display defects in epithelial organization, a decrease in pancreatic progenitor epithelium, a delay in normal tubular remodeling and a concomitant reduction in pancreatic branching. Together, this study establishes a baseline reference for future identification of pancreatic regulatory genes and it reveals novel aspects of epithelial organogenesis.

**Stratification and polarity in the pancreatic epithelium**

Formation of pancreatic branches and their lumens is preceded, in both the dorsal and ventral buds, by epithelial stratification and shifts in cell polarity. Interestingly, the process of stratification coincides with the ‘protodifferentiated state’ in pancreas, when the pool of multipotent progenitors is expanding (Cleaver and MacDonald, 2009). The epithelium then resolves, as the progenitor pool diminishes and cells differentiate into endocrine, exocrine and ductal lineages. It is possible that transient stratification is integral to accumulating the proper pool of progenitors required to give rise to the mature pancreas.

Once a pool of unpolarized body cells has accumulated, these cells almost immediately begin to reacquire polarity and rearrange to form microlumens. The first evidence of polarity re-establishment within stratified epithelium is the emergence of the apical tight junction component ZO-1 and of the apical/lumen marker Muc1. Proper localized accumulation of these two molecules and other apical proteins in pancreatic stratified epithelium has been shown to depend on Cdc42 function (Kesavan et al., 2009). It is only later during lumen formation that standard polarity molecules such as aPKC accumulate, followed by ezrin, Par3/6 and laminin in resolving branches.

During reacquisition of polarity by body cells (E10.0-10.75), individual cells experienced asynchronous apical constriction, each forming a tight collar of ZO-1. Localized ZO-1 in single cells is followed by cell shape changes in neighboring cells, resulting in the formation of a rosette of cells with apical ends gathered together between them. Interestingly, focal ZO-1 aggregation in body cells and rosette formation closely resembled development of the intestinal lumen in zebrafish (Home-Badovinac et al., 2001). In both cases, initially scattered junctions between epithelial cells undergo reorganization, forming small foci at cell-cell interfaces. These foci merge with one another until there is a single cluster at the center of each rosette, allowing lumens to emerge at the center of these highly polarized clusters. We speculate that pancreatic lumens emerge as proposed in the zebrafish gut lumen, including rearrangement of cell junctions and expansion of apical membrane domains.

Although the cellular mechanisms underlying pancreatic stratification are unknown, it is conceivable that initially cap cells undergo asymmetric cell division, in a manner similar to skin stratification (Lechler and Fuchs, 2005). It will be interesting to elucidate the cellular process of pancreatic epithelial stratification, to identify origins and mechanisms underlying body cell generation and polarity shifts, and to determine whether the plane of division has an impact on cell fate.

Interestingly, the stratified pancreatic bud displays similarities to that of early mammary and salivary glands (Mailleux et al., 2008; Sternlicht, 2006; Tucker, 2007; Walker et al., 2008). All three generate transiently stratified epithelium and form lumens de novo. However, the underlying mechanisms of tubulogenesis and branch formation are likely to be different. Both mammary and salivary gland lumens are formed by apoptotic clearance of body cells (Mailleux et al., 2008; Tucker, 2007). By contrast, pancreatic lumens form independently of apoptosis (data not shown). In addition, in the mammary and salivary gland, de novo lumens form after the organ has already branched, whereas in the pancreas the reverse is true: a ductal plexus appears before branching (Hogg et al., 1983; Mailleux et al., 2008; Sternlicht, 2006; Tucker, 2007).

**Pancreatic branching results from ductal plexus remodeling**

Our work thus demonstrates a new mechanism of branch formation that occurs in a highly unusual manner, from the ‘inside out’, with lumens forming within a thick multilayered epithelium significantly earlier than branches appear. Externally recognizable pancreatic branches emerge later, following complex remodeling of the lumen plexus rather than simple extension of epithelial buds. Although the geometric process underlying this conversion remains unclear, it is possible that cellular mechanisms such as epithelial convergent extension or regression are at play.

Pancreatic ductal remodeling is reminiscent of the cellular changes observed during blood vessel formation (Risau and Flamme, 1995). Embryonic vessels initially form a homogeneous plexus of tubes that subsequently ‘remodels’ into hierarchical arrangements of large and small arteries and veins. In large part, these events are driven by the hemodynamic flow of blood within primitive vessels (Jones et al., 2006). It remains an unresolved issue whether flow of exocrine secretions in embryonic pre-ductal tubules is significant enough to play any role in pancreatic ductal plexus remodeling.

