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Impaired Spleen Formation Perturbs Morphogenesis of the Gastric Lobe of the Pancreas

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Abstract

Despite the extensive use of the mouse as a model for studies of pancreas development and disease, the development of the gastric pancreatic lobe has been largely overlooked. In this study we use optical projection tomography to provide a detailed three-dimensional and quantitative description of pancreatic growth dynamics in the mouse. Hereby, we describe the epithelial and mesenchymal events leading to the formation of the gastric lobe of the pancreas. We show that this structure forms by perpendicular growth from the dorsal pancreatic epithelium into a distinct lateral domain of the dorsal pancreatic mesenchyme. Our data support a role for spleen organogenesis in the establishment of this mesenchymal domain and in mice displaying perturbed spleen development, including Dh +/-, Bapx1 -/- and Sox11 -/-, gastric lobe development is disturbed. We further show that the expression profile of markers for multipotent progenitors is delayed in the gastric lobe as compared to the splenic and duodenal pancreatic lobes. Altogether, this study provides new information regarding the developmental dynamics underlying the formation of the gastric lobe of the pancreas and recognizes lobular heterogeneities regarding the time course of pancreatic cellular differentiation. Collectively, these data are likely to constitute important elements in future interpretations of the developing and/or diseased pancreas.

Introduction

The mouse pancreas has been extensively studied as a model for embryonic patterning and branching-morphogenesis. With the development of transgene technology the mouse has also become the outstanding system for research on the genetics underlying pancreas related diseases, including diabetic disorders and cancer. An accurate description of normal pancreas development and constitution is hence indispensable to fully appraise potential aberrations relating to these research areas. Traditionally, the pancreas is described to form by a dorsal and ventral evagination of the gut tube epithelium resulting in the formation of a dorsal (DP) and ventral (VP) pancreatic bud. During subsequent development these buds grow, branch, differentiate and eventually fuse to form the adult exocrine and endocrine compound gland (for reviews see [1,2,3]). A number of signalling molecules expressed in the pancreas-associated mesenchyme are important for induction of the pancreatic program and subsequent growth and differentiation of the pancreatic epithelium [1,2]. However, the molecular mechanisms directing later pancreatic morphogenesis are poorly understood. The gastric lobe (GL) of the pancreas ([4] and Figure 1A) is conserved in a number of rodent species including hamster, rat and mouse, and has been suggested to correspond to the auricle or “ear of the pancreas” in humans [5]. Given its distinct spatial localization and significant contribution to the overall pancreatic mass (Figure 1A–C), surprisingly few records of this structure are to be found in the literature and very limited information regarding its embryonic development has been presented. It has been demonstrated that pancreatic mesenchymal morphogenesis is independent on growth of the pancreatic epithelium and the pancreatic mesenchyme has been suggested to provide patterning information for pancreatic branching [6,7]. A recent study addressing epithelial dynamics during pancreatic branching morphogenesis outlined the morphogenesis of the GL epithelium during development [6]. However, limited information exists regarding the gross mesenchymal morphogenesis during pancreas development. We have previously demonstrated the possibility to perform detailed, high contrast, studies of the developing pancreatic epithelium and its associated mesenchyme by optical projection tomography (OPT) [8,9]. In this study, we provide a quantitative and three-dimensional (3D) description of pancreas development and investigate the morphological events underlying the formation of the GL [10]. Based on analyses of normal and transgenic animals, we propose a model in which proper initiation of spleen formation is key for the formation of this prominent feature of the rodent pancreas.

