Essential roles of zebrafish *bmp2a*, *fgf10* and *fgf24* in the specification of the ventral pancreas

running title: role of BMP & FGF in pancreas formation

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Abbreviations
BMP, Bone Morphogenic Protein; EHD, extrahepatic ducts, EPD, extrapancreatic duct; FGF, Fibroblasts Growth Factor; VPB, ventral pancreatic bud; HB, hepatic bud;

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Summary

In vertebrates, pancreas and liver arise from bipotential progenitors located in the embryonic gut endoderm. BMP and FGF signalling pathways have been shown to induce hepatic specification while repressing pancreatic fate. Here, we show that BMP and FGF factors also play crucial function, at slightly later stages, in the specification of the ventral pancreas. By analyzing the pancreatic markers *pdx1*, *ptf1a* and *hlxb9la* in different zebrafish models of BMP loss-of-function, we demonstrate that the BMP pathway is required between 20-24 hpf to specify the ventral pancreatic bud. Knockdown experiments show that *bmp2a*, expressed in the lateral plate mesoderm at these stages, is essential for ventral pancreas specification. *Bmp2a* action is not restricted to the pancreatic domain and is also required for the proper expression of hepatic markers. By contrast, through the analysis of *fgf10-/-; fgf24-/-* embryos, we unveil the specific role of these two FGF ligands in the induction of the ventral pancreas and in the repression of the hepatic fate. These mutants display ventral pancreas agenesis and ectopic masses of hepatocytes. Overall, these data highlight the dynamic role of BMP and FGF in the patterning of the hepatopancreatic region.

Introduction

Pancreas is an endodermal organ composed of endocrine and exocrine tissues that respectively produce hormones and digestive enzymes. During embryogenesis, pancreas arises from two buds developing from the dorsal and ventral aspects of the gut epithelium. The ventral pancreatic bud appears adjacent to the liver bud and previous studies on mouse embryonic explants and in zebrafish have revealed the presence of bipotential endodermal progenitors that can give rise to both organs (Chung et al., 2008; Deutsch et al., 2001; Rossi et al., 2001). This cell fate decision is controlled by extrinsic factors released by the neighbouring lateral plate mesoderm (LPM). Identification of such inducing stimuli is critical to design novel cellular therapies for pathologies affecting these organs.

In zebrafish, specification of the pancreatic region can be detected as early as 14 hours post fertilization (hpf) through the activation of *pdx1* expression in midtrunk endoderm (Biemar et al., 2001). The first *pdx1* expressing cells which are located near the medial line of the embryo just under the notochord will delaminate to form the dorsal pancreatic bud by 24 hpf and will generate the first pancreatic endocrine cells. The first signs of hepatic development occur at 22 hpf with the activation of *prox1* and *hhex* expression in a segment of the gut endoderm anterior to the dorsal pancreatic bud (Ober et al., 2006). These *prox1+*/*hhex+* cells produce an outgrowth on the left side of the intestinal rod. The formation of this hepatic bud is quickly followed by the specification of the ventral pancreatic bud which appears adjacent and posterior to the liver (Field et al., 2003a). Indeed, *ptf1a* and *hlxb9la* (also named *mnr2a)*, the two earliest markers of the ventral pancreas are detected at 32 hpf between the liver and dorsal pancreatic buds (Wendik et al., 2004; Zecchin et al., 2004). The *pdx1* homeobox gene, expressed in endocrine cells of the dorsal bud, is also detected in the adjacent segment of intestinal rod which encompasses the prospective ventral pancreatic bud. In zebrafish, the ventral pancreatic bud (VPB) generates the whole exocrine tissue comprising the acinar and ductal cells. The ventral and dorsal pancreatic buds eventually merge by 52 hpf to form the pancreas (Field et al., 2003a; Field et al., 2003b).

The ventral pancreatic and hepatic buds are induced by the adjacent mesodermal tissues through the release of signalling molecules such as Fibroblast Growth Factors (FGF) and Bone Morphogenic Proteins (BMPs). However, conflicting data have been reported, on the effect of these factors, revealing either an inducing or repressing activity. For example, experiments on mouse embryonic explants have shown that FGF from the cardiac mesoderm and BMP from the septum transversum are essential for the induction of liver markers and block pancreatic specification (Deutsch et al., 2001; Rossi et al., 2001). Similarly, FGFs and BMPs have also been shown in zebrafish to be essential for hepatic induction (Shin et al., 2007) and *bmp2b* can specify liver at the expense of pancreas (Chung et al., 2008). On the other hand, experiments with chicken embryonic explants indicate that BMP from the LPM is required for development of the ventral pancreas (Kumar et al., 2003) and we have reported that FGF signalling is essential to specify the ventral pancreatic bud in zebrafish embryos (Manfroid et al., 2007). These contradictory results could be explained by a highly dynamic change in the inductive network. Indeed, Wandzioch *et al* have recently demonstrated that, while BMPs repress pancreatic specification at 3-4S in mouse embryos, they promote pancreatic fate a few hours later at 5-6S (Wandzioch and Zaret, 2009). Another explanation could be that distinct members of the FGF and/or BMP ligand family have different activities. Thus, a better understanding of liver and ventral pancreas development will require the identification of the BMP and FGF ligands expressed near the prospective hepatopancreatic region and their mutual relation.

In the present study, we show the crucial role of BMP pathway after 20 hpf for the specification of the ventral pancreatic bud in zebrafish embryos. We identify *bmp2a* as a crucial player in this induction and demonstrate its requirement for the activation of the first markers of the ventral pancreas (*ptf1a* and *hlxb9la*) as well as of the liver (*prox1* and *hhex*). In contrast, by analyzing these pancreatic and hepatic markers in the double *fgf10-/-;fgf24-/-* mutants, we find that both FGF10 and FGF24 ligands have complete opposed effect on the two organs, inducing all pancreatic markers while repressing hepatic markers. Thus, our study provides new insights into the molecular mechanisms that initiate development of the liver and pancreas.

