

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

Isabelle Manfred^{1,*}, François Delporte¹, Ariane Baudhuin¹, Patrick Motte², Carl J. Neumann³, Marianne L. Voz¹, Joseph A. Martial¹ and Bernard Peers¹

In amniotes, the pancreatic mesenchyme plays a crucial role in pancreatic epithelium growth, notably through the secretion of fibroblast growth factors. However, the factors involved in the formation of the pancreatic mesenchyme are still largely unknown. In this study, we characterize, in zebrafish embryos, the pancreatic lateral plate mesoderm, which is located adjacent to the ventral pancreatic bud and is essential for its specification and growth. We firstly show that the endoderm, by expressing the *fgf24* gene at early stages, triggers the patterning of the pancreatic lateral plate mesoderm. Based on the expression of *isl1*, *fgf10* and *meis* genes, this tissue is analogous to the murine pancreatic mesenchyme. Secondly, Fgf10 acts redundantly with Fgf24 in the pancreatic lateral plate mesoderm and they are both required to specify the ventral pancreas. Our results unveil sequential signaling between the endoderm and mesoderm that is critical for the specification and growth of the ventral pancreas, and explain why the zebrafish ventral pancreatic bud generates the whole exocrine tissue.

KEY WORDS: Zebrafish, Pancreas, FGF, Signaling, Endoderm, Mesoderm, *ptf1a*

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. *Hlx9/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 that interacts with the pancreatic endoderm and induces the budding, the growth and the branching of the pancreatic

epithelium (Golosow and Grobstein, 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 *mnr2a* 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

¹GIGA-Research–Unité de Biologie Moléculaire et Génie Génétique, Tour B34, Université de Liège, B-4000 Sart Tilman, Belgium. ²Laboratoire de Biologie Cellulaire Végétale, Cellule d’Appui Technologique en Microscopie, Université de Liège, Institut de Botanique, Bâtiment B22, B-4000 Sart-Tilman, Belgium. ³European Molecular Biology Laboratory (EMBL), Meyerhofstrasse 1, D-69117 Heidelberg, Germany.

*Author for correspondence (e-mail: isabelle.manfred@ulg.ac.be)

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 *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 adjacent to the ventral pancreatic bud, that we named pancreatic LPM, expresses *isll*, *meis3* and *fgf10* and plays a role in ventral pancreas induction and growth. In addition, we uncover a novel, earlier and pivotal function of the FGF signaling in the specification of the pancreatic LPM. Indeed, transient endodermal *fgf24* expression is critical for the patterning of the pancreatic LPM, which is required for the subsequent induction of the ventral pancreatic bud. Our study also reveals that *fgf10* and *fgf24* display a redundant activity in patterning the pancreatic LPM.

MATERIALS AND METHODS

Embryos

Zebrafish (*Danio rerio*) were raised and cared for according to standard protocols (Westerfield, 1995). Wild-type embryos from the AB strain were used and staged according to Kimmel (Kimmel et al., 1995). The *ikarus* (*ika^{lx118}* mutant allele of *fgf24*) and *daedalus* (*dae^{lxbbo}* mutant allele of *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 $\mu\text{g}/\mu\text{l}$. 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 *isll* and *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 *fgf24* and *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 *fgf10* and MO *fgf24* each. MO sequences are: splice inhibition MO *isll*, ATGCAATGCCTACTGCCATTTGTA (E3I3); translation inhibition MO *meis3*, AGCTCACACTCACTGACGGAGGA; *fgf10* and *fgf24* MO were described previously: splice *fgf10*, MO GAAAATGATGCTCACCGCCCCGTAG (E2I2 MO) (Norton et al., 2005); *fgf24* MO, AGGAGACTCCCGTACCGTACTTGCC (E3I3 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 [anti-DIG-AP (Roche) or anti-DNP-AP (Vector Laboratories)] and with NBT/BCIP or Fast Red (Invitrogen) as substrate.

Fluorescent labeling was performed as described (Mavropoulos et al., 2005). The DIG- and DNP-labeled probes were either revealed by Tyramide-Cy3 or Tyramide-FITC using the Perkin Elmer TSA kit and peroxidase linked to the first revealed probe was inactivated by a 90-minute incubation with 2% H_2O_2 .

The riboprobes used were *isll* (Korz et al., 1993), *meis3* [accession number AF222995 (Sagerstrom et al., 2001)], *fgf10* (EST fd11d03.x1, clone MPMGp609F0649Q, RZPD), *fgf24* (Fischer et al., 2003), *neurod* (Korz et al., 1998), *pdx1* (Milewski et al., 1998), *foxa1* (Odenthal and Nusslein-Volhard, 1998), *ceruloplasmin* (Korz et al., 2001), *trypsin* (Biemar et al., 2001) and *ptf1a* (Zecchin et al., 2004).

For vibratome sections, embryos were embedded in 4% SeaPlaque agarose (Tebu) for fluorescent-labeled in situ hybridization on thick (150 μm) 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 \times (NA 1.2) Plan-Apo water-immersion objective at 1024 \times 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 μm . 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.