Results

Spatial and quantitative dynamics of pancreas morphogenesis

To assess the developmental growth dynamics of the GL we analysed gut segments including the stomach, duodenum, pancreas and spleen between embryonic day 10.5 (e10.5) and
postnatal day 0 (P0) in normal C57Bl/6 mice based on the signal from E-cadherin antibodies and endogenous tissue fluorescence for epithelium and mesenchyme respectively. The hereby-generated 3D time-line of pancreas organogenesis (Figure 1D–Q and Video S1, Video S2, Video S3, Video S4, Video S5, Video S6 and Video S7) revealed that the GL begins to form by perpendicular growth from the stalk of the DP epithelium around e13.5. During subsequent development, the GL epithelium colonizes a mesenchymal domain extending from the proximal DP mesenchyme overlying the pyloric sphincter and pyloric antrum (Figure 1G–J and N–Q and Video S4, Video S5, Video S6 and Video S7). Quantitative OPT analyses demonstrated that from e15.5 onwards, the GL epithelium constitutes more than 10% of the total pancreatic epithelial volume and that its growth rate is similar to that of the DP and VP (Figure 1B–C). Thus, the GL represents a significant portion of the pancreas that is formed by lateral growth from the stalk of the dorsal pancreatic anlage approximately four days later than the formation of the dorsal pancreatic bud around e9.5.

GL morphogenesis is perturbed in mice displaying disturbed spleen development

The stomach and spleno-pancreatic mesenchyme constitute two separately inducible cell populations. During splenogenesis precursors located in the latter population expand in an anterior direction guided by signals originating from the anterior aspect of the stomach [11]. This process coincides with a condensation of the spleen mesenchyme across the length of the left side of the greater curvature of the stomach [8]. Our data reveal that growth of the GL epithelium is initiated at a stage when the DP mesenchyme and the developing spleen are morphologically distinct and leftward growth of both structures is well established. To elucidate the morphological events underlying the formation of the GL we focused our investigation on the gut-associated...
mesenchyme, before and around the time for initial GL formation (Figure 1L–O). From e11.5 onwards, a morphologically distinguishable spleen anlage can be recognized in the distal spleno-pancreatic mesenchyme (the spatial localization of the spleen anlage was verified by immunohistochemical analyses for Tlx1/Hox11 (Figure S1)). As development advances, spleen progenitors condense and move in an anterior direction over the greater curvature of the stomach. This process results in an apparent separation of the DP mesenchyme into two distinct domains, disjoined by a wedge of primordial spleen cells (Figure 1N). The hereby-formed perpendicular mesenchymal domain gradually becomes separated from the splenic primordium and the distal DP mesenchyme and at around e13.5 lateral growth from the stalk of the DP epithelium into this domain was observed (Figure 1N).

Given the morphological indication of a role for spleen organogenesis in establishment of the GL mesenchyme we next investigated the impact of disturbed spleen development on GL formation. The transcriptional hierarchy governing spleen development is not fully understood. Available data, obtained primarily by assessments of knock out mice, suggest that at least two parallel genetic pathways are at play during early spleen development [12] and a number of mouse mutants displaying varying degrees of impaired spleen formation have been described. These include mice deficient for Tfx1 (Hox11) [13,14], Ncx2.3 [15], Ncx3.2 (Bapx1) [16,17], Tcf21 (POD1/Copula1) [18], Wt1 [19], Barx1 [20], Sox11 [21], Pbx1 [22] and the Dh mutant [17,23]. Of these, Tfx1, Tcf21, Wt1, Ncx2.3, Pbx1, and Barx1 null mice have been demonstrated to form, at least initially, a defined splenic primordium. For example, in the Tfx1 mutants, the spleen primordium develops normally until e13.5 but fails to expand beyond this stage [13]. In contrast, Dh+/− mutants do not form a recognizable spleen primordium [17] and in Bapx1−/− embryos the spleen mesenchyme does not condense to form a morphologically distinct spleen anlage [8,17]. Hence, these mutants display some of the most severe aberrations of spleen organogenesis described. OPT generated iso-surface renderings revealed that the GL epithelial domain was pronounced, and in Sox3.2 mice the structure fails to form a SMP, although less pronounced, and in Dh+/− mice the structure fails to develop [17]. In Sox11−/− mutants however the SMP appears to form normally (Figure S3). Collectively, these results suggest that the mechanisms directing asymmetric growth of the DP are not involved in GL formation other than as a possible indirect result of a potential role for the SMP in spleen development [17].

