Reciprocal endoderm-mesoderm interactions mediated by fgf24 and fgf10 govern pancreas development

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INTRODUCTION

In amniotes, the pancreas originates from the thickening of the endodermal epithelium leading to the formation of a dorsal and a ventral bud that grow into the surrounding stromal tissue. The two pancreatic buds eventually fuse and both give rise to endocrine and exocrine cells. Pdx1 and Ptf1a are among the first markers of the pancreatic commitment (Kawaguchi et al., 2002; Jonsson et al., 1994). Pancreas specification occurs in a stepwise fashion as a result of interactions of the endoderm with mesodermal neighboring tissues (Kumar and Melton, 2003). Formation of the dorsal pancreatic bud first requires signals from the notochord, which repress sonic hedgehog (Shh) expression in the dorsal midgut endoderm, allowing activation of Pdx1 expression (Hebrok et al., 1998; Kim et al., 1997). Then, interactions between the dorsal pancreatic endoderm and the aorta are required to maintain high expression of Pdx1 and Ptf1a and to stimulate the endocrine cell differentiation program (Lammert et al., 2003; Yoshitomi and Zaret, 2004). The ventral pancreatic bud is in contact with neither the notochord nor the dorsal aorta but instead receives instructive signals from the adjacent lateral plate mesoderm (LPM) (Kumar et al., 2003). Although Pdx1 and Ptf1a are activated in both the ventral and dorsal pancreatic endoderm in amniotes, specific regulatory mechanisms underlie the development of each of these buds as inactivation of several genes affects the initial steps of formation of only one of the two buds (i.e. Hlxb9/Mnx1, Hnf6/Onecut1) (Jacquemin et al., 2003; Li et al., 1999).

After induction of the ventral and dorsal pancreatic territories, a portion of the LPM will generate the pancreatic mesenchyme and induces the budding, the growth and the branching of the pancreatic epithelium (Golosow and Groebstein, 1962; Spooner et al., 1970; Pictet et al., 1972; Ahlgren et al., 1996; Bhushan et al., 2001; Ye et al., 2005). The mesenchymal cells surrounding the dorsal pancreatic epithelium are characterized by the expression of the LIM homeodomain gene Isl1 and of Fgf10, which encodes a member of the fibroblast growth factor (FGF) family, and both genes are required for accurate pancreas organogenesis. In Isl1−/− embryos, which display agenesis of the dorsal pancreas, the mesenchyme fails to condense around the dorsal pancreatic epithelium (Ahlgren et al., 1997). In Fgf10−/− embryos, the specification of the pancreatic epithelium occurs normally, but it subsequently fails to undergo normal growth and branching owing to reduced proliferation of the epithelial progenitors (Bhushan et al., 2001). In addition, Fgf10 was shown to be essential in maintaining Ptf1a expression in the dorsal pancreatic bud (Jacquemin et al., 2006). To date, the determinants that specifically pattern the LPM into the pancreatic mesenchyme are unknown.

In zebrafish, the pancreas also derives from two endodermal anlagen (Field et al., 2003). Formation of the first pancreatic anlagen, present by 24 hours post fertilization (hpf) on the dorsal side of the developing gut, requires interactions with the notochord (Biemar et al., 2001). In contrast to the amniotes, this dorsal bud expresses pdx1 but not ptf1a and generates only endocrine cells. The second pancreatic bud appears by 32 hpf from the ventral aspect of the developing gut in a position slightly anterior to the dorsal bud and on the left part of the zebrafish embryo (Field et al., 2003). The ventral pancreatic bud expresses pdx1, ptf1a and mmr2a and will generate the whole pancreatic exocrine tissue and a small number of endocrine cells (Lin et al., 2004; Wendik et al., 2004; Zecchin et al., 2004). The ventral bud will grow and by 54 hpf eventually envelops the single islet derived from the dorsal pancreatic bud.

The role of the LPM for the specification or the growth of the pancreatic buds has not been investigated yet in zebrafish. The LPM has been implicated in the leftward bend of the developing intestine, known as ‘gut looping’ (Horne-Badovinac et al., 2003). In this process, taking place between 26 and 30 hpf, the left and right LPM migrate asymmetrically toward the midline pushing the gut on the left side of the embryo. The LPM has also been shown to play a
crucial function in the specification of the liver, which is formed just anteriorly to the ventral pancreatic bud. This induction is mediated by expression of \textit{wnt2bb} in the LPM adjacent to the pre-hepatic endoderm (Ober et al., 2006).

The present study was designed (1) to determine whether a tissue equivalent to the pancreatic mesenchyme described in the amniotes is also present in zebrafish embryos; (2) to investigate how this tissue is established; and (3) to explore its function in pancreatic development. We found that, in zebrafish embryos, the LPM is established; and (3) to explore its function in pancreatic is also present in zebrafish embryos; (2) to investigate how this tissue is

For vibratome sections, embryos were embedded in 4% SeaPlaque agarose (Tebu) for fluorescent-labeled in situ hybridization on thick (150 \( \mu \)m) sections. The nuclei were stained on the sections by TO-PRO-3 iodide (642/661 nm, Invitrogen). Sections were mounted in ProLong Gold Antifade Reagent (Invitrogen).