Results

Requirement of BMP signalling in the specification of the ventral pancreatic bud

Involvement of BMP signalling in liver bud specification has been previously investigated with *laf/alk8* mutants (Chung et al., 2008). In addition, Chung *et al* have shown that VPB outgrowth was severely affected in *alk8* mutants and *alk8* morphants have a hypoplasic ventral pancreas at 3 days post fertilization (dpf) (Chung et al., 2010). However, it was unknown if BMP signalling was necessary for the first steps of VPB development. Thus, we evaluated VPB specification by analyzing the expression of *ptf1a* and *hlxb9la (mnr2a)*, the two first VPB markers. At 32 hpf, *ptf1a* and *hlxb9la* were absent in the pancreatic region of *alk8* mutants whereas their expression in the neural tube remained normal (Figure 1A-B and 1E-F). However, expression of these two markers was detected a few hours later at around 38 hpf in the pancreatic region of *alk8* mutants, although in very few cells and at a reduced level compared to wild type siblings (Figure 1C-D and 1G-H). While *hlxb9la* was strongly reduced in the prospective VPB of *alk8* mutants at 38 hpf, its expression in the dorsal pancreatic bud stayed unchanged (see arrowhead in Figure 1H). In contrast to *ptf1a* and *hlxb9la*, the pan-pancreatic marker *pdx1* was not reduced in the prospective ventral bud at 30 hpf (Figure 1I-J). Taken together, these results indicate that BMP signalling is required for the proper activation of the two ventral pancreatic genes *ptf1a* and *hlxb9la* but not for the pan-pancreatic gene *pdx1*. These data suggest that the severe pancreas hypoplasia recently reported at 72 hpf in the *alk8* mutant is not only due to an outgrowth defect of the VPB but also results from a severe delay in its specification.

BMP signalling is required at 20 hpf to induce ventral bud specification

To determine more precisely the time-window during which the BMP signalling is required to specify the VPB, we blocked this pathway using the transgenic fish *Tg(hsp70l:dnBmpr-GFP)* (Pyati et al., 2005) expressing a dominant negative form of the BMP receptor fused to GFP under the control of the inducible heat shock promoter hsp70. These fish were crossed with *Tg(ptf1a:eGFP)* fish and embryos from this cross were exposed to one heat shock, at 16 hpf (n=88), 20 hpf (n=103), 24 hpf (n=104) or 28 hpf (n=100). Expression of the dominant negative BMP receptor was verified after the heat shock by following the induced ubiquitous GFP expression, being strong after 3 hours and weak 24 hours after induction. Expression of ptf1a:eGFP in the ventral pancreatic region was analyzed at 48 hpf. Heat shocked embryos could be sorted in three kinds of phenotype: unaffected (Figure 2A), strongly reduced (Figure 2B) and absent (Figure 2C) in the pancreatic region while other expression domains such as eyes and brain remained unaltered. The strongest inhibition of ptf1a:eGFP in the VPB was observed in *Tg(hsp701:dnBmpr-GFP)* embryos heat shocked at 16 or 20 hpf as 75 % of transgenic embryos displayed no GFP in the VPB (Figure 2D). By contrast, heat shocking of embryos lacking the *hsp70:dnBmpr-GFP* transgene had no effect on the expression of ptf1a:GFP. Blocking BMP signalling from 24 hpf in the double transgenic embryos still had

an inhibitory effect on VPB specification but the proportion of affected embryos was smaller compared to 16 and 20 hpf. This proportion was further reduced when the heat shock was performed at 28 hpf. These data indicate that BMP activity is required from 20 hpf onward for the specification of VPB. In order to determine whether, in wild type embryos, the BMP pathway is activated in endodermal cells during this time-window, we performed an immunostaining at 22 hpf with anti-phosphorylated Smad1/5/8 antibody and counterstained with anti-Pdx1 antibody to label pancreatic cells (Figure 2E-G). Nuclear phospho-Smad 1/5/8 staining was indeed detected in some Pdx1+ ventral cells indicating that BMP pathway is activate in pancreatic cells at 22 hpf.

As for *alk8* mutants, we examined the expression of other pancreatic markers in embryos heat shocked at 20 hpf. Similarly to *ptf1a* gene, no expression of *hlxb9la* could be detected in the VPB when analyzed at 48hpf (Figure 2H and 2I). Moreover, *trypsin,* a marker of the acinar pancreatic tissue, the major VPB derivative, is totally absent at 3 dpf (Figure 2J and 2K). Despite the absence of *ptf1a* expression at 32 hpf after BMP inhibition, *pdx1* expression was maintained, as observed in the *alk8* mutants (Figure 2L and 2M). Taken together, theses results indicate that activation of the BMP pathway in endodermal cells after 20 hpf is crucial for the induction of the early ventral pancreatic markers *ptf1a* and *hlxb9la* and for further acinar differentiation.

bmp2a is expressed in LPM adjacent to the ventral pancreatic bud and is involved in its specification

The LPM has been shown in vertebrates to be essential for proper specification of liver and pancreas (Chung et al., 2008; Manfroid et al., 2007). Zebrafish *bmp2a* and *bmp2b* have been both reported to be expressed in LPM at 24 hpf (Chocron et al., 2007; Chung et al., 2008). *bmp2b* is expressed in LPM since 10somites-stage and is required for hepatic specification at the expense of pancreatic fate while *bmp2a* seems activated at later stages. Thus, we investigated more closely the expression of *bmp2a* in the LPM adjacent to the prospective VPB when the BMP pathway is required for VPB specification. *bmp2a* expression starts to be detected in the LPM from 22 hpf at the level of the third somite, ie in the hepatopancreatic region (Figure 3A), and its expression increases at 24 hpf (Figure 3C) To confirm that *bmp2a* was expressed adjacent to the pancreatic region, a double *in situ* hybridization with a *pdx1* probe was performed. *Bmp2a* was expressed just next and on both sides of the *pdx1* expression domain (Figure 3B). Expression of *bmp2a* in LPM is also adjacent to the hepatic anlagen as highlighted by *hhex* staining (shown at 24 hpf in Figure 3C). Furthermore, *bmp2a* is expressed more ventrally compared to *bmp2b* (Figure 3D) and more closely to the prospective VPB and the hepatic bud. Thus, these results show that *bmp2a* is expressed at the correct time and place to be the BMP ligand responsible for proper ventral pancreatic bud specification.