RESULTS

isll, *fgf10* and *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 *isll* and *fgf10* genes (Ahlgren et al., 1997; Bhushan et al., 2001). Also, strong expression of the murine *meis* 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 zebrafish *isll*, *fgf10* and *meis3* genes in the pancreatic region. At 24 hpf, *isll* expression was detected only in the endocrine cells of the dorsal bud located on the midline (Fig. 1A), as previously reported (Biemar et al., 2001). At 28 hpf, *isll* 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 *isll* anterior domains overlap and relocate progressively on the left side of the embryo. This additional *isll* 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, *fgf10* and *meis3*. We noticed *fgf10* expression in two domains in the same region as *isll* at 28 hpf (Fig. 1B). At later stages, these two *fgf10* domains also move to the left side of the embryos. At 24 hpf, *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 *meis3* strengthens in this region at 38 hpf (Fig. 1C). Since *isll* additional expression was located anterior and not closely juxtaposed to the dorsal bud, we compared the expression of the *isll*, *fgf10* and *meis3* genes with *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 *isll*, *meis3* and *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, *meis3*

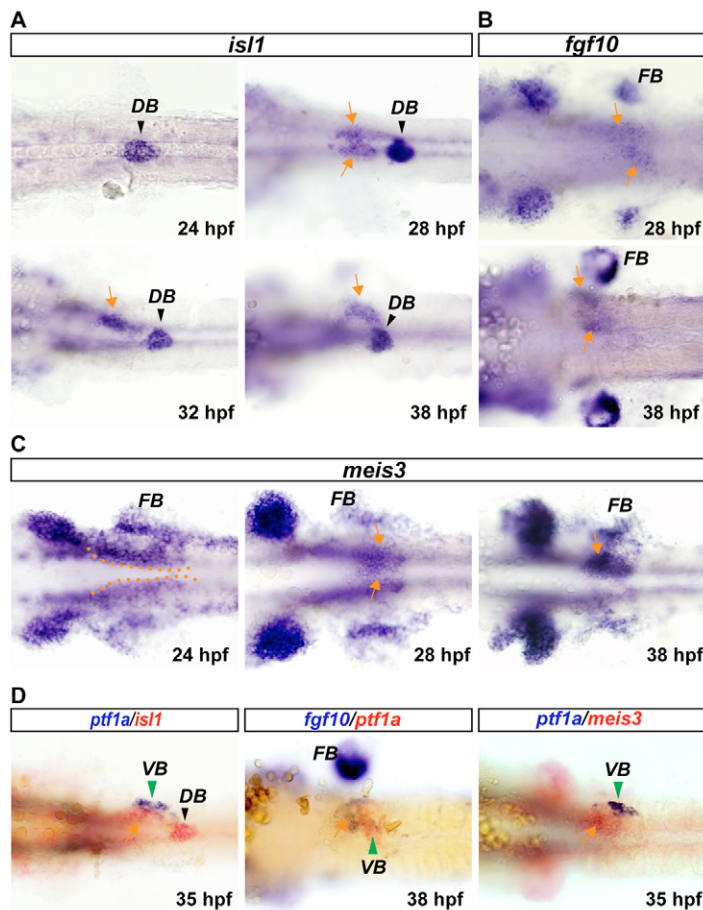


Fig. 1. *is11*, *fgf10* and *meis3* expression adjacent to the pancreatic ventral bud. Embryos analyzed by whole-mount in situ hybridization for the expression of *is11*, *fgf10* and *meis3*. Images are ventral views of the trunk embryo, with the anterior oriented to left and the left side of the embryo to the top. **(A)** *is11* expression from 24 to 36 hpf. **(B)** *fgf10* expression at 28 and 36 hpf. **(C)** *meis3* expression from 24 to 36 hpf. Dotted yellow lines highlight the bilateral expression domain. **(D)** Double-labeled whole-mount in situ hybridization showing the expression of *is11*, *fgf10* and *meis3* compared with *ptf1a*. Yellow arrows indicate expression of *is11*, *meis3* and *fgf10* in a tissue adjacent to the ventral pancreatic bud. *DB*, dorsal pancreatic bud; *FB*, pectoral fin bud; *VB*, ventral pancreatic bud. Magnification, 200 \times .

was expressed in a broad region contiguous to the gut that included the domain labeled by *is11* (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). *is11* and *meis3* were expressed in the left and right LPM, with *meis3* expression greater than *is11*, 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 *is11*-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 *is11* 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 *is11*. These findings are consistent with our working hypothesis that the LPM adjacent to the ventral bud, expressing *is11*, *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 *is11* and *meis3* reduces the exocrine pancreatic tissue

To investigate the function of the pancreatic LPM expressing the *is11* and *meis3* genes in the development of the ventral pancreatic bud, which gives rise to the exocrine pancreatic tissue, morpholino (MO) antisense oligonucleotides against *is11* or *meis3* were injected into one-cell-stage embryos. Knockdown of *is11* 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 *is11* 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 *is11* 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