As demonstrated in mice deficient for Pdx1(Ipf1), the morphogenesis of the DP mesenchyme is uncoupled from that of DP epithelium [7]. In those mutants, in which the pancreatic epithelium is growth arrested around e10.5, the spleen develops normally and the DP mesenchyme grows and develops morphologically and functionally independently of the DP epithelium [7]. Analyses of Pdx1−/− embryos revealed that similarly to the DP mesenchyme, the GL mesenchyme essentially occupies its normal space and position in the absence of a developing pancreatic epithelium. These data show that establishment of the GL mesenchyme, as well as the spatial information for its morphogenesis, is independent of pancreatic epithelial morphogenesis (arrowsheads Figure 2I). Collectively, in support of the notion that spleen formation from within the mesenchyme overlying the dorsal aspect of the pancreas is required for formation of the GL mesenchymal domain, and thereby the GL epithelium; Dh+/−, Bapx1−/− and Sox11−/− mice all display varying degrees of impaired GL development.

Gastric lobe formation appears independent on the mechanisms that direct L-R asymmetry during early spleno-pancreatic development

Leftward growth of the spleno-pancreatic (dorsal) region is mediated by a transient columnar mesodermally derived cell layer, the splanchic mesodermal plate (SMP), which is under influence of the left-right genetic cascade. Leftward growth of the SMP itself appears to be driven by cell proliferation but it is also a source of growth factors such as FGF10, which is suggested to be a chemotactic factor for pancreatic growth [17]. To elucidate if similar mechanisms are at play also during GL formation we screened the mesenchyme surrounding the presumptive GL between e12.5–14.5. Hereby, we could neither detect a columnar cell layer analogous to the SMP nor increased mitotic activity or expression of FGF10 in the GL mesenchyme (Figure S3). The SMP is also suggested to play a role in the induction of the splenic mesenchyme [17]. Bapx1−/− mice form a SMP, although less pronounced, and in Dh+/− mice the structure fails to develop [17]. In Sox11−/− mutants however the SMP appears to form normally (Figure S3). Collectively, these results suggest that the mechanisms directing asymmetric growth of the DP are not involved in GL formation other than as a possible indirect result of a potential role for the SMP in spleen development [17].

The gastric lobe displays a prolonged capacity to maintain markers for multipotent progenitor cells

Initial growth of the GL epithelium into the GL mesenchyme, around e13.5, coincides with an important hallmark of pancreas development. It was recently proposed that pancreas organogenesis is guided by multipotent progenitors that give rise to the endocrine, exocrine and ductal cell lineages of the pancreas. These cells, located in the tip of the branching DP, express Pdx1 and CPA1 but are negative for the exocrine marker Amylase. However, around e13.5 they begin to express markers for exocrine differentiation (Amylase) and subsequently give rise only to exocrine progeny [24]. The GL lobe contains all major pancreatic cell lineages. Consequently, unless the tip cells of the branching GL epithelium would maintain this multipotent potential for a longer time period than the DP epithelium from which they are derived, the GL-derived cell lineages would be likely to have a different descent. By performing triple labelling for Pdx1, CPA1 and Amylase at e12.5 to e13.5, in the ventral and dorsal pancreatic lobes we could verify that Pdx1+, CPA1+ and Amylase+ (Figure S4) cells were present in the dorsal and ventral lobes until e13.5 whereas at e14.5 the majority of the cells were Pdx1+, CPA1+ and
Amylase+ and CPA1+ cells of this lobe were maintained until e14.5 (Figure S4). These results suggest that the cellular differentiation of the developing pancreas from a temporal point of view is not uniform, even between the dorsally derived lobes.