Although our observations support lateral branching as the principle mechanism of pancreatic branching, as observed using live imaging of explanted pancreatic buds (Puri and Hembro, 2007), we refine the concept here, showing it occurs not only by epithelial extension, but in coordination with epithelial remodeling. We also note that branch ‘tips’, presumed by the nature of their name to represent structures that grow by tubular extension, in fact do not generate individual branches. Our observations support a different mechanism by which MPC-containing ‘tip’ structures instead divide and multiply, fueling growth of the remodeling epithelium by a longitudinal contribution.
Predictable trends in branch patterning
Our data also show that developing pancreatic branches display predictable trends in morphology. Comparing hundreds of individual pancreata, a baseline model of gross branch patterning was constructed. Although our analysis focused on the dorsal pancreatic bud, the ventral pancreas undergoes a similar branching process, with onset occurring (as the dorsal pancreas) just before secondary transition at about E12.5. We note that owing to the unusual sequential nature of internal lumen formation preceding external branch formation, patterning of branches could only be assessed starting at E13.5. Analysis of early ‘branching’ patterns (E10.5-11.5) was inherently precluded during ductal plexus formation, as the outer cap epithelium is initially smooth.

An interesting question that arises is how pancreatic branches acquire their patterning information. We speculate that the mesenchyme is a likely candidate signaling tissue, as we observed distinct regionalization of the mesenchyme and localized expression of various factors. Indeed, mesenchymal domains were altered in EphB mutants (see Fig. S8 in the supplementary material), suggesting reciprocal signaling between mesenchyme and epithelium. Further analysis of branching patterns upon experimental disruption of these mesenchymal factors will be required to further our understanding of pancreatic branch patterning.

EphB signaling is required for epithelial morphogenesis in the pancreas
As EphB2/B3 receptors are expressed in pre-ductal epithelium (see Fig. S8 in the supplementary material) (van Eyll et al., 2006) and Eph/ephrin signaling influences epithelial cell behavior (Holmberg et al., 2006), we analyzed pancreatic branching in the absence of EphB signaling. Indeed, key morphological features of the developing pancreas were defective in the absence of EphB signaling. We found early disruption of epithelial rosettes and microlumens, delay in ductal remodeling, as well as reduced Ptf1a (progenitor epithelium), resulting in later abrogation of pancreatic branches and reduction of endocrine/exocrine mass. Together, these data indicate that branching defects were probably the result of defective epithelial morphogenesis, and suggest that differentiation of pancreatic cell lineages depends on proper architecture, remodeling and extension of the initial epithelium into a branched organ.

The molecular basis for EphB mutant epithelial defects appears distinct from cell polarity regulation. In EphB mutants, although ZO-1 is increased and disorganized in body cells, it is nonetheless localized apically as expected. In addition, morphological defects observed are distinct from those in the recently described pancreatic deletion of Cdc42 (Kesavan et al., 2009). Although the lack of Cdc42 function in the epithelium results in failure of epithelial integrity and microlumen coalescence, absence of EphB signaling results instead in epithelial disorganization, affecting subsequent lumen and branching development.

These defects are probably due to altered epithelial adhesion in EphB mutants. Decrease in junctional β-catenin between epithelial cells in EphB mutants may be accompanied by an increase in cytoplasmic β-catenin, and possibly β-catenin signaling. Mice expressing a constitutively active form of β-catenin in pancreatic epithelium, PdxCreαβcatactive mice display epithelial dysmorphogenesis, decreased E-cadherin expression and concomitant defects in exocrine branches (Heiser et al., 2006). Similarly, EphB2lacZlacZ/EphB3−/− mutants exhibited decreased junctional E-cadherin levels, along with pancreatic hypoplasia and decreased branching. However, EphB2lacZlacZ/EphB3−/− mutant pancreata did not exhibit the cyst formation seen in PdxCreαβcatactive mice (data not shown), reflecting a less severe pancreatic phenotype. Taken together, these data demonstrate that EphB signaling regulates pancreatic epithelial morphogenesis, including microlumen formation and acinar development, potentially via regulation of epithelial cell adhesion and β-catenin protein availability.

A cellular model for pancreatic lumen ontogeny and branching
Our study reports the unusual epithelial dynamics that underlie pancreas morphogenesis. It also provides the first analysis of murine pancreatic branching and overall morphology during embryonic development, and therefore sets a baseline for the future evaluation of pancreatic structure in mouse mutants, for the identification of regulatory genes. Given that both islet endocrine cells and the entire exocrine glandular tree originate in the protodifferentiated pancreatic epithelium, elucidating its ontogeny and changing architecture will advance our understanding of the developmental steps important to its cell lineages, both exocrine and endocrine, and their progenitors.

Acknowledgements
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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.052993/-/DC1

References
Epithelial dynamics of the branching pancreas


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**Blunt tail**

**Type I: No/short left lateral branches**

**Uniform morphology**

**Bifurcated tail**

**Type II: Few left lateral branches**

~ 2 types

**Type III: Multiple/Long left lateral branches**

~ 3 types

**Short**

~ 2 types

**Long**

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ephrinB2 (EB2) - blood vessel
EphB2/EphB3 - epithelium
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