In order to test the role of *bmp2a* in VPB specification, we performed a knockdown of its expression using a morpholino targeting the splice donor site at the *bmp2a* exon 1-intron 1 junction. The efficiency of this knockdown was checked by performing a RT-PCR on RNA extracted from injected embryos using primers annealing to exons 1 and 2 of *bmp2a*. The amplified cDNA was shorter in *bmp2a* morphants compared to control embryos and sequencing of this fragment revealed that the *bmp2a* morpholino induces a deletion of 86 bp in exon1 coding sequence (Figure 4D). This deletion induces a frameshift leading to a truncated protein of 101 aa instead of 386 aa. When the ventral pancreatic markers *ptf1a* and *hlxb9la* were analyzed at 36-38 hpf in *bmp2a* morphants, absence or strong reduction of expression was noted in 69% and 73% of *bmp2a* morphants for these 2 genes respectively, (n=85 and n= 91)(Figure 4A-B). This effect was specific to the VPB and expression of

hlxb9la in dorsal pancreatic bud remained unaffected (see arrowheads in Figure 4B). Expression of *insulin* and *pdx1* was not modified at 30 hpf (data not shown). As observed in *alk8* mutants, expression of *ptf1a* and *hlxb9la* was detected at later stages (48 hpf) in almost all *bmp2a* morphants but significantly reduced compared to control embryos (data not shown). Thus, these data clearly demonstrate the involvement of *bmp2a* in VPB development, its knockdown leading to a strong delay in VPB specification.

As several studies have shown a role of BMP in induction of the hepatic bud (Huang et al., 2008; Shin et al., 2007) and as *bmp2a* is expressed adjacent to the *hhex* expression domain (Figure 3C), we also analyzed the hepatic markers *hhex* and *prox1* in the *bmp2a* morphants. While the expression of these two hepatic genes was still detected, their expression in the hepatic bud was clearly reduced in 44% of morphants (n=153) for *hhex* (Figure 4C) and in 55% of morphants for *prox1* (n=64). These two genes were also expressed at normal level in the dorsal pancreatic bud. Thus, *bmp2a* is not only crucial for the specification of the VPB but is also involved in hepatic development, albeit it is less essential than for the VPB.

To verify that the delay of the VPB specification caused by *bmp2a* morpholino is actually due to the loss of BMP2a activity, we next assessed whether induced ectopic BMP expression could rescue this defect. To activate BMP expression specifically at 20 hpf, we used the *Tg(hsp70l:bmp2b)* transgenic line allowing induction of *bmp2b* expression upon heat shock. *bmp2a* morpholinos were injected in eggs obtained from an outcross between *Tg(hsp70l:bmp2b)* and wild type fish. Then, injected embryos were heat shocked at 20 hpf and *hlxb9la* expression was analyzed by ISH at 38 hpf. Identification of transgenic and non-transgenic embryos was performed after *hlxb9la* staining by genotyping. *Tg(hsp70l:bmp2b)* embryos also display loss of *hlxb9la* expression in rhombomeres 5 and 6 (see arrowheads in Figure 4E and 4F). The rescue of *hlxb9la* expression in *bmp2a* morphants is shown in Figure 4G. The percentage of embryos exhibiting absence of *hlxb9la* expression in the pancreatic region was drastically reduced in the transgenic embryos (12%, n=32) compared to the non transgenic embryos (31%, n=38). Also, the percentage of embryos with normal *hlxb9la* expression in the VPB was significantly higher in the transgenic (50%) compared to non transgenic (21%) embryos. Thus, overexpression of *bmp2b* at 20 hpf can partially rescue the knockdown of *bmp2a* and indicates that the delay in VPB specification caused by the *bmp2a* morpholino is specific.

fgf10 and *24* are specifically involved in ventral pancreatic specification

As the specification of the ventral pancreas and the development of the neighbouring hepatic bud both rely on *bmp2a*, other extrinsic factors must determine the choice between the pancreatic or hepatic fate. We recently reported by morpholino knockdown experiments that *fgf10* acts redundantly with *fgf24* to control the formation of the pancreatic exocrine tissue (Manfroid et al., 2007). However, the role of these two FGF ligands on hepatic specification has not been investigated. Therefore, we generated *fgf10; fgf24* compound mutants and examined in detail the development of the entire hepatopancreatic region. Analysis of *trypsin* and *transferin (tfa)* expression at 3 dpf indicated a complete lack of the pancreatic exocrine tissue in the double mutants while the liver was not reduced (Figure 5A), confirming our previous knockdown data. The absence of pancreatic exocrine tissue was due to a defect in VPB specification as expression of *ptf1a* and *hlxb9la* was not detected in most double *fgf10; fgf24* mutants at 48 hpf (Figure 5B and data not shown). As the VPB also gives rise to the pancreatic ducts, their formation was next analyzed in the compound mutants. Immunolabelling with Prox1, HNF4α and 2F11 antibodies revealed complete lack of the extrapancreatic duct (EPD) connecting the pancreas to the intestine in the mutants at 50 hpf (red arrowhead). Indeed, while the 2F11+ ductal cells delineating the extrahepatic duct (EHD)

still make the junction between the hepatic bud and intestine in the mutants, extrapancreatic ductal cells could not be detected between the pancreatic islet, derived from the dorsal pancreatic bud (also 2F11+), and the intestine (Figure 5D). In addition, the hepatic domain appeared posteriorly expanded towards the pancreatic endocrine islet in all mutants examined (green arrowheads) (n=6 for wild type embryos and n=8 for mutant embryos). Indeed, while the distance from the anterior limit of the hepatic bud to the islet did not significantly change (125 µm in wild type embryos and 127 µm in mutants, yellow dotted lines), the length of the hepatic domain increased (108 µm in mutants compared to 75 µm in wild type embryos, white dotted lines). When the double *fgf10; fgf24* mutants were analyzed at 3 dpf, no pancreatic acinar cell (Prox1+) could be detected around the islet as well as no intra- and extra- pancreatic ducts (Figure 5E and F). In addition, masses of ectopic hepatocyte-like (Prox1+/HNF4α+/2F11-) cells and biliary-like cells (Prox1+/HNF4α-/2F11+ cells) were observed contiguous to the hepatopancreatic duct remnants and also in direct contact with the gut (Figure 5E and F). As ectopic *tfa* expression was also noticed in this region (see white arrowhead in Figure 5A), this supports ectopic hepatic differentiation occurring posterior to the hepatic domain. Thus, all these data demonstrate a complete lack of pancreatic acinar and pancreatic ductal cells in the *fgf10; fgf24* mutants, while hepatic cell differentiation seems increased. These results led us to examine whether, in the double mutants, specification of the hepatic bud was favoured at the expense of the ventral pancreas. To that goal, we examined the expression of the hepatic marker *prox1* and of the pancreatic marker *pdx1* at 29 hpf *i.e.* just prior to the initiation of ventral pancreas specification (Figure 5C). Interestingly, *pdx1* labelling in the ventral aspect of the pancreatic domain was dramatically reduced in all the double mutants while the *prox1*+ hepatic domain was expanded posteriorly and reached the dorsal pancreas (green arrowheads, n=4). This observation suggests that, in the absence of FGF10/FGF24 signalling, hepatic progenitors are specified at the expense of the ventral pancreatic progenitors.