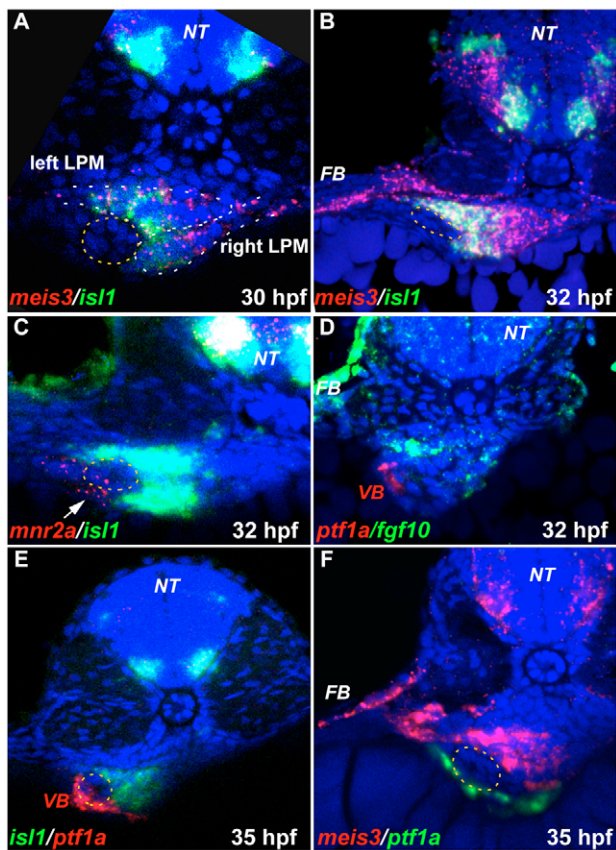


Fig. 2. *isl1* and *meis3* label the LPM next to the developing ventral pancreatic bud. Confocal analysis of transverse sections of embryos stained by fluorescent whole-mount in situ hybridization with two probes (red and green) through the pancreatic region. Nuclear staining was achieved with TO-PRO-3 (633 nm) and artificially colored in blue. The left side of the embryo is situated to the left in all panels. (A,B) *meis3* (red) and *isl1* (green) expression at 30 hpf (A) and 32 hpf (B). (C) *isl1* expression with *mnr2a* at 32 hpf. (D) Expression of *fgf10* (green) and *ptf1a* (red) at 32 hpf. (E) *isl1* (green) and *ptf1a* (red) expression at 35 hpf. (F) *meis3* (red) and *ptf1a* (green) expression at 35 hpf. On transverse section in A, the white dotted lines highlight the left and right LPM. In A-F, the yellow dotted lines encircle the gut tube. The white arrows indicate the appearing ventral bud cells. Owing to the low levels of *mnr2a* and *fgf10* expression, the views in C and D are flat stacking of several consecutive optical sections. VB, ventral pancreatic bud; DB, dorsal pancreatic bud; FB, pectoral fin bud; NT, neural tube.

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

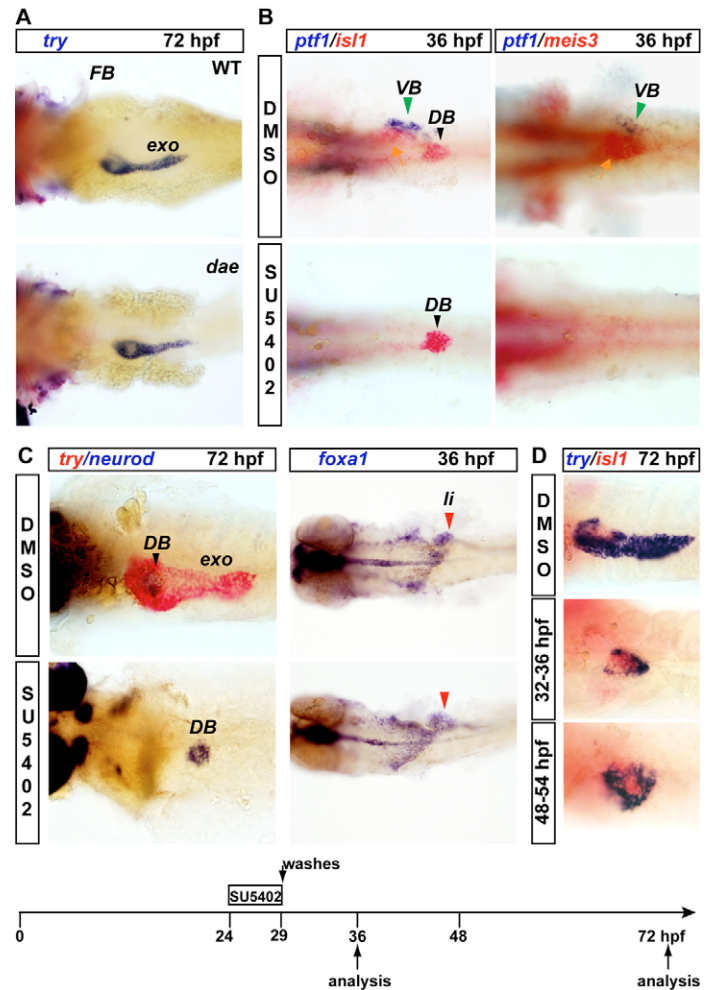


Fig. 3. Inhibition of FGF signaling, but not Fgf10, impairs specification of the ventral pancreatic bud and the expression of *isl1* and *meis3* in the adjacent pancreatic LPM. (A) *trypsin* (*try*) expression analysis in wild-type embryos (WT, top) and in *fgf10*^{-/-} mutants (*dae*, bottom) at 72 hpf. Note the underdeveloped pectoral fin bud in the *dae* mutant. (B) Expression of *ptf1a* (blue), *isl1* and *meis3* (red) at 36 hpf in embryos treated with the FGF signaling inhibitor SU5402 from 24 to 29 hpf. (C) Expression of *trypsin* (red) and *neurod* (blue) at 72 hpf after the same treatment as in B and analysis of the endodermal marker *foxa1* at 36 hpf upon SU5402 treatment. Note that the liver and the rest of the endoderm are clearly labeled whereas, at this stage, the pancreas is almost undetectable. The SU5402 treatment analyzed in B and C is schematized at the bottom of the panel. (D) *trypsin* (blue) and *isl1* (red) expression at 72 hpf in embryos exposed to SU5402 from 32 to 36 hpf and from 48 to 54 hpf. The green arrowhead indicates the ventral pancreatic bud (VB); the black arrowhead indicates the dorsal bud (DB); and the yellow arrow indicates the pancreatic LPM adjacent to the ventral bud. *exo*, exocrine tissue; *li*, liver; FB, pectoral fin bud.