**Discussion**

The pancreatic epithelium is shaped by distinct stepwise cellular mechanisms to form a functional branching organ, and it was recently shown that the gross branch patterns show predictable trends during development [6,25]. In this report we provide a 3D and quantitative description of the morphological events underlying normal pancreatic organogenesis in the mouse. Based on these data and analyses of mouse mutants displaying disturbed spleen development, we propose a model in which organogenesis of a neighbouring organ - the spleen - is required for normal morphogenesis of the dorsal pancreatic region and for the formation of the gastric pancreatic lobe in particular (Figure 3). In the examined mice mutants, we cannot rule out the possibility that the lack of gene function itself contribute to the perturbance of GL morphogenesis. However, given that the most severe splenic phenotype also coincides with the most pronounced perturbance of GL morphogenesis it seems reasonable that spleen formation is an important mediator of mesenchymal morphogenesis in the dorsal pancreatic region. Mesenchymal signals are important stimulators of pancreatic growth, branching and differentiation [26] and heterogeneous gene expression patterns have been described in the pancreas associated mesenchyme during different stages of development [6,17]. However, the developmental significance of these observations is not fully understood. The question of whether unique signalling molecules and/or pathways are at play in the GL mesenchyme, which contribute to the formation of the GL epithelium therefore remains an open issue and is beyond the scope of this study. It is clear that the early steps of pancreatic epithelial development may commence with a variety of mesenchymal sources [27,28,29]. As suggested by this
study, the spatial organization of the pancreatic mesenchyme, and the GL mesenchymal domain in particular, is mediated by spleen formation. Therefore, it is likely that this process, by itself is a key element in the establishment of the GL epithelial domain. As mentioned earlier, the auricle of the human pancreas has been suggested to be a reminiscence of the gastric lobe of the pancreas in rodents [5]. Although the location of this structure, as well as its close interaction with the gastroepiploic vessels, appear similar in both mice and humans, the issue of whether comparable morphological processes, as those described in this report, are at play during development of the human pancreas needs to be determined. Given the ethical and practical constraints associated with experimentation on human material, to investigate this issue will be a much challenging enterprise.

The herein described lobular heterogeneity regarding the temporal capacity to maintain Pdx1⁺, CPA1⁺ and Amylase⁻ cells may reflect a general delay in development of the GL. However, it also indicates that the local environment, even within the dorsal pancreatic region, influences the development of lobular characteristics of the pancreas. Hence, our data suggest that the dorsal, ventral and gastric pancreatic lobes together may provide an inherent system for comparative assessments of how cellular differentiation is coordinated within the pancreas. As to what extent this heterogeneity may influence other developmental or functional aspects of the pancreas remain a question for further investigation. Nevertheless, it seems likely that the close interplay between the splenic and pancreatic mesenchyme during early development described in this report must be taken into careful consideration in future assessments of the rodent pancreas.

### Material and Methods

#### Ethics statement

All experiments were performed in compliance with the relevant national and institutional laws and guidelines. The study was...
approved by the Ethical Committee of Animal Research, Northern Sweden. Ethical approval ID A-8-2010.

Animals
Mice were maintained as heterozygotes and/or bred and in the respective animal facility of: Umeå University, Sweden (Bapx1, Pdx1, C57Bl/6 (Taconic, Denmark)); MRC, Human Genetics Unit, Edinburgh, UK (Dh); Universität Erlangen Nuernberg, Germany (Sox11).

Tissue preparation, immunohistochemistry and in situ hybridisation
Embryonic gut segments were dissected free, fixed in 4% paraformaldehyde and prepared for cryo-sectioning or whole-mount immunohistochemistry. Staining of cryosections was performed according to standard protocols. Cryosections with a thickness of 8 μm were obtained and blocked in 10% serum (from the same species in which the secondary antibody was derived) followed by antibody incubation. The adult pancreatic lobes were separated before OPT scanning. Antibody incubation time was reduced for embryonic tissues (24 h) and freeze-thawing was omitted. No bleaching of autofluorescence was performed for reduced for embryonic tissues (24 h) and freeze-thawing was omitted. No bleaching of autofluorescence was performed for embryonic stages up to e14.5 [9]. Primary antibodies used were: Rabbit α-Hox11 (Santa Cruz Biotecnoity), Rabbit α-Cpa1 (MediQip), Phalloidin (Molecular probes), Rabbit α-PH3 (Millipore), Rat α-E-cad (Zymed), Phalloidin-FITC (Sigma), Goat α-Pdx1 (Abcore), Guinea Pig α-Pdx1 (Abcam), Sheep α-Amy (Abcam). Primary antibodies were visualized with Alexa 488, 594 and 633-conjugated secondary antibodies (Molecular Probes). DIG-label in situ hybridization was performed according to standard protocols. FGF10 probe was made from full length cDNA of RIKEN clone 9430031A18.