As *bmp2a, fgf24* and *fgf 10* are involved in specification of the VPB, we finally tested whether cross-regulation occurs between these extrinsic factors. As *fgf10* expression starts in the LPM about 5 hours after the onset of *bmp2a* expression (Manfroid et al., 2007), the activation of *bmp2a* cannot be controlled by *fgf10*. As previously reported, the *fgf24* gene displays a dynamic expression pattern, being expressed in the gut endoderm before 24 hpf, then endodermal expression progressively decreases at the level of the pancreas while it appears in the adjacent pancreatic LPM. Expression of *bmp2a* was not modified in *fgf24* mutants at 24 hpf (data no shown). Inversely, *fgf24* and *fgf10* expression was analyzed in *bmp2a* morphants. In order to accurately discern *fgf24* expression in the endoderm and in the LPM, double *in situ* hybridization was performed with *fgf24* and *pdx1* probes. Comparison of wild type and *bmp2a* morphant embryos at 32 hpf revealed that the expression of *fgf24* is strongly decreased in the pancreatic LPM while the level in the endoderm remains high (Figure S1A). In contrast, *fgf10* expression in the LPM was not affected in *bmp2a* morphants (Figure S1B). All these data indicate that *bmp2a* acts not only on the endoderm but also on the mesoderm where it is involved in the activation of *fgf24* expression.

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Discussion

Conflicting data have been reported on the effect of BMP and FGF signals on pancreas development either showing a negative effect (Chung et al., 2008; Deutsch et al., 2001; Rossi et al., 2001; Shin et al., 2007; Spagnoli and Brivanlou, 2008; Tehrani and Lin, 2011) or a positive effect (Kumar et al., 2003; Manfroid et al., 2007; Wandzioch and Zaret, 2009). Studies on mouse embryonic explants have first indicated that these two signalling pathways act on endodermal progenitors to favour hepatic differentiation while restricting formation of pancreatic cells (Rossi et al., 2001). Such role of the BMP pathway has also been confirmed

in zebrafish; indeed, BMP2b secreted from the LPM around 14 hpf acts on endodermal cells to induce hepatic fate at the expense of pancreas (Chung et al., 2008). In the present study, we show that, a few hours later at around 20-24 hpf, the BMP pathway plays a different role and is actually essential for the specification of the ventral pancreatic bud. Furthermore, we identify BMP2a as a major BMP ligand responsible for this induction process. Similarly, FGF signalling has been shown to play a crucial role in development of liver in vertebrate (Deutsch et al., 2001) and notably in zebrafish embryos between 18 and 22 hpf, *i.e.* just before hepatic specification (Shin et al., 2007). Here, we demonstrate that *fgf10* and *fgf24*, also expressed in the LPM around 26 hpf and required for ventral pancreas specification, impair hepatic development. Finally, we show that *bmp2a* positively regulates *fgf24* expression in the LPM. In conclusion, our data underscore the importance of the developmental stage in the response to these extrinsic signals.

BMP signalling is crucial for ventral pancreatic specification

A recent analysis of the *alk8* zebrafish mutants has revealed an increase of endocrine pancreatic β-cells but also a severe hypoplasy of the ventral pancreas (Chung et al., 2010). In this study, we have focused our analyses on the first steps of ventral pancreatic bud development by analyzing three early pancreatic markers, *pdx1, ptf1a* and *hlxb9la*. We show that these three pancreatic regulatory genes are differently controlled in response to BMP signalling. Indeed, while *pdx1* was not significantly affected in the *alk8* mutants, expression of *ptf1a* and *hlxb9la* was not detected at 32 hpf and strongly reduced after 38 hpf in these mutants. A similar regulation was observed when BMP pathway was disrupted by two other means: first, by blocking BMP receptors after 20 hpf by using the *Tg(hsp70l:dnbmpr-GFP)* line, secondly, by the knockdown of *bmp2a*.

The delay in the formation of the *ptf1a*+ cells was longer after induction of the dominant negative receptor dnBMPR-GFP than in *alk8* mutants and *bmp2a* morphants (a 16 hours delay compared to 6 hours). Such differences in phenotype severity are probably due to compensation by others BMP receptors I and other BMP ligands expressed at later stages. *bmp2a* expression in the LPM coincides in timing and space with the requirement of BMP for ventral pancreas specification. It is interesting to remind that its paralog, *bmp2b*, expressed a few hours before, triggers an opposite action on pancreas specification, indeed favouring hepatic fate at the expense of pancreatic fate. This is not due to different biological activities of the two BMP2 proteins, as *bmp2a* morphant defects can be partially rescued by *bmp2b* overexpression, but rather results from a difference in their timing of expression. We can speculate that, after the genome duplication that occurred early during teleost evolution, each copy of the ancestral *bmp2* gene retained distinct regulatory sequences driving expression in the LPM either at early (for *bmp2b*) or later (for *bmp2a*) developmental stages. As in zebrafish, the critical stages of liver specification precede ventral pancreas specification, the role of the two *bmp2* paralogs were split, *bmp2b* acting on liver specification and *bmp2a* acting on ventral pancreas specification and on the maintenance of hepatic genes *hhex* and *prox1*.