**Fluorescent imaging**

Confocal imaging was performed using a Leica TCS SP2 inverted confocal laser microscope (Leica Microsystems, Germany). Digitized images were acquired using a 63× (NA 1.2) Plan-Apo water-immersion objective at 1024×1024 pixel resolution. For multicolor imaging, FITC was visualized by using an excitation wavelength of 488 nm and the emission light was dispersed and recorded at 500-535 nm. Cy3 was detected by using an excitation wavelength of 543 nm and the fluorescence emission was dispersed and recorded at 555-620 nm. TO-PRO-3 iodide was detected by using an excitation wavelength of 633 nm and the fluorescence emission was dispersed and recorded at 650-750 nm. The acquisition was set up to avoid any cross-talk of the three fluorescence emissions. Series of optical sections were carried out to analyze the spatial distribution of fluorescence, and for each embryo, they were recorded with a Z-step ranging between 1 and 2 \( \mu \)m. Image processing, including background subtraction, was performed with Leica software (version 2.5). Captured images were exported as TIFF and further processed using Adobe Photoshop and Illustrator CS2 for figure mounting.

**MATERIALS AND METHODS**

Embryos

Zebrafish (\textit{Danio rerio}) were raised and cared for according to standard protocols (Westefield, 1995). Wild-type embryos from the AB strain were used and staged according to Kimmel (Kimmel et al., 1995). The \textit{ikaros} (\textit{ika}) mutant allele of \textit{fgf24} and \textit{daedalus} (\textit{dau}) mutant allele of \textit{fgf10} embryos were provided in methanol by C. Neumann (Fischer et al., 2003; Norton et al., 2005).

**Morpholino design and injection**

Morpholino oligonucleotides (MO) were synthesized by Gene Tools (Corvallis, OR). Each MO was resuspended in Danieau’s solution at the stock concentration of 8 \( \mu \)g/\( \mu \)l. For injections, they were diluted in Danieau’s solution at the given concentration. Rhodamine dextran was added at 0.5% to the samples to check injection efficiency. For \textit{isl1} and \textit{meis3} MO, optimal doses, 1.5 ng per injection, defined as the highest dose that did not significantly increase mortality or cause overt non-specific necrosis, were determined empirically. For \textit{fgf24} and \textit{fgf10} MO injections, the lower dose resulting in the absence of pectoral fin buds was determined to 2 ng/injection for each MO and was the condition used in our experiments (Fischer et al., 2003; Norton et al., 2005). Double knockdown experiments were achieved by injecting 1 ng of MO \textit{fgf10} and MO \textit{fgf24} each. MO sequences are: splice inhibition MO \textit{isl1}, ATGCAATGCCTACT-CTGCCATTGTA (E313); translation inhibition MO \textit{meis3}, AGCTCA-CACTCTGCTACGAGGAGA; \textit{fgf10} and \textit{fgf24} MO were described previously: splice \textit{fgf10}, MO GAAAATGTAGTCTCAGCGCCTCGTAG (E212 MO) (Norton et al., 2005); \textit{fgf24} MO, AGGAGACTCCCTGACCGTACG-TACTGGCC (E313 MO) (Draper et al., 2003). Presented MO data were collected from at least three reproducible and independent experiments.

**Whole-mount in situ hybridization**

Single- and double-labeled in situ hybridization (ISH) were performed as described (Hauptmann and Gerster, 1994). Digoxigenin or DNP incorporated in the riboprobes were localized immunohistochemically with an antibody conjugated to alkaline phosphatase \([\text{anti-DIG-AP (Roche) or anti-DNP-AP (Vector Laboratories)}]\) and with NBT/BCIP or Fast Red (Invitrogen) as substrate.

**RESULTS**

\textit{isl1}, \textit{fgf10} and \textit{meis3} are expressed in the LPM adjacent to the emerging ventral pancreas

In mouse, the mesenchyme surrounding the dorsal pancreatic bud is characterized by the expression of \textit{isl1} and \textit{fgf10} genes (Ahlgren et al., 1997; Bhushan et al., 2001). Also, strong expression of the murine \textit{meis3} genes, encoding homeodomain proteins of the TALE family, has been observed in the pancreatic mesenchymal cells (Zhang et al., 2006; Oulad-Abdelghani et al., 1997). In an attempt to identify a tissue analogous to the murine pancreatic mesenchyme in zebrafish embryos, we analyzed in detail the expression pattern of the zebrfish \textit{isl1}, \textit{fgf10} and \textit{meis3} genes in the pancreatic region. At 24 hpf, \textit{isl1} expression was detected only in the endocrine cells of the dorsal bud located on the midline (Fig. 1A), as previously reported (Biemiar et al., 2001). At 28 hpf, \textit{isl1} expression begins in two bilateral domains just anterior to the dorsal pancreatic bud (orange arrows in Fig. 1A). At later stages (32 and 38 hpf, Fig. 1A), these two \textit{isl1} anterior domains overlap and relocate progressively on the left side of the embryo. This additional \textit{isl1} expression domain led us to ask whether it could be analogous to the murine pancreatic mesenchyme. We thus examined two other genes described in the mouse mesenchyme, \textit{fgf10} and \textit{meis3}. We noticed \textit{fgf10} expression in two domains in the same region as \textit{isl1} at 28 hpf (Fig. 1B). At later stages, these two \textit{fgf10} domains also move to the left side of the embryos. At 24 hpf, \textit{meis3} is expressed as two lateral stripes in the same region (Fig. 1C). These two stripes reached the midline at about 30 hpf and afterwards relocated to the left side of the embryo. Expression of \textit{meis3} strengthens in this region at 38 hpf (Fig. 1C). Since \textit{isl1} additional expression was located anterior and not closely juxtaposed to the dorsal bud, we compared the expression of the \textit{isl1}, \textit{fgf10} and \textit{meis3} genes with \textit{ptf1a}, the first ventral pancreatic bud marker (Lin et al., 2004; Zecchin et al., 2004) which appeared by 32 hpf in a position anterior to the dorsal pancreatic bud. Double in situ hybridization revealed that \textit{isl1}, \textit{meis3} and \textit{fgf10} expression domain is contiguous with the ventral pancreatic bud (Fig. 1D).