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

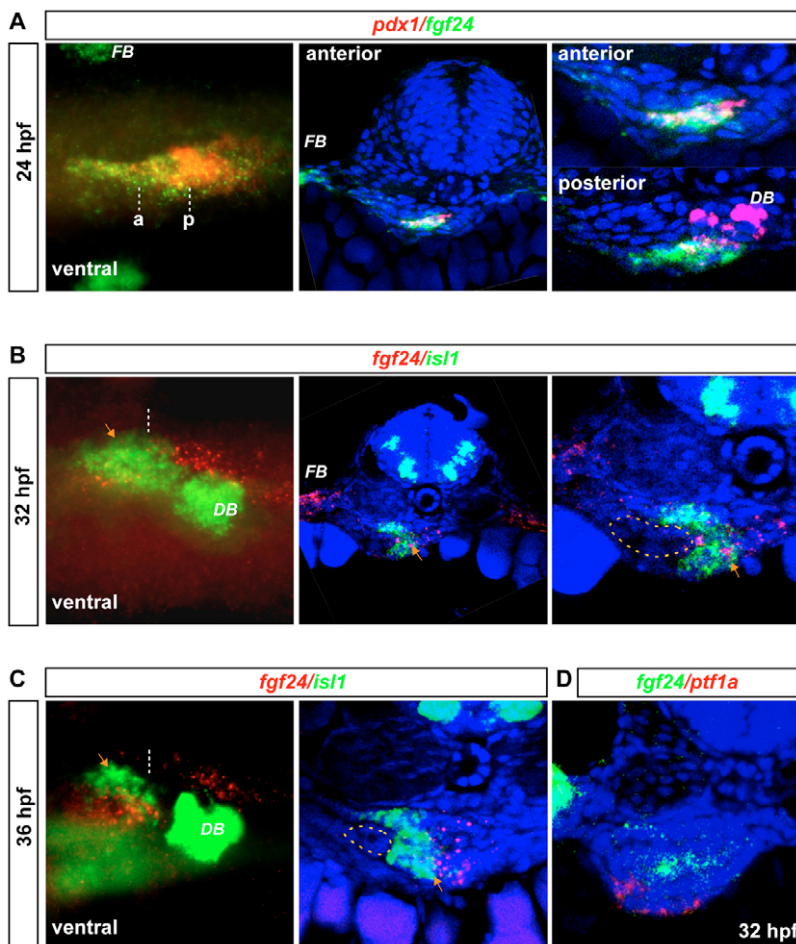


Fig. 4. *fgf24* is expressed in the pancreatic endoderm and in the pancreatic LPM prior to and during ventral pancreatic bud formation. (A) *fgf24* (green) expression analysis by fluorescent whole-mount in situ hybridization at 24 hpf with *pdx1* probe (red). A whole-mount ventral view (epifluorescence microscopy) is shown on the left panel (anterior to the left and left side of the embryo up) with anterior (a) and posterior (p) level of section. The transverse sections were analyzed by confocal microscopy, through the anterior pancreatic domain (middle panel, global view; right panel, close-up) and more posteriorly, through the dorsal pancreatic bud (right panel, close-up). (B) Expression of *fgf24* (red) compared with *isl1* (green) at 32 hpf (transverse section, close-up in the right panel). Expression in the pancreatic LPM is indicated by orange arrows. (C) *fgf24* and *isl1* expression at 36 hpf. *fgf24* expression appears as small red grains owing to its weak expression. (D) *fgf24* (green) and *ptf1a* (red) expression at 32 hpf. The images of transverse sections presented in B and C are flat stacking of several consecutive optical sections. DB, dorsal pancreatic bud; FB, pectoral fin bud.

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 bud specification, *ptf1a* expression was not activated at 33 hpf in 92% of *fgf24* mutant embryos compared with the normal *ptf1a* expression in 91% 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