***fgf10* and *24* specify the VPB at the expense of the hepatic fate**

One important finding of our study is the role *fgf10* and *fgf24* in the induction of ventral pancreas while they restrict hepatic competence. Activation of *ptf1a* and *hlxb9la* genes in endoderm is not detected at 32 hpf in the double *fgf10;fgf24* mutants and the *pdx1*+ domain becomes significantly reduced while the *prox1*+ hepatic domain extends posteriorly. Only a delay in *ptf1a* and *hlxb9la* endodermal expression was observed either in *fgf24* homozygous

mutants or in some double heterozygotes (data not shown), but expression of these two genes reappears later around 44 hpf (data not shown). This indicates that a correct level of FGF is required for proper timing of ventral pancreas specification. At 3 dpf, the *fgf10-/-; fgf24-/-* larvae possess only the pancreatic endocrine islet, which is derived from the dorsal bud, and no pancreatic exocrine tissue. The liver bud in these mutant larvae is apparently normal but ectopic masses of hepatocytes develop in the endoderm just posterior to the liver, near the remnant of the hepatopancreatic ducts and closed to the junction with the intestine. The actual origin of these hepatocytes is unclear; they may derive from the posterior extension of the *prox1+* hepatic domain observed at 30 hpf (Figure 3C). Two zebrafish studies have recently described defects highly similar to the *fgf10; fgf24* mutant phenotype as they showed that the endoderm posterior to the hepatic domain, corresponding to the prospective intestinal bulb and pancreas, can give rise to ectopic hepatocytes upon Wnt overexpression (Poulain and Ober, 2011; Shin et al., 2011). Interestingly, this hepatic competence can be induced by Wnt overexpression at 26 hpf, thus following liver specification in the hepatic progenitor domain at 22 hpf. Moreover, it is negatively regulated with time by FGF signalling (Shin et al., 2011) and *fgf10a* (here referred to as *fgf10*) was shown to be partially responsible of this inhibitory effect. Our present data indicate that the combined loss of *fgf10* and *fgf24*, both genes being expressed in the LPM after liver specification, is sufficient to trigger hepatic cell differentiation posterior to the normal hepatic domain without artificial Wnt overexpression. Whether Wnt signalling is activated in the *fgf10; fgf24* compound mutants remains to be elucidated.

Our data showing the ability of FGF signalling to induce pancreatic fate versus hepatic fate contrast drastically with previous data on mouse embryonic explants demonstrating the opposite effect of FGF (Deutsch et al., 2001). This difference does not seem to be due to intrinsic biological activities of FGF10 and FGF24 protein ligands as the phenotype of the *fgf10;fgf24* mutant can be almost recapitulated in wild type zebrafish embryos treated with FGF inhibitor SU5402 between 24 hpf and 48 hpf (data not shown and (Manfroid et al., 2007)). In contrast, if FGF signalling is blocked at earlier stage (*i.e*. from 18 hpf onward), liver specification is disrupted (Shin et al., 2007). This again underscores the importance of developmental stage in the response to extrinsic factors.

From data of this and other zebrafish studies, we can build a model where hepatic versus pancreatic commitment is controlled by the sequential action of distinct extrinsic factors (Figure 6). First, b*mp2b* expressed in the LPM from 14 hpf promotes hepatic fate at the expense of pancreatic fate notably through a down-regulation of *pdx1* in gut endoderm (Chung et al., 2008; Shin et al., 2007). Then, around 18-22 hpf, a FGF signal, whose exact identity is still unknown, is required to activate *hhex* and *prox1* genes in hepatoblasts (Shin et al., 2007). Activation of these two hepatic genes also necessitates Wnt2bb expression from the LPM from 22 hpf (Ober et al., 2006). BMP2A is also secreted from that stage to allow activation of *ptf1a* and *hlxb9la* genes in the VPB and to maintain high expression level of *hhex* and *prox1* in hepatoblasts. Secretion of FGF10 and FGF24 from around 24-26 hpf is essential for the onset of *ptf1a* and *hlxb9la* expression determining ventral pancreatic bud. In the absence of these two FGF ligands, the *prox1/hhex+* hepatic anlagen expands posteriorly at the expense of *pdx1+* cells. Finally, our results also reveal that *bmp2a* stimulates *fgf24* expression in the pancreatic LPM. As *fgf24* expression in the LPM, reflecting proper pancreatic LPM patterning (Manfroid et al., 2007), is important for VPB specification, this thereby demonstrates that cross-talks between BMP and FGF pathways contribute to coordinated liver and pancreas development.

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). We used the following mutant and transgenic lines: *laf*^{tm110b} (Mintzer et al., 2001), *Tg(hsp70l:dnBmpr-GFP)* (Pyati et al., 2005), *Tg(ptf1a:eGFP)* (Godinho et al., 2007) and *Tg(hsp70l:bmp2b)* (Chocron et al., 2007).

Heat shock conditions

Embryos were heat shocked at various stages by transferring them into a pre-warmed falcon containing egg water in a water bath.. *Tg(hsp70l:dnBmpr-GFP*) embryos were heat shocked for 30 minutes at 40°C; *Tg(hsp70l:bmp2b)* embryos for 30 minutes at 37°C. After heat shock, embryos were transferred at 28°C, and then were harvested between 30 and 72 hpf. Hemizygous *Tg(hsp70l:dnBmpr-GFP)* embryos were sorted 3 hours after heat shock based on GFP positive expression. Hemizygous *Tg(hsp70l:bmp2b)* embryos were tested for presence of the *hsp70l:bmp2b* transgene by PCR using genomic DNA after *in situ* hybridization, using the primers previously used by Shin *et al* (Shin et al., 2007). In our hands, a nested PCR was needed and the following primers were used: 5'-GCAAAAGGCCAGGAACCGTAA-3' and 5'- GCACACAGCCCAGCTTGGAGC-3'.