To refine the identification of these expression domains, transverse sections through the pancreatic region were performed slightly anterior to the dorsal pancreatic bud. By 30-32 hpf, \textit{meis3}
was expressed in a broad region contiguous to the gut that included the domain labeled by isl1 (Fig. 2A,B). At this antero-posterior level, the gut undergoes a looping to the left caused by the right LPM that pushes the gut toward the left side of the embryo, whereas the left LPM migrates dorsally to the endoderm (Horne-Badovinac et al., 2003). isl1 and meis3 were expressed in the left and right LPM, with meis3 expression greater than isl1, the former being limited to the region of the LPM abutting the right and dorsal sides of the gut. We never detected meis3 expression in the endoderm (see Fig. S1 in the supplementary material). At 32 hpf, the first cells of the ventral pancreatic bud, detected by low expression of ptf1a and mnr2a genes (Wendik et al., 2004), originated ventrolaterally from the gut (Fig. 2C). At 35 hpf, the expression of these two markers increased within these pancreatic cells which then migrated ventrally to the gut toward the endocrine islet situated more posteriorly (Fig. 2D,E,F). At 32 hpf, a few meis3 or isl1-expressing cells of the left LPM contacted the dorsal-most mnr2a-labeled cells (Fig. 2B,C). At 35 hpf, cell-cell contacts were also observed ventrally between migrating cells of the ventral bud and cells expressing meis3 and isl1 in the right LPM (Fig. 2E,F). Similarly, fgf10 was found in the LPM (Fig. 2D). Like meis3, fgf10 in the LPM was more broadly expressed than isl1. These findings are consistent with our working hypothesis that the LPM adjacent to the ventral bud, expressing isl1, fgf10 and meis3, could be the functional equivalent of the mesenchyme surrounding the murine dorsal pancreatic bud. This tissue, which we named the pancreatic LPM, could release regulatory signals controlling the specification and/or growth of the zebrafish ventral pancreatic bud and hence, the formation of the exocrine pancreas.

Knockdown of isl1 and meis3 reduces the exocrine pancreatic tissue
To investigate the function of the pancreatic LPM expressing the isl1 and meis3 genes in the development of the ventral pancreatic bud, which gives rise to the exocrine pancreatic tissue, morpholino (MO) antisense oligonucleotides against isl1 or meis3 were injected into one-cell-stage embryos. Knockdown of isl1 or meis3 resulted in a partial loss of the exocrine tissue as revealed by the restricted trypsin (try) domain at 72 hpf in the majority of injected embryos (see Fig. S2 in the supplementary material), whereas injection of the control MO had no effect on the exocrine pancreas. This result is in agreement with the reduction of the exocrine tissue recently reported after injection of a different isl1 MO in elaA:GFP embryos (Wan et al., 2006). We could not detect any effect on the activation of ptf1a or mnr2a in these morphants at 36 hpf (data not shown). These results indicate that isl1 and meis3 are not essential for the specification of the pancreatic ventral bud but rather influence the subsequent growth of the exocrine tissue. Thus, the role of the pancreatic LPM adjacent to the ventral pancreatic anlagen consists at least in promoting the growth of the exocrine tissue.

FGF signaling, but not Fgf10, is essential for induction of the ventral pancreatic bud and for differentiation of pancreatic LPM
We described above the expression of fgf10 in the pancreatic LPM. Since Fgf10 secreted by the pancreatic mesenchyme is crucial for the growth of the associated pancreatic bud in mouse embryos, we next determined whether, in zebrafish embryos, the effect of the pancreatic LPM on the growth of the ventral pancreatic bud is driven...
by Fgf10. To this purpose, we analyzed the pancreatic exocrine tissue in fgf10 morphants and in fgf10\(^{-/-}\) daedalus (dae) mutant embryos (Norton et al., 2005). In contrast to results with meis3 and isl1, knockdown or mutation of fgf10 did not disturb the development of the pancreatic exocrine tissue (Fig. 3A and data not shown).