Optical projection tomography, volumetric quantification and image analysis
OPT analysis was performed essentially as described [9,30]. Each specimen was scanned using the Bioptonics 3001 OPT scanner with a resolution of 1024×1024 pixels and reconstructed with the NRecon version 1.6.1.0 (Skyscan) software. Quantification of the pancreatic epithelium during development was made using the quantitation module for Volocity version 5.4.1 (Perkin Elmer). Reconstructed image stacks were digitally cropped to include the dorsal, ventral and gastric lobe epithelium respectively. The epithelial volumes were calculated by applying a “find objects by intensity” task to select voxels above a specified intensity. The intensity threshold value was manually determined for each image stack. A kernel filter (fine filter, 3 x 3 voxels) was used to remove background noise pixels with intensities above the selected threshold. All iso-surface reconstructions of embryonic gut segments were made using the visualization module for Volocity (Perkin Elmer), version 5.4.1. All measured volumes, adult and embryonic, were finally exported to the Excel 2007 (Microsoft Corporation, Redmond, Washington) software for statistical analysis (descriptive statistics). OPT images were exported as screenshots from iso-surface reconstructions in Volocity and processed in Photoshop CS2 version 9.0.2 (Adobe). All image adjustments were applied equally to images and occasional artefacts such as fibers or dust were digitally removed.

Supporting Information
Figure S1 OPT based assessments of Thx1/Hox11 expression determining the spatial localization of the spleen primordium during embryonic development. A through I: OPT generated iso-surface reconstructions of gut segments including the stomach, duodenum, spleen and pancreas at e11.5 (A, D, G), e12.5 (B, E, H) and e13.5 (C, F, I) based on the signal from E-cadherin antibodies (epithelium, green in A to C), the signal from tissue autofluorescence (mesenchyme, A to I) and the signal from Tlx1 antibodies (spleen primordium, red in A to F). Compare with pseudo coloring in fig. 1 I to K.

Figure S2 Early failure to form GL mesenchymal domain in Dh +/+ and Bapx1 −/− mice. OPT generated iso-surface reconstructions of gut segments including the stomach, duodenum, spleen and pancreas at e12.5 in normal C57/B6 (A and D), Dh+/− (B and E) and Bapx1−/− (C and F) mice. Reconstructions are based on the signal from E-cadherin antibodies (epithelium – light grey, A to C) and the signal from tissue autofluorescence (mesenchyme – dark grey, A to F). At e12.5, spleen condensation has mediated the formation of a GL mesenchymal domain lateral to the main bulk of dorsal pancreatic mesenchyme in wild-type mice (A and E). In Dh +/+ −/− mice (B and F), the complete absence of the spleen primordium prevents formation of a GL mesenchymal domain. In the Bapx1 −/− mice, morphogenesis of the GL mesenchymal domain is perturbed by the failure of the early spleen primordium to condense and dislocate from the pancreatic epithelium. The GL mesenchyme is indicated with arrowheads in E. Dorsal and ventral pancreatic epithelium have been pseudocolored red and green respectively in A to C. The pancreatic epithelial outline has been indicated with a white line in D to F. The specimens are not depicted to scale.

Figure S3 The gastric lobe mesenchyme does not display characteristics analogous to those of the SMP during early leftward growth of the dorsal pancreas and spleen. Sections of GL mesenchyme stained with phalloidin (red - A, B) and antibodies against phospho-histone H3 (red, D-E) and E-cadherin (green, D-E). (C) Iso-surface reconstruction of e12.5 gut segment based on tissue autofluorescence (mesenchyme) indicating the plane of section in (A-B, D-E and G-I). (F) Schematic representation of section plane shown in (C). Arrow indicates direction of GL growth. (G-I) In situ hybridization showing absence of FGF10 expression in GL mesenchyme between e12.5 and e14.5. J-K Sox11 −/− embryos display normal SMP (arrowheads) morphology at e10.5 as shown by phalloidin (green) and Pdx1staining (red). Abbreviations; dp, dorsal pancreas; gln, gastric lobe mesenchyme; ps, pyloric sphincter/posterior stomach epithelium; vp, ventral pancreas.