Whole-mount *in situ* hybridization and immunochemistry

Visible *in situ* hybridization (ISH) was performed as described (Hauptmann and Gerster, 1994). Fluorescent labeling was performed as described (Mavropoulos et al., 2005).
The riboprobes used were *ptf1a* (Zecchin et al., 2004), *hlxb9la* (Wendik et al., 2004), *trypsin* (Biemar et al., 2001), *pdx1* (Milewski et al., 1998) *bmp2a* (Thisse and Thisse, 2005), *tfa* (Mudumana et al., 2004), *prox1* (Glasgow and Tomarev, 1998) and *hhex* (Ho et al., 1999).
Whole-mount immunohistochemistry was described in (Dong et al., 2007). We used the following antibodies: polyclonal rabbit anti-Prox1 (1:1000, Chemicon), polyclonal rabbit anti-phospho-Smad (1:200, Cell Signaling), polyclonal guinea pig against zebrafish Pdx1 (1:200, gift from C. Wright), polyclonal goat anti-HNF4α (1:100, Santa Cruz Biotechnology), monoclonal mouse 2F11 (Crosnier et al., 2005) (1:1000, gift from J. Lewis) and fluorescently conjugated Alexa antibodies (Molecular Probes).
Fluorescent images were acquired with a Leica SP2 or Olympus FluoView FV1000 confocal microscopes and 3D Blend projections were performed with the Imaris software (Bitplane).

Injection of morpholino antisense oligonucleotides

Morpholino oligonucleotides (MO) were purchased by Gene Tools, LLC. Each MO was resuspended in Danieau's solution at the stock concentration of 8 µg/µl. For injections, they were further diluted in Danieau's solution at 4ng/nl with 0.5% Rhodamine dextran to check injection efficiency. The splice inhibition MO *bmp2a* (AGTAAACACTTGCTTACCATCATGG) targets the exon1-intron1 boundary.

Control of the morpholino efficiency

Zebrafish embryos were injected at one-cell stage with Mo Bmp2a (4ng/embryo) and mRNAs were extracted at 30 hpf. RT-PCR were performed on 1 µg of mRNAs. The primers used for PCR amplification were the followings: *bmp2a* exon 1 (upstream primer; 5'-GGCTCCAGTGGACTCGTTCCTCA-3'), *bmp2a* exon 2 (downstream primer, 5'-CTCCTGAAGAGAACCGGACGGCCT-3') and PCR cycles were performed as follows: 40 cycles of 30 sec at 94°C, 30 sec at 55°C and 30 sec at 72°C, followed by a final 7-min extension at 72°C. Amplified cDNAs were analyzed by gel electrophoresis and sequencing.

Genotyping

Genotyping was performed on genomic DNA extracted from adult tails or tails obtained from embryos processed through *in situ* hybridization or immunohistochemistry. The *ikarus* mutation in the *fgf24* locus generates a restriction site for the AccI endonuclease. The PCR fragment obtained with forward 5'-CTGTCAGTCCCACAGCAGTGGACCA-3' and reverse 5'-CCATGTAGATTTTATTACATGTAGGT-3' primers (615 pb) digested by AccI produces two fragments (185 and 430 pb) in the mutant. The *daedalus* mutation in *fgf10* generates a SNP that was identified by the TaqMan SNP Genotyping Assays (Applied Biosystem). The region encompassing the mutation was amplified with the forward primer dae-SNp1F 5'-CCGAGCTCCAGGACAATGTG-3' and reverse primer dae-SNp1R 5'-GCAGGACAGACGGAACCA-3'. Allelic discrimination was performed by dae-SNp1V2 VIC primer 5'-CCCTTAGTCACTTTCCATTT-3' (wild type allele) and dae-SNp1M2 FAM primer 5'-CCTTAGTCACTTACCATTT-3' (mutant allele) according to the manufacturer.

Acknowledgements

The authors thank Dr S. Ormenese, G. Moraes and the "GIGA-Cell Imaging" facility, Dr. W. Coppieters and the “GIGA-GenoTranscriptomics” facility and Dr. M. Winandy and the "GIGA-Zebrafish Facility".
I.M. was supported by the FNRS-FRS and by the "Action de Recherches Concertées" (University of Liège). B.P. and M.L.V. are Chercheurs qualifiés FNRS. N.D. and V.V.B. are funded by the WALEO (Région Wallonne). F.N. has a postdoctoral fellowship from the University of Liège. This work was funded by the Belgian State's "Interuniversity Attraction Poles" Program (SSTC, PAI) and by the 6th European Union Framework Program (BetaCellTherapy Integrated Project).

References

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.

Chocron, S., Verhoeven, M. C., Rentzsch, F., Hammerschmidt, M. and Bakkers, J. (2007). Zebrafish Bmp4 regulates left-right asymmetry at two distinct developmental time points. *Dev Biol* **305**, 577-88.

Chung, W. S., Andersson, O., Row, R., Kimelman, D. and Stainier, D. Y. (2010). Suppression of Alk8-mediated Bmp signaling cell-autonomously induces pancreatic beta-cells in zebrafish. *Proc Natl Acad Sci U S A* **107**, 1142-7.

Chung, W. S., Shin, C. H. and Stainier, D. Y. (2008). Bmp2 signaling regulates the hepatic versus pancreatic fate decision. *Dev Cell* **15**, 738-48.

Crosnier, C., Vargesson, N., Gschmeissner, S., Ariza-McNaughton, L., Morrison, A. and Lewis, J. (2005). Delta-Notch signalling controls commitment to a secretory fate in the zebrafish intestine. *Development* **132**, 1093-104.

Deutsch, G., Jung, J., Zheng, M., Lora, J. and Zaret, K. S. (2001). A bipotential precursor population for pancreas and liver within the embryonic endoderm. *Development* **128**, 871-81.

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.

Field, H. A., Dong, P. D., Beis, D. and Stainier, D. Y. (2003a). Formation of the digestive system in zebrafish. II. Pancreas morphogenesis. *Dev Biol* **261**, 197-208.

Field, H. A., Ober, E. A., Roeser, T. and Stainier, D. Y. (2003b). Formation of the digestive system in zebrafish. I. Liver morphogenesis. *Dev Biol* **253**, 279-90.

Glasgow, E. and Tomarev, S. I. (1998). Restricted expression of the homeobox gene prox 1 in developing zebrafish. *Mech Dev* **76**, 175-8.

Godinho, L., Williams, P. R., Claassen, Y., Provost, E., Leach, S. D., Kamermans, M. and Wong, R. O. (2007). Nonapical symmetric divisions underlie horizontal cell layer formation in the developing retina in vivo. *Neuron* **56**, 597-603.

Hauptmann, G. and Gerster, T. (1994). Two-color whole-mount in situ hybridization to vertebrate and Drosophila embryos. *Trends Genet* **10**, 266.