In view of the importance of FGFs in pancreatic development in mouse, we assessed whether other FGFs might be involved in this process in zebrafish embryos by using the pharmacological FGF receptor inhibitor, SU5402 (Mohammadi et al., 1997). Embryos were treated with the inhibitor from 24 to 29 hpf, just before the specification of the ventral pancreatic bud, and then washed extensively. The expression of the early exocrine marker ptf1a, analyzed at 36 hpf, was completely abrogated upon treatment in 100% embryos compared with control embryos (Fig. 3B). Concomitantly, the expression of meis3 and isl1 in the pancreatic LPM was also lost. In all the treated embryos, the dorsal bud, which was labeled with isl1, was not altered. Consistently with the loss of ptf1a at 36 hpf, the expression of the exocrine marker trypsin was lost at 76 hpf whereas the endocrine islet generated by the dorsal bud, highlighted by the endocrine marker neurod, developed...
correctly (Fig. 3C). The expression of the endoderm marker foxa1 showed that the gross morphology of the endoderm appeared normal (Fig. 3C). A shorter treatment from 26 to 29 hpf gave the same results (not shown) indicating that FGF-dependent events critical for the ventral bud induction occur within this period. By contrast, when embryos were exposed to the FGF inhibitor at later stages, from 29 to 32 hpf, 32 to 36 hpf or from 48 to 54 hpf, trypsin expression persisted but the size of the exocrine tissue was strongly reduced (Fig. 3D and data not shown). All these observations imply that the FGF activity is required between 26 hpf and 29 hpf for the specification of the ventral pancreatic anlagen and of the pancreatic LPM, whereas at later stages, the FGF activity is also crucial for the growth of the exocrine tissue.

**Role of fgf24 in the specification of the ventral pancreatic bud**

As blocking the FGF signaling completely prevented the development of the ventral pancreatic bud whereas mutation of the fgf10 gene did not disrupt this tissue, we hypothesized the involvement of other FGF gene(s) in the development of the exocrine tissue. In the search for such genes, we identified a second fgf10 gene in the zebrafish genome, fgf10b, also recently reported as fgf25 (Katoh and Katoh, 2005). However, analysis of fgf10b revealed no expression in the pancreatic region from 1 to 3 dpf (data not shown). Beside several other FGF genes tested (fgf7, fgf11, fgf18), we found fgf24 expressed in the pancreatic region. fgf24 is a new member of the Fgf8/17/18 subclass of FGF ligands for which there is no ortholog among the 22 known Fgfs in the mouse-human family (Draper et al., 2003; Fischer et al., 2003; Itoh and Ornitz, 2004). fgf24 is also critical for the initiation of the pectoral fin buds and acts upstream fgf10 in this process (Draper et al., 2003; Fischer et al., 2003). We examined more thoroughly its expression in the pancreatic region from 24 to 36 hpf, during the specification of the ventral pancreatic bud. At 24 hpf, we detected fgf24 expression within the pancreatic endoderm, as revealed by co-labelling with the pancreatic marker pdx1 (Fig. 4A). The pdx1 domain consists of the prospective ventral pancreatic bud anteriorly, of the dorsal bud posteriorly, and of the region of the gut attached to the dorsal bud. fgf24 pancreatic expression was specifically found in the prospective ventral bud but not in the dorsal bud. fgf24 is also expressed in more posterior parts of the gut (not shown) (Draper et al., 2003). After 30 hpf, the expression in the prospective ventral bud decreased progressively whereas a slight staining becomes visible in the pancreatic LPM adjacent to the prospective ventral pancreatic bud, as revealed by its co-expression with isl1 (Fig. 4B,D). At 36 hpf, fgf24 expression in the pancreatic region was restricted to the pancreatic LPM whereas its endodermal expression domain was retained only in the posterior gut (Fig. 4C).

The role of fgf24 in pancreas development was next determined by knockdown using an fgf24 antisense MO and by analyzing the fgf24−/− ikarus (ika) mutant embryos (Fischer et al., 2003). In both cases, fgf24 loss of function was assessed by the absence of pectoral fin buds (Fig. 5A), whereas the overall morphology of the fgf24 morphants at 3 days post fertilization (dpf), like the ika mutants, was not altered. Similar results were obtained with fgf24 ika mutants and with the fgf24 morphants. Inactivation of fgf24 function resulted in
a significant reduction of the pancreatic exocrine compartment at 3 dpf in most fgf24 morphant or mutant embryos (Fig. 5B), whereas expression of ceruloplasmin (cp) in the liver was normal. Moreover, ptf1a expression was not detected at 36 hpf in about one third of fgf24 morphant or ika mutant embryos (Fig. 5C) and was reduced in the remaining mutant/morphant embryos. Since differentiation of trypsin-expressing cells, albeit diminished, still occurs in all fgf24 mutant embryos at 3 dpf, one hypothesis was that the onset of the ventral bud specification might be delayed in ika embryos. We thus examined the expression of ptf1a at later stages of development. ptf1a was indeed expressed at 50 hpf in all the fgf24 mutant and morphant embryos, although the level of expression was significantly reduced (Fig. 5E). In support of the delay of the ventral morphant embryos, although the level of expression was not detected at 36 hpf in about one third of ptf1a expression was not detected at 36 hpf in about one third of the heterozygotes or wild-type siblings (not shown). Taken together, these results demonstrate that the specification of the ventral pancreatic bud is postponed rather than prevented in the absence of fgf24.