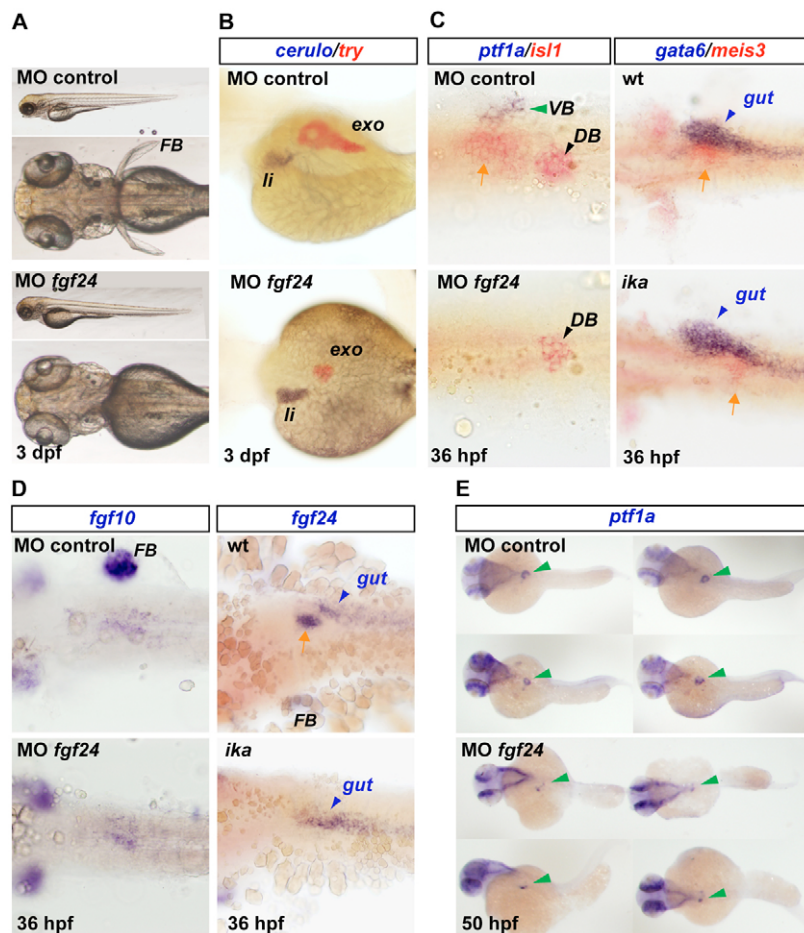


Fig. 5. *fgf24* is required for the specification of the ventral pancreatic bud. (A) General morphology of a MO *fgf24*-injected embryo at 3 dpf compared with a control injected embryo. Identical results were obtained with mutants and morphants and illustration in morphants or mutants is stated on the 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* mutants (*cas*) embryos, which are devoid of endoderm (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

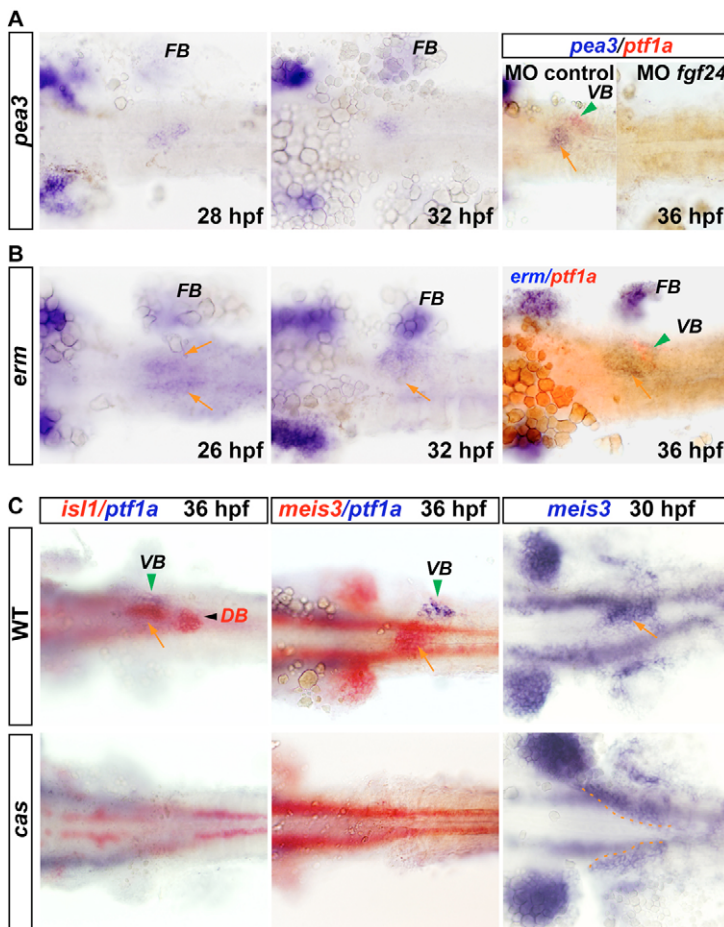


Fig. 6. The pancreatic LPM is a target of *fgf24* expressed within the endoderm. (A,B) Expression analysis of the FGF target genes *pea3* (A) and *erm* (B) just before and during specification of the ventral pancreatic bud from 26 to 36 hpf.

Right panel in A, *pea3* (blue) and *ptf1a* (red) expression in *fgf24* loss-of-function embryos. The green arrowheads indicate the ventral pancreatic bud (VB) and the orange arrows point to the pancreatic LPM. Right panel in B, *erm* (blue) and *ptf1a* (red) expression at 36 hpf. (C) Expression of *ptf1a* (blue) and *meis3* (30 and 36 hpf) and *isl1* (36 hpf) in red in *casanova* (*cas*) mutants. The yellow dotted lines underline the LPM labeled by *meis3*. DB, dorsal pancreatic bud; FB, pectoral fin buds.