Ho, C. Y., Houart, C., Wilson, S. W. and Stainier, D. Y. (1999). A role for the extraembryonic yolk syncytial layer in patterning the zebrafish embryo suggested by properties of the hex gene. *Curr Biol* **9**, 1131-4.

Huang, H., Ruan, H., Aw, M. Y., Hussain, A., Guo, L., Gao, C., Qian, F., Leung, T., Song, H., Kimelman, D. et al. (2008). Mypt1-mediated spatial positioning of Bmp2-producing cells is essential for liver organogenesis. *Development* **135**, 3209-18.

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.

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**, 109-22.

Manfroid, I., Delporte, F., Baudhuin, A., Motte, P., Neumann, C. J., Voz, M. L., Martial, J. A. and Peers, B. (2007). Reciprocal endoderm-mesoderm interactions mediated by fgf24 and fgf10 govern pancreas development. *Development* **134**, 4011-21.

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**, 211-23.

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**, 1440-9.

Mintzer, K. A., Lee, M. A., Runke, G., Trout, J., Whitman, M. and Mullins, M. C. (2001). Lost-a-fin encodes a type I BMP receptor, Alk8, acting maternally and zygotically in dorsoventral pattern formation. *Development* **128**, 859-69.

Mudumana, S. P., Wan, H., Singh, M., Korzh, V. and Gong, Z. (2004). Expression analyses of zebrafish transferrin, ifabp, and elastaseB mRNAs as differentiation markers for the three major endodermal organs: liver, intestine, and exocrine pancreas. *Dev Dyn* **230**, 165-73.

Ober, E. A., Verkade, H., Field, H. A. and Stainier, D. Y. (2006). Mesodermal Wnt2b signalling positively regulates liver specification. *Nature* **442**, 688-91.

Poulain, M. and Ober, E. A. (2011). Interplay between Wnt2 and Wnt2bb controls multiple steps of early foregut-derived organ development. *Development*.

Pyati, U. J., Webb, A. E. and Kimelman, D. (2005). Transgenic zebrafish reveal stage-specific roles for Bmp signaling in ventral and posterior mesoderm development. *Development* **132**, 2333-43.

Rossi, J. M., Dunn, N. R., Hogan, B. L. and Zaret, K. S. (2001). Distinct mesodermal signals, including BMPs from the septum transversum mesenchyme, are required in combination for hepatogenesis from the endoderm. *Genes Dev* **15**, 1998-2009.

Shin, D., Lee, Y., Poss, K. D. and Stainier, D. Y. (2011). Restriction of hepatic competence by Fgf signaling. *Development* **138**, 1339-48.

Shin, D., Shin, C. H., Tucker, J., Ober, E. A., Rentzsch, F., Poss, K. D., Hammerschmidt, M., Mullins, M. C. and Stainier, D. Y. (2007). Bmp and Fgf signaling are essential for liver specification in zebrafish. *Development* **134**, 2041-50.

Spagnoli, F. M. and Brivanlou, A. H. (2008). The Gata5 target, TGIF2, defines the pancreatic region by modulating BMP signals within the endoderm. *Development* **135**, 451-61.

Tehrani, Z. and Lin, S. (2011). Antagonistic interactions of hedgehog, Bmp and retinoic acid signals control zebrafish endocrine pancreas development. *Development* **138**, 631-40.

Thisse, C. and Thisse, B. (2005). High Throughput Expression Analysis of ZF-Models Consortium Clones. *ZFIN Direct Data Submission (http://zfin.org)*.

Wandzioch, E. and Zaret, K. S. (2009). Dynamic signaling network for the specification of embryonic pancreas and liver progenitors. *Science* **324**, 1707-10.

Wendik, B., Maier, E. and Meyer, D. (2004). Zebrafish mnx genes in endocrine and exocrine pancreas formation. *Dev Biol* **268**, 372-83.

Westerfield, M. (1995). The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (Danio rerio), 3rd Edition. *Eugene, OR, University of Oregon Press,385*.

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**, 174-84.

Figure Legends

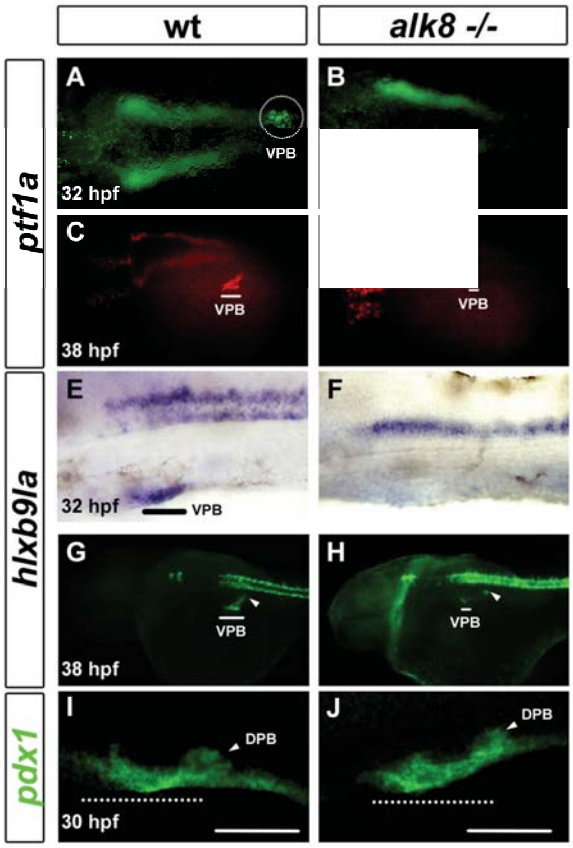

Figure 1
Decrease in BMP signalling leads to a delay in VPB specification and to a reduction in *ptf1a*+ and *hlxb9la*+ VPB progenitors
(A and B) Ventral view of *ptf1a* expression in wild type (wt) sibling compared to *alk8* mutant (*alk8*-/-) at 32 hpf. (C and D) Lateral view of *ptf1a* expression in wt and *alk8* mutant at 36-38 hpf. (E-J) Lateral view of *hlxb9la* expression in wt and *alk8* mutant at 32 hpf (E and H)and 36-38 hpf. (I andJ). Lateral view of *pdx1* expression in wt and *alk8* mutant. White and black brackets, white dot circle underlines the ventral pancreatic bud (VPB); white dot brackets indicate the prospective ventral pancreatic bud; white arrowheads indicate the dorsal pancreatic bud (DPB).