We next addressed the influence of fgf24 on the pancreatic LPM. At 36 hpf, the fgf24 morphant/mutant embryos with no ptf1a expression in the endoderm were also devoid of isl1 expression in the LPM; the remaining fgf24 morphant/mutant embryos exhibited decreased isl1 expression (Fig. 5C). Despite these defects, isl1 expression in the dorsal bud (i.e. endocrine islet) was not altered in any of the mutant embryos, consistent with the absence of fgf24 in this part of the pancreas. The strong expression of meis3 normally observed at 36 hpf in the pancreatic LPM was also reduced in most fgf24 morphant/mutant embryos while the gut marker gata6 was not altered (Fig. 5C). Since fgf24 and fgf10 are also markers of the pancreatic LPM, their expression at 36 hpf was determined in the morphants/mutants. fgf24 expression was completely absent in about half of the morphants and mutants (Fig. 5D), showing that fgf24 is involved in its own activation in the pancreatic LPM. By contrast, fgf10 expression in this tissue appeared unchanged compared with that in control embryos.

Thus, it can be inferred from all these data that fgf24 plays a role in the patterning of the pancreatic LPM and in the proper specification of the pancreatic ventral bud. However, given that the defects displayed by fgf24 mutants/morphants result from a delay of the ventral bud specification whereas a complete and persistent absence of ventral bud was caused by the pharmacological inhibition of FGF signaling, these findings suggest the involvement of another FGF factor.

The pancreatic LPM is a direct target tissue of fgf24 expressed within the endoderm

Because the loss of fgf24 function affects both the ventral pancreatic bud and the adjacent LPM, we next asked which of these tissues is a direct target of fgf24. To that end, the expression of pea3 and erm genes – two well-known direct targets of the FGF signaling pathway – has been analyzed in detail (Fig. 6). pea3 expression started by 28 hpf in the pancreatic region and was clearly detected within the pancreatic LPM at 36 hpf, as revealed by double labeling with ptf1a probes (Fig. 6A). erm onset of expression started earlier, from 26 hpf, within the pancreatic LPM (at 26-28 hpf, the right and left pancreatic LPM gene expression in this tissue appeared unchanged compared with that in control embryos.

Fig. 5. fgf24 is required for the specification of the ventral pancreatic bud. (A) General morphology of a MO fgf24-injected embryo at 3 dpf compared with a control injected embryo. Identical results were obtained with mutants and morphants and illustration in morphants or mutants is stated on the images. (B) trypsin (try) and ceruloplasmin (cp) expression in fgf24 loss-of-function embryos at 3 dpf. trypsin was reduced in 80% (n=121) of embryos, whereas cp was not affected. (C) Expression of the ventral bud marker ptf1a and of the pancreatic LPM marker isl1, or of the gut marker gata6 and the pancreatic LPM gene meis3 at 36 hpf. ptf1a was absent in 32% and reduced in 59% of the embryos (n=146). isl1 was absent in 17% or reduced in 80% (n=81) of embryos, and meis3 was reduced in 72% (n=95). The orange arrows indicate expression in the pancreatic LPM. (D) fgf10 and fgf24 expression at 36 hpf in the pancreatic region in fgf24 loss-of-function embryos. fgf24 was repressed in the pancreatic LPM in 52% of the embryos (n=96). (E) ptf1a (green arrowheads) was expressed at 50 hpf in both control and the fgf24 loss-of-function embryos. DB, dorsal pancreatic bud; FB, pectoral fin buds; VB, ventral pancreatic bud.
left LPM have not yet joined up) (Fig. 6B and see Fig. S3 in the supplementary material). *erm* and *pea3* expression in the pancreatic LPM was strongly reduced in the *fgf24* morphant embryos (Fig. 6A and data not shown) thereby establishing that *erm* and *pea3* are activated by the FGF24 signaling in this tissue. These results clearly indicate that the pancreatic LPM is a direct target of FGF24 signaling.

As *fgf24* was expressed within the endoderm between 24 hpf and 28 hpf, this raised the hypothesis that an important and early function of *fgf24* is to mediate a signaling from the endoderm to the mesoderm in order to specify the pancreatic LPM. To further investigate the role of the endoderm in patterning the pancreatic LPM, we analyzed the pancreatic LPM markers in *casanova* (cas) mutants (Alexander et al., 1999). *isl1* and *meis3* expression was absent in the LPM at 36 hpf (Fig. 6C). *pea3*, *erm* and *fgf24* were also lost in the LPM of *cas* mutants (data not shown), indicating that the expression of the pancreatic LPM markers relies on a signal provided by the endoderm. At 30 hpf, by contrast, *meis3* expression in *cas* mutants resembled the 24 hpf expression pattern characterized by two bilateral labeled stripes (compare Fig. 6C with Fig. 1C). The presence of the endoderm after 24 hpf is therefore required for the maintenance and the up-regulation of *meis3* in the LPM and for the activation of *isl1*, *fgf24*, *erm* and *pea3* in the same tissue. This observation supports the fact that the LPM and the endoderm come in close contact by 24-26 hpf (Horne-Badovinac et al., 2003) and with our present data showing that SU5402 blocks the expression of the pancreatic LPM markers when applied between 26 and 29 hpf.