Figure S4 The gastric lobe of the pancreas display a prolonged maintenance of markers for multipotent progenitor cells. (A through J) Ventral (A to D), dorsal (E to G) and gastric (I and J) pancreas between e12.5 to 15.5 stained for Pdx1 (red), Carboxypeptidase A1 (CPA1, blue) and Amylase (Amy, green). At e14.5 the absolute majority of tip cells in the dorsal and ventral lobe have lost their progenitor potential and are; Pdx1+, CPA1+, Amy− (arrows in C and G). In contrast, the gastric lobe tip cells are Pdx1+, CPA1+, Amy+ at the same stage and display an expression profile similar to the dorsal and ventral pancreas at e12.5 (arrowheads in I).

Video S1 Pancreatic epithelial and mesenchymal morphology at e10.5. OPT generated movie sequence depicting a gut segment including the stomach, duodenum, lung and pancreas with associated mesenchyme. Volume rendering of the mesenchyme.
is based on the signal from tissue autofluorescence (grey). Iso-surface rendering of the epithelium is based on the signal from E-cadherin antibody staining (yellow). At e10.5, the initial evagination of the foregut epithelium have developed into distinct ventral and dorsal pancreatic buds.

(MP4)

**Video S2** Pancreatic epithelial and mesenchymal morphology at e11.5. OPT generated movie sequence depicting a gut segment including the stomach, duodenum and pancreas with associated mesenchyme. Volume rendering of the mesenchyme is based on the signal from tissue autofluorescence (grey). Iso-surface rendering of the epithelium is based on the signal from E-cadherin antibody staining (yellow). At this stage the spleen anlage is morphologically recognizable in the mesenchyme covering the dorsal aspect of the pancreas.

(MP4)

**Video S3** Pancreatic epithelial and mesenchymal morphology at e12.5. OPT generated movie sequence depicting a gut segment including the stomach, duodenum and pancreas with associated mesenchyme. Volume rendering of the mesenchyme is based on the signal from tissue autofluorescence (grey). Iso-surface rendering of the epithelium is based on the signal from E-cadherin antibody staining (yellow). As the stomach and duodenum rotate during development the dorsal and ventral pancreas starts to become positioned on the same side. The spleen anlage is morphologically distinct and starts to become separated from the dorsal pancreatic mesenchyme.

(MP4)

**Video S4** Pancreatic epithelial and mesenchymal morphology at e13.5. OPT generated movie sequence depicting a gut segment including the stomach, duodenum and pancreas with associated mesenchyme. Volume rendering of the mesenchyme is based on the signal from tissue autofluorescence (grey). Iso-surface rendering of the epithelium is based on the signal from E-cadherin antibody staining (yellow). At this stage lateral growth from the stalk of the dorsal pancreatic epithelium into gastric lobe mesenchymal domain is observed. Note the fusion of the dorsal and ventral pancreatic ducts at some distance from the duodenum.

(MP4)

**Video S5** Pancreatic epithelial and mesenchymal morphology at e14.5. OPT generated movie sequence depicting a gut segment including the stomach, duodenum and pancreas with associated mesenchyme. Volume rendering of the mesenchyme is based on the signal from tissue autofluorescence (grey). Iso-surface rendering of the epithelium is based on the signal from E-cadherin antibody staining (yellow). At this stage the gastric lobe essentially occupies its adult position and shape.

(MP4)

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**Author Contributions**

Conceived and designed the experiments: AH UA. Performed the experiments: AH AUE. Analyzed the data: AH UA. Contributed reagents/materials/analysis tools: ES REH. Wrote the paper: AH UA. Commented on the manuscript and discussed the results: ES REH.

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