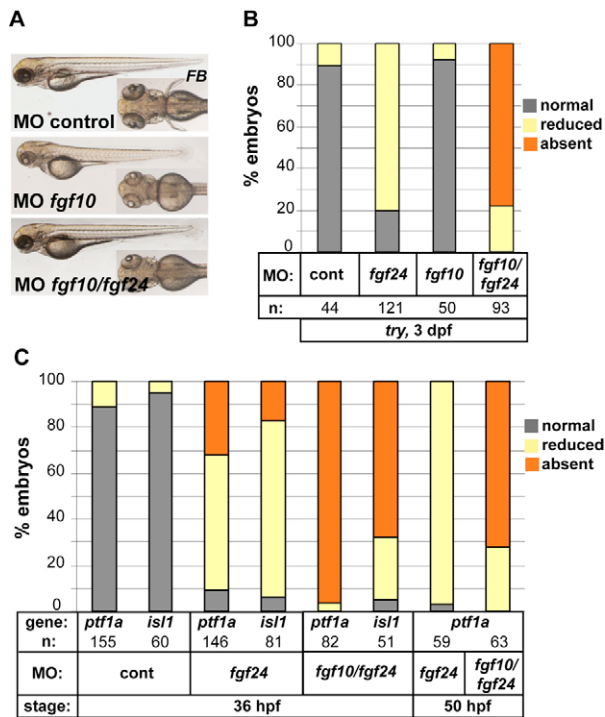


Fig. 7. *fgf24* and *fgf10* cooperate to specify the ventral pancreatic bud. (A) Overall morphology of *fgf10* and *fgf10/fgf24* morphants at 3 dpf. Note the absence of pectoral fin buds in both morphants. (B) *trypsin* (*try*) expression at 3 dpf in embryos injected with MO control, MO *fgf24*, MO *fgf10* and with a combination of MO *fgf10* and MO *fgf24*. Data are presented as the percentage of embryos displaying normal, reduced, or absent expression of *trypsin*. (C) Expression analysis reported as in B, as the percentage of embryos expressing *ptf1a* in the ventral pancreatic bud and the pancreatic LPM marker *isl1* in MO-injected embryos at 36 and 50 hpf. *FB*, pectoral fin bud; n, number of analyzed injected embryos.

fgf24 mutants/morphants (Fig. 5E) was severely impaired after *fgf10/fgf24* double knockdown (Fig. 7C). To determine whether these defects correlate with changes in the pancreatic LPM next to the ventral bud, we analyzed *isl1* expression at 36 hpf. Within the pancreatic LPM, *isl1* was almost undetectable in most double morphants whereas it was unaffected in the pancreatic dorsal bud at this stage (Fig. 7C). Similar observations were made when we examined *meis3* and *fgf24* expression (data not shown). The pancreatic phenotype of the double *fgf24/fgf10* morphants was similar to the defects presented by SU5402-treated embryos (Fig. 3B). Thus, all these data reveal a redundant function of *fgf10* and *fgf24* in the development of the pancreatic ventral bud.

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).

The endoderm patterns the pancreatic LPM via FGF signaling

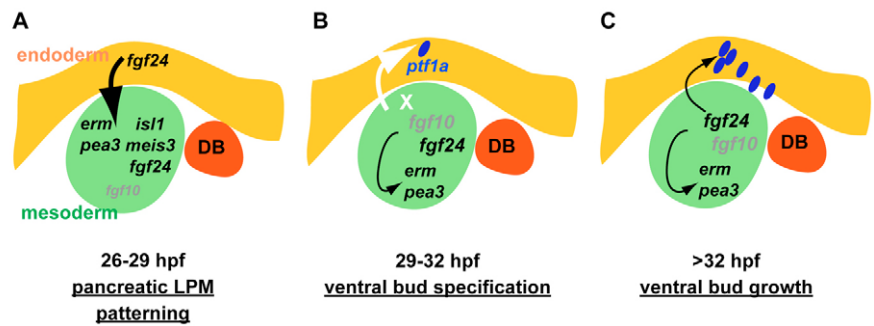
A major finding from the present work is that the endoderm specifies the pancreatic LPM (Fig. 8A). This has been confirmed by multiple approaches. Indeed, *casanova* mutant embryos, which lack endoderm, display defects in the patterning of the pancreatic LPM: *isl1* and *fgf24* genes are not activated 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 fulfil 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

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 FGF-independent (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 knockdown 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 FGF activity with SU5402 after 29 hpf, when *fgf24* and *fgf10* expression by the pancreatic LPM starts, does not suppress *ptf1a* expression. 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 *Fgf10* (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). *Fgf10*, 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 (diIorio 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 *isll* and surrounding the dorsal pancreatic epithelium. In zebrafish, *fgf10* and *isll* 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 *isll* 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 *isll* or *meis3* provoked a significant reduction of the exocrine tissue at late stages, whereas the initial activation of *ptfla* 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 *ptfla* expression was also noticed (Dong et al., 2007). Nevertheless, the affect 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.

We thank D. Y. R. Stainier for *casanova* and *heart and soul* mutant lines; R. Scharfmann for critical reading of the manuscript; S. A. Harvey for the *fgf24* cDNA; I. Kreins for help with some experiments. I.M. is a Chargée de recherches and B.P. is a Chercheur qualifié at the Belgian National Fund for Research (FRS-FNRS). This work was funded by the Belgian State Program on 'Interuniversity Poles of Attraction' (SSTC, PAI p5/35) and by the 6th European Union Framework Program (Beta-Cell Therapy Integrated Project). P.M. was funded by FRFC 2.4542.00 and 2.4540.06, and les Fonds spéciaux pour la recherche ULg.