Taken together, all these findings support the idea that endodermal expression of *fgf24* plays a key role in patterning the adjacent pancreatic LPM.

**fgf24 and fgf10 cooperate for the specification of the ventral pancreatic bud**

The fact that the defects caused by *fgf24* loss of function are less penetrant and less severe than those caused by SU5402 suggests a compensation effect from other FGF factors. FGF10 could be such a factor. Indeed, while mutation of *fgf10* causes no obvious defect in exocrine pancreas development (Fig. 3A), *fgf10* is expressed in the pancreatic LPM after 28 hpf (Fig. 1B and Fig. 2D) and its function could be masked by *fgf24* also expressed in this tissue. However, our present results reveal that *fgf10* alone is not mandatory for exocrine pancreas development, which derives from the ventral pancreatic bud. Therefore, a putative redundancy between *fgf24* and *fgf10* could mask a role of *fgf10* in the development of the ventral bud. To address this possibility, double knockdowns were performed by injecting a combination of MO *fgf10* and MO *fgf24* (Fig. 7). The single and double morphant embryos were characterized by a loss of pectoral fins as expected, although their overall morphology was not drastically affected (Fig. 7A). Strikingly, *trypsin* expression at 3 dpf was dramatically repressed in the majority of the double *fgf10/fgf24* morphant embryos (Fig. 7B), as observed upon SU5402 treatment (Fig. 3C), whereas only a weak reduction of *trypsin* was observed in the *fgf24* morphants and mutants. None of the double *fgf10/fgf24* morphant embryos exhibited *ptf1a* expression at 36 hpf (Fig. 7C). The delayed *ptf1a* expression observed at 50 hpf in the
Thus, all these data reveal a redundant function of fgf24 in the development of the pancreatic ventral bud. It has been shown that the patterning of the pancreatic LPM relies on FGF signaling. Indeed, blocking the FGF signaling by the FGFR inhibitor SU5402 suppressed the expression of the pancreatic LPM markers isl1 and meis3. To be efficient, this blocking has to be performed in a very restricted time window, that is, between 24 and 29 hpf or even 26 and 29 hpf; later exposures having no effect. Finally, we show that FGF24 released by the endoderm during this period is involved in this patterning.

The endoderm patterns the pancreatic LPM via FGF signaling

A major finding from the present work is that the endoderm specifies the pancreatic LPM (Fig. 8A). This has been confirmed by multiple approaches. Indeed, casanova mutant embryos, which lack endoderm, display defects in the patterning of the pancreatic LPM: isl1 and fgf24 genes are not activated and meis3 expression is not maintained in the pancreatic LPM of these mutants. The loss of gene expression is the result of failed specification rather than of the absence of LPM since the LPM is present in zebrafish mutants devoid of endoderm (Horne-Badovinac et al., 2003). Furthermore, we showed that the patterning of the pancreatic LPM relies on FGF signaling. Indeed, blocking the FGF signaling by the FGFR inhibitor SU5402 suppressed the expression of the pancreatic LPM markers isl1 and meis3. To be efficient, this blocking has to be performed in a very restricted time window, that is, between 24 and 29 hpf or even 26 and 29 hpf; later exposures having no effect. Finally, we show that FGF24 released by the endoderm during this period is involved in this patterning. Indeed, fgf24 expression is restricted to the endoderm at these stages (<30 hpf) and fgf24 morphant/mutant embryos display significantly reduced expression of isl1, meis3 and fgf24 in the pancreatic LPM and these results were confirmed using the fgf24 mutants. By contrast, fgf10 expression appears to be independent from fgf24 function.

Another important finding of our study is that fgf24-mediated signaling from the endoderm triggered the expression of FGF signaling targets erm and pea3 in the adjacent pancreatic LPM. Indeed, erm expression can be detected in the pancreatic LPM at 26 hpf, but not within the pancreatic endoderm. This stage coincides with the appearance of isl1 expression and precedes the strengthening of meis3 expression in this tissue—the LPM patterning process. Moreover, the expression of erm and pea3 was lost in casanova mutant embryos (not shown) as well as in fgf24 morphants suggesting that FGF24 released by the endoderm initiates an FGF cascade in the adjacent tissue via transcription factors such as Erm and Pea3 that will in turn control isl1 and meis3 transcription, either directly or indirectly.

In mouse, signaling factors involved in the specification of the pancreatic mesenchyme have not yet been identified. As the fgf24 gene is not present in mammals (Fischer et al., 2003; Draper et al., 2003; Itoh and Ornitz, 2004), another member of the FGF family should fulfill this role in the mouse. fgf24 belongs to the FGF8/17/18 subfamily. Although there is no report of expression of these Fgf genes in the developing pancreas, it would be interesting to closely examine their expression in this tissue and to investigate a putative function in patterning the murine pancreatic mesenchyme.

Previous mouse data shown the importance of another signaling molecule, Shh, in endoderm-driven patterning of the mesoderm during the development of the gastro-intestinal tract. Shh is expressed in the whole gut endoderm, except at the level of the prepancreatic endoderm (Kim et al., 1997). It has been shown that ectopic expression of Shh within the pancreatic epithelium converts the pancreatic mesenchyme into duodenal mesoderm and represses pancreas development from the endoderm (Apelqvist et al., 1997). As in mouse, zebrafish shh expression in the endoderm is excluded

DISCUSSION

Although numerous studies have shown the importance of the mesoderm in the regionalization of the gut, our findings reveal that endoderm-to-mesoderm signaling mediated by FGF is a primary event governing pancreatic development. We propose a model in which the zebrafish ventral pancreas development occurs in three successive steps (see Fig. 8). The first phase, taking place between 24 and 30 hpf, consists of the patterning by the pancreatic endoderm of the adjacent LPM through the secretion of Fgf24 (see Fig. 8A): fgf24 stimulates its own expression in the pancreatic LPM as well as that of isl1 and meis3. In the second phase (30–32 hpf), the pancreatic LPM instructs the endoderm to induce the expression of ptf1a gene thereby specifying the ventral pancreatic bud (see Fig. 8B). During the last step, from 32 hpf onwards, FGF signals secreted by the pancreatic LPM promote the ventral bud growth (see Fig. 8C).
Fig. 8. Three-step model for endoderm-mesoderm cross-talk controlling formation of the zebrafish ventral pancreatic bud. (A) First phase, 26-29 hpf. Early fgf24 expression within the region of the endoderm (orange) that will give rise to the pancreatic ventral bud patterns the adjacent LPM (green) into the pancreatic LPM. erm is a probable direct Fgf24 transcriptional target within the LPM. The pancreatic LPM is characterized by pea3, erm, isl1, meis3, fgf24 and fgf10 expression. fgf10 (light gray) is weakly expressed in the pancreatic LPM at these stages. (B) Second phase, 29-32 hpf. The pancreatic LPM triggers the induction at 32 hpf of ptf1a expression in endodermal cells (ptf1a-positive cells schematized in blue). fgf24 expression is restricted within the pancreatic LPM and, at the same time, fgf10 expression increases in the same tissue. pea3 and erm expression in the pancreatic LPM indicates that FGF signaling is active in this tissue. As SU5402 exposures after 26-29 hpf do not abrogate ventral bud specification, this suggests that this step is FGFindendent (signal X and white arrow), fgf10 and fgf24 are functionally redundant in patterning the pancreatic LPM, and therefore in specifying the ventral bud. At the same time, fgf24 expression disappears from the pancreatic endoderm and both fgf10 and fgf24 are expressed in the pancreatic LPM. (C) Third phase, after 32 hpf. Since SU5402 treatments after ventral bud specification limit the size of the exocrine tissue, we propose that FGF genes, perhaps fgf24 and fgf10, could be involved in mesoderm-to-endoderm communication promoting ventral pancreas growth. DB, dorsal pancreatic bud; VB, ventral pancreatic bud.

from the pancreas (Roy et al., 2001). A putative functional interaction between shh and fgf during zebrafish pancreatic development remains to be determined.

The zebrafish pancreatic LPM induces the pancreatic ventral bud and promotes its subsequent growth

In addition to the endoderm-to-mesoderm signal discussed above, our results also highlight a subsequent mesoderm-endoderm interaction during the second phase that initiates ptf1a expression at 32 hpf in the ventral bud (Fig. 8B). This was suggested by the key observation of the redundant function of fgf10 and fgf24 in the patterning of the pancreatic LPM and in the specification of the ventral pancreatic bud. The combined knockout of both genes led to a complete loss of isl1, fgf24 and meis3 expression in the pancreatic LPM and to the concomitant absence of ptf1a expression within the endoderm (Fig. 7C and data not shown), whereas fgf24 loss of function alone only delayed the induction of ptf1a and reduced isl1 expression in a fraction of the embryos. As fgf10 and fgf24 expression colocalizes in the pancreatic LPM from 30 hpf, the simplest interpretation is that the redundant activities of Fgf24 and Fgf10 are required to accurately pattern the pancreatic LPM, which subsequently will send a signal to the endoderm for activating ptf1a expression correctly at 32 hpf (factor X in Fig. 8B). Nevertheless, it is unlikely that Fgf24 and Fgf10 act directly upon the endoderm to induce the expression of ptf1a at 32 hpf because blocking Pgf activity with SU5402 after 29 hpf, when fgf24 and fgf10 expression by the pancreatic LPM starts, does not suppress ptf1a expression in a similar mesoderm-to-endoderm signal has been reported in chick embryos, where the LPM instructs the endoderm to differentiate into pancreatic endoderm (Kumar et al., 2003). BMPs and activin factors were proposed to be potential mesodermal signals implicated in this regulation. Interestingly, FGF10 and FGF8 were not able to stimulate pancreatic induction in chick embryos, strengthening our findings that FGFs are not involved in the second phase of the zebrafish ventral bud specification.

However, at later stages (after specification of the ventral bud), Fgf24 and Fgf10 or a distinct FGF could have an effect on the endoderm to control ventral pancreatic growth as SU5402 treatments of embryos at these stages reduce the exocrine pancreatic tissue. Consistent with this model, fgf10 expression in mesenchymal cells within the pancreatic region has been reported at later stages (Dong et al., 2007). In addition, we observed the expression of fgfr2, known to encode for the main receptor of FGF10 in other species, in both the endoderm and mesoderm during ventral pancreas development (see Fig. S4 in the supplementary material).