Supplementary material

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

References

- Ahlgren, U., Jonsson, J. and Edlund, H. (1996). The morphogenesis of the pancreatic mesenchyme is uncoupled from that of the pancreatic epithelium in IPF1/PDX1-deficient mice. *Development* **122**, 1409–1416.
- Ahlgren, U., Pfaff, S. L., Jessell, T. M., Edlund, T. and Edlund, H. (1997). Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells. *Nature* **385**, 257–260.
- Alexander, J., Rothenberg, M., Henry, G. L. and Stainier, D. Y. (1999). *casanova* plays an early and essential role in endoderm formation in zebrafish. *Dev. Biol.* **215**, 343–357.
- Apelqvist, A., Ahlgren, U. and Edlund, H. (1997). Sonic hedgehog directs specialised mesoderm differentiation in the intestine and pancreas. *Curr. Biol.* **7**, 801–804.
- Bhushan, A., Itoh, N., Kato, S., Thiery, J. P., Czernichow, P., Bellusci, S. and Scharfmann, R. (2001). Fgf10 is essential for maintaining the proliferative capacity of epithelial progenitor cells during early pancreatic organogenesis. *Development* **128**, 5109–5117.
- Biemar, F., Argenton, F., Schmidtke, R., Epperlein, S., Peers, B. and Driever, W. (2001). Pancreas development in zebrafish: early dispersed appearance of endocrine hormone expressing cells and their convergence to form the definitive islet. *Dev. Biol.* **230**, 189–203.
- dilorio, P., Alexa, K., Choe, S. K., Etheridge, L. and Sagerstrom, C. G. (2007). TALE-family homeodomain proteins regulate endodermal sonic hedgehog expression and pattern the anterior endoderm. *Dev. Biol.* **304**, 221–231.
- Dong, P. D., Munson, C. A., Norton, W., Crosnier, C., Pan, X., Gong, Z., Neumann, C. J. and Stainier, D. Y. (2007). Fgf10 regulates hepatopancreatic ductal system patterning and differentiation. *Nat. Genet.* **39**, 397–402.
- Draper, B. W., Stock, D. W. and Kimmel, C. B. (2003). Zebrafish *fgf24* functions with *fgf8* to promote posterior mesodermal development. *Development* **130**, 4639–4654.
- Field, H. A., Dong, P. D., Beis, D. and Stainier, D. Y. (2003). Formation of the digestive system in zebrafish. II. Pancreas morphogenesis. *Dev. Biol.* **261**, 197–208.
- Fischer, S., Draper, B. W. and Neumann, C. J. (2003). The zebrafish *fgf24* mutant identifies an additional level of Fgf signaling involved in vertebrate forelimb initiation. *Development* **130**, 3515–3524.
- Gittes, G. K., Galante, P. E., Hanahan, D., Rutter, W. J. and Debase, H. T. (1996). Lineage-specific morphogenesis in the developing pancreas: role of mesenchymal factors. *Development* **122**, 439–447.
- Golosow, N. and Grobstein, C. (1962). Epitheliomesenchymal interaction in pancreatic morphogenesis. *Dev. Biol.* **4**, 242–255.
- Hauptmann, G. and Gerster, T. (1994). Two-color whole-mount *in situ* hybridization to vertebrate and *Drosophila* embryos. *Trends Genet.* **10**, 266.
- Hebrok, M., Kim, S. K. and Melton, D. A. (1998). Notochord repression of endodermal Sonic hedgehog permits pancreas development. *Genes Dev.* **12**, 1705–1713.
- Horne-Badovinac, S., Rebagliati, M. and Stainier, D. Y. (2003). A cellular framework for gut-looping morphogenesis in zebrafish. *Science* **302**, 662–665.
- Itoh, N. and Ornitz, D. M. (2004). Evolution of the Fgf and Fgfr gene families. *Trends Genet.* **20**, 563–569.
- Jacquemin, P., Lemaigre, F. P. and Rousseau, G. G. (2003). The *Oncut* transcription factor HNF-6 (OC-1) is required for timely specification of the pancreas and acts upstream of *Pdx-1* in the specification cascade. *Dev. Biol.* **258**, 105–116.
- Jacquemin, P., Yoshitomi, H., Kashima, Y., Rousseau, G. G., Lemaigre, F. P. and Zaret, K. S. (2006). An endothelial-mesenchymal relay pathway regulates early phases of pancreas development. *Dev. Biol.* **290**, 189–199.
- Jonsson, J., Carlsson, L., Edlund, T. and Edlund, H. (1994). Insulin-promoter-factor 1 is required for pancreas development in mice. *Nature* **371**, 606–609.
- Katoh, Y. and Katoh, M. (2005). Comparative genomics on FGF7, FGF10, FGF22 orthologs, and identification of *fgf25*. *Int. J. Mol. Med.* **16**, 767–770.
- Kawaguchi, Y., Cooper, B., Gannon, M., Ray, M., MacDonald, R. J. and Wright, C. V. (2002). The role of the transcriptional regulator Ptf1a in converting intestinal to pancreatic progenitors. *Nat. Genet.* **32**, 128–134.
- Kim, S. K., Hebrok, M. and Melton, D. A. (1997). Notochord to endoderm signaling is required for pancreas development. *Development* **124**, 4243–4252.
- Kimmel, C. B., Ballard, W. W., Kimmel, S. R., Ullmann, B. and Schilling, T. F. (1995). Stages of embryonic development of the zebrafish. *Dev. Dyn.* **203**, 253–310.
- Korz, S., Emelyanov, A. and Korzh, V. (2001). Developmental analysis of ceruloplasmin gene and liver formation in zebrafish. *Mech. Dev.* **103**, 137–139.
- Korz, V., Edlund, T. and Thor, S. (1993). Zebrafish primary neurons initiate expression of the LIM homeodomain protein *Isl-1* at the end of gastrulation. *Development* **118**, 417–425.
- Korz, V., Slepstova, I., Liao, J., He, J. and Gong, Z. (1998). Expression of

- zebrafish bHLH genes *ngn1* and *nrd* defines distinct stages of neural differentiation. *Dev. Dyn.* **213**, 92-104.
- Kumar, M. and Melton, D.** (2003). Pancreas specification: a budding question. *Curr. Opin. Genet. Dev.* **13**, 401-407.
- Kumar, M., Jordan, N., Melton, D. and Grapin-Botton, A.** (2003). Signals from lateral plate mesoderm instruct endoderm toward a pancreatic fate. *Dev. Biol.* **259**, 109-122.
- Lammert, E., Cleaver, O. and Melton, D.** (2003). Role of endothelial cells in early pancreas and liver development. *Mech. Dev.* **120**, 59-64.
- Li, H., Arber, S., Jessell, T. M. and Edlund, H.** (1999). Selective agenesis of the dorsal pancreas in mice lacking homeobox gene *Hlx9*. *Nat. Genet.* **23**, 67-70.
- Li, Z., Manna, P., Kobayashi, H., Spilde, T., Bhatia, A., Preuett, B., Prasadani, K., Hembree, M. and Gittes, G. K.** (2004). Multifaceted pancreatic mesenchymal control of epithelial lineage selection. *Dev. Biol.* **269**, 252-263.
- Lin, J. W., Biankin, A. V., Horb, M. E., Ghosh, B., Prasad, N. B., Yee, N. S., Pack, M. A. and Leach, S. D.** (2004). Differential requirement for *ptf1a* in endocrine and exocrine lineages of developing zebrafish pancreas. *Dev. Biol.* **270**, 474-486.
- Mavropoulos, A., Devos, N., Biemar, F., Zecchin, E., Argenton, F., Edlund, H., Motte, P., Martial, J. A. and Peers, B.** (2005). *sox4b* is a key player of pancreatic alpha cell differentiation in zebrafish. *Dev. Biol.* **285**, 211-223.
- Milewski, W. M., Duguay, S. J., Chan, S. J. and Steiner, D. F.** (1998). Conservation of PDX-1 structure, function, and expression in zebrafish. *Endocrinology* **139**, 1440-1449.
- Miralles, F., Czernichow, P., Ozaki, K., Itoh, N. and Scharfmann, R.** (1999). Signaling through fibroblast growth factor receptor 2b plays a key role in the development of the exocrine pancreas. *Proc. Natl. Acad. Sci. USA* **96**, 6267-6272.
- Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P., Yeh, B. K., Hubbard, S. R. and Schlessinger, J.** (1997). Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* **276**, 955-960.
- Norton, W. H., Ledin, J., Grandel, H. and Neumann, C. J.** (2005). HSPG synthesis by zebrafish *Ext2* and *Extl3* is required for *Fgf10* signalling during limb development. *Development* **132**, 4963-4973.
- Ober, E. A., Verkade, H., Field, H. A. and Stainier, D. Y.** (2006). Mesodermal *Wnt2b* signalling positively regulates liver specification. *Nature* **442**, 688-691.
- Odenthal, J. and Nusslein-Volhard, C.** (1998). fork head domain genes in zebrafish. *Dev. Genes Evol.* **208**, 245-258.
- Oulad-Abdelghani, M., Chazaud, C., Bouillet, P., Sapin, V., Chambon, P. and Dolle, P.** (1997). *Meis2*, a novel mouse Pbx-related homeobox gene induced by retinoic acid during differentiation of P19 embryonal carcinoma cells. *Dev. Dyn.* **210**, 173-183.
- Pictet, R. L., Clark, W. R., Williams, R. H. and Rutter, W. J.** (1972). An ultrastructural analysis of the developing embryonic pancreas. *Dev. Biol.* **29**, 436-467.
- Roy, S., Qiao, T., Wolff, C. and Ingham, P. W.** (2001). Hedgehog signaling pathway is essential for pancreas specification in the zebrafish embryo. *Curr. Biol.* **11**, 1358-1363.
- Sagerstrom, C. G., Kao, B. A., Lane, M. E. and Sive, H.** (2001). Isolation and characterization of posteriorly restricted genes in the zebrafish gastrula. *Dev. Dyn.* **220**, 402-408.
- Spooner, B. S., Walther, B. T. and Rutter, W. J.** (1970). The development of the dorsal and ventral mammalian pancreas in vivo and in vitro. *J. Cell Biol.* **47**, 235-246.
- Wan, H., Korzh, S., Li, Z., Mudumana, S. P., Korzh, V., Jiang, Y. J., Lin, S. and Gong, Z.** (2006). Analyses of pancreas development by generation of *gfp* transgenic zebrafish using an exocrine pancreas-specific elastaseA gene promoter. *Exp. Cell Res.* **312**, 1526-1539.
- Wendik, B., Maier, E. and Meyer, D.** (2004). Zebrafish *mnx* genes in endocrine and exocrine pancreas formation. *Dev. Biol.* **268**, 372-383.
- Westerfield, M.** (1995). *The Zebrafish Book: Guide for the Laboratory Use of Zebrafish (Danio Rerio)*. Eugene, OR: University of Oregon Press.
- Ye, F., Duvillie, B. and Scharfmann, R.** (2005). Fibroblast growth factors 7 and 10 are expressed in the human embryonic pancreatic mesenchyme and promote the proliferation of embryonic pancreatic epithelial cells. *Diabetologia* **48**, 277-281.
- Yoshitomi, H. and Zaret, K. S.** (2004). Endothelial cell interactions initiate dorsal pancreas development by selectively inducing the transcription factor *Ptf1a*. *Development* **131**, 807-817.
- Zecchin, E., Mavropoulos, A., Devos, N., Filippi, A., Tiso, N., Meyer, D., Peers, B., Bortolussi, M. and Argenton, F.** (2004). Evolutionary conserved role of *ptf1a* in the specification of exocrine pancreatic fates. *Dev. Biol.* **268**, 174-184.
- Zhang, X., Rowan, S., Yue, Y., Heaney, S., Pan, Y., Brendolan, A., Selleri, L. and Maas, R. L.** (2006). *Pax6* is regulated by *Meis* and *Pbx* homeoproteins during pancreatic development. *Dev. Biol.* **300**, 748-757.