Another argument supporting the role of the pancreatic LPM in the specification of the ventral pancreatic bud was provided by the analysis of heart and soul (has) zebrafish mutant embryos. In these mutants, two ventral pancreatic buds are specified on each side of the embryonic gut (Field et al., 2003). Analysis of isl1, meis3 and fgf10 expression in has mutant embryos indicated that the left and right pancreatic LPM do not migrate to the midline but stay in a lateral position contacting the gut on each side (data not shown) (Horne-Badovinac et al., 2003). Thus, this bilateral contact between endoderm and the pancreatic LPM may explain the duplicated ventral pancreatic buds.

In mouse, the pancreatic mesenchyme is crucial for the growth of the pancreatic tissue by stimulating the proliferation of pancreatic progenitor cells. This mesenchyme expresses Isl1 (Ahlgren et al., 1997), Meis genes (Zhang et al., 2006) and Fg410 (Bhushan et al., 2001). Isl1 expression is limited in the murine pancreatic mesenchyme surrounding the dorsal pancreatic bud and mutation of Isl1 consistently affects formation of this bud (Ahlgren et al., 1997). Fg410, expressed in both the dorsal and ventral pancreatic mesenchyme, is crucial for the growth of both dorsal and ventral pancreatic epithelium (Bhushan et al., 2001; Ye et al., 2005). In the present study, we identified in zebrafish, the pancreatic LPM, analogous to the dorsal pancreatic mesenchyme surrounding the murine dorsal bud, on the basis of the expression of isl1, fgf10 and meis3. However, this tissue is rather contiguous to the ventral rather than the dorsal bud. Furthermore, our data clearly indicate that this pancreatic LPM is crucial for the specification and the growth of the pancreatic ventral bud. Our finding that meis3 is exclusively expressed in the LPM contradicts a recent report of meis3 expression in the endoderm (dilorio et al., 2007). However, neither transverse section nor double staining with endodermal marker was analyzed in that study. In addition, our data are consistent with the strong expression of meis genes in the mouse dorsal bud mesenchyme (Zhang et al., 2006). Nevertheless, it is possible that meis3 is...
expressed within the endoderm, albeit at a much lower level than in the LPM, as it was not detectable by in situ hybridization or earlier during gastrulation.

In amniotes, the LPM gives rise to the dorsal pancreatic mesenchyme expressing fgf10 and is11 and surrounding the dorsal pancreatic epithelium. In zebrafish, fgf10 and is11 are already expressed in the LPM. This tissue is just lying on the prospective ventral anlagen instead of surrounding the epithelium as observed in mouse. Later, cells expressing is11 and fgf10 form a mesenchyme contacting the hepatopancreatic ducts (Dong et al., 2007).

Our study shows that, after specification of the ventral pancreatic bud (32 hpf), the pancreatic LPM stimulates the growth of this bud. Indeed, knockdown of is11 or meis3 provoked a significant reduction of the exocrine tissue at late stages, whereas the initial activation of ptf1a was unaffected. This growth effect could be mediated by FGF signaling (Fig. 8C). This hypothesis is supported by two observations. First, treatment of zebrafish embryos with the FGF inhibitor SU5402 after 32 hpf led to a significant reduction in exocrine tissue (see Fig. 3D). Second, fgf24 mutation/knockdown, detected in the pancreatic LPM after 32 hpf, also limits the expansion of the exocrine tissue in most embryos at late stages. However, as the exocrine tissue is not drastically affected in some fgf24 morphant/mutant embryos, it is highly probable that, in addition to fgf24, other FGF genes (such as fgf10) control exocrine growth. In contrast to data obtained in mice, mutation of fgf10 alone does not affect the growth of the zebrafish pancreatic buds. Our result is in agreement with a recent study describing the role of zebrafish fgf10 in the establishment of the hepatopancreatic duct system at late stages (60-80 hpf) and the lack of effect on ptf1a expression was also noticed (Dong et al., 2007). Nevertheless, the effect of fgf10 on pancreatic bud growth could be masked by a redundant fgf24 activity.

In amniotes and amphibians, both the ventral and dorsal pancreatic buds generate exocrine tissue. However, in zebrafish embryos, the dorsal pancreatic bud seems to give rise only to endocrine cells and the whole exocrine tissue appears to derive from the ventral pancreatic bud. Our present data could explain such a difference. Indeed, previous studies performed in rodents, using cultured pancreatic epithelium in absence or presence of mesenchyme, showed that the pancreatic mesenchyme plays a specific pro-exocrine effect (Ahlgren et al., 1997; Miralles et al., 1999; Li et al., 2004; Gittes et al., 1996). In those studies, cultures of pancreatic epithelium without any mesenchyme could lead to the differentiation of endocrine cells but never of exocrine cells. However, co-cultures of pancreatic epithelium with mesenchyme give rise to large amount of exocrine cells. Since, in zebrafish embryos, the pancreatic LPM, which seems to play an equivalent function of the murine pancreatic mesenchyme, is located adjacent to the ventral pancreatic bud, this may explain why pancreatic endocrine and exocrine tissues are generated by the different buds in zebrafish. In mammals, both the ventral and dorsal buds are in direct contact with mesenchymal cells derived from the LPM.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/22/4011/DC1

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