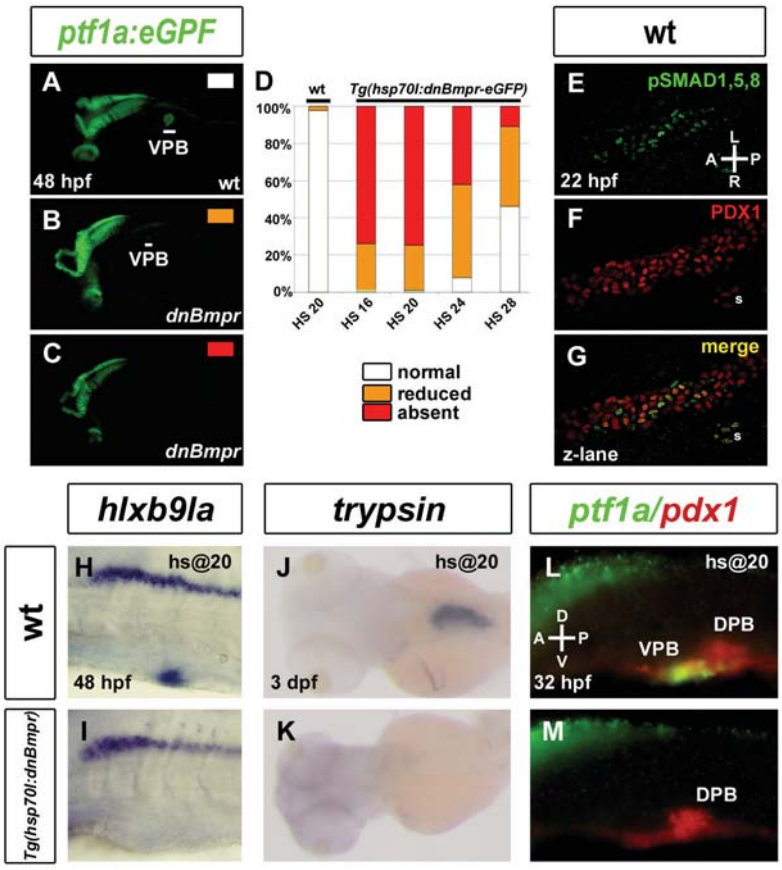

Figure 2

BMP signalling is essential at 20 hpf for VPB specification

(A-C, H-M) Embryos obtained from outcrossing a hemizygous *Tg(hsp70l:dnBmpr-GFP)* zebrafish with homozygous *Tg(ptf1a:eGFP)* were heat shocked at 20hpf and harvested at 30 hpf (L and M) 48 hpf (A-C, H-I) and 3 dpf (J,K) and examined for ptf1a:eGFP fluorescence (A-C), *hlxb9la* (H and I), *trypsin* (J and K), *ptf1a* and *pdx1* (L and M) expression. (D) Graph quantifying ptf1a:GFP expression in wild type (wt) and *Tg(hsp70l:dnBmpr-GFP)* heat shocked embryos at 16 hpf, 20 hpf, 24 hpf and 28 hpf. Data are presented as the percentage of embryos displaying normal (white), reduced (orange), or absent (red) ptf1a:GFP fluorescence. (E-G) Confocal z-lane showing activation of the BMP pathway in pancreatic cells revealed by immunostaining with anti-pSmad 1/5/8 (green) (E) and anti-Pdx1 (red) (H) antibodies. Double pSmad 1/5/8 + and Pdx1+ cells are observed on the merge picture (G). White brackets underline the VPB. DPB, dorsal pancreatic bud; s, somite.

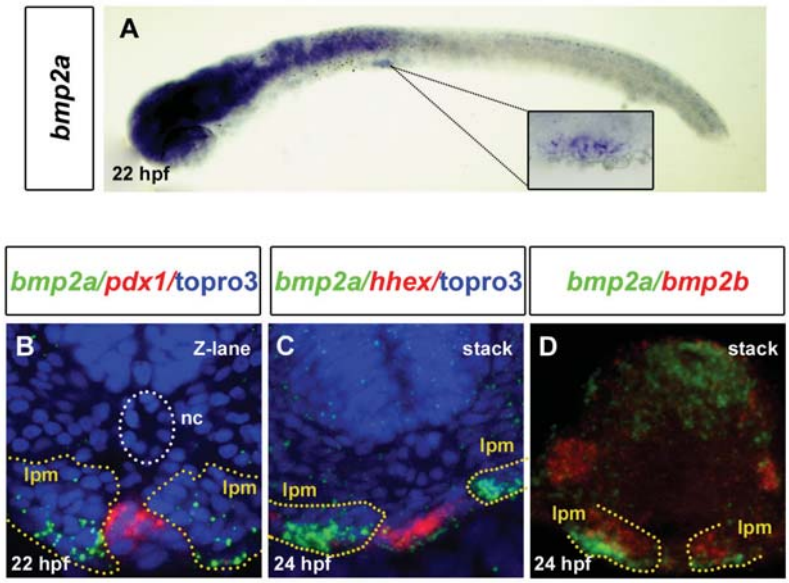

Figure 3

***bmp2a* is expressed in the LPM adjacent to the hepatopancreatic endoderm at 22 hpf**

(A)Lateral view showing *bmp2a* expression in the LPM starting at 22 hpf . The square shows a close-up of the region pointed by the dot-lines. (B) *bmp2a* (green) and *pdx1* (red) expression analyzed by fluorescent whole-mount *in situ* hybridization at 22 hpf. The transverse sections were analyzed by confocal microscopy. (C) *bmp2a (green)* and *hhex* (red) expression analyzed at 24 hpf with. (D) *bmp2a* (green) and *bmp2b* (red) expression comparison at 24 hpf. The images of transverse sections are flat stacking of several consecutive optical sections. TO-PRO-3 (blue) labels nuclei. Yellow and white dots delineate the LPM and the notochord (nc), respectively. FB, Fin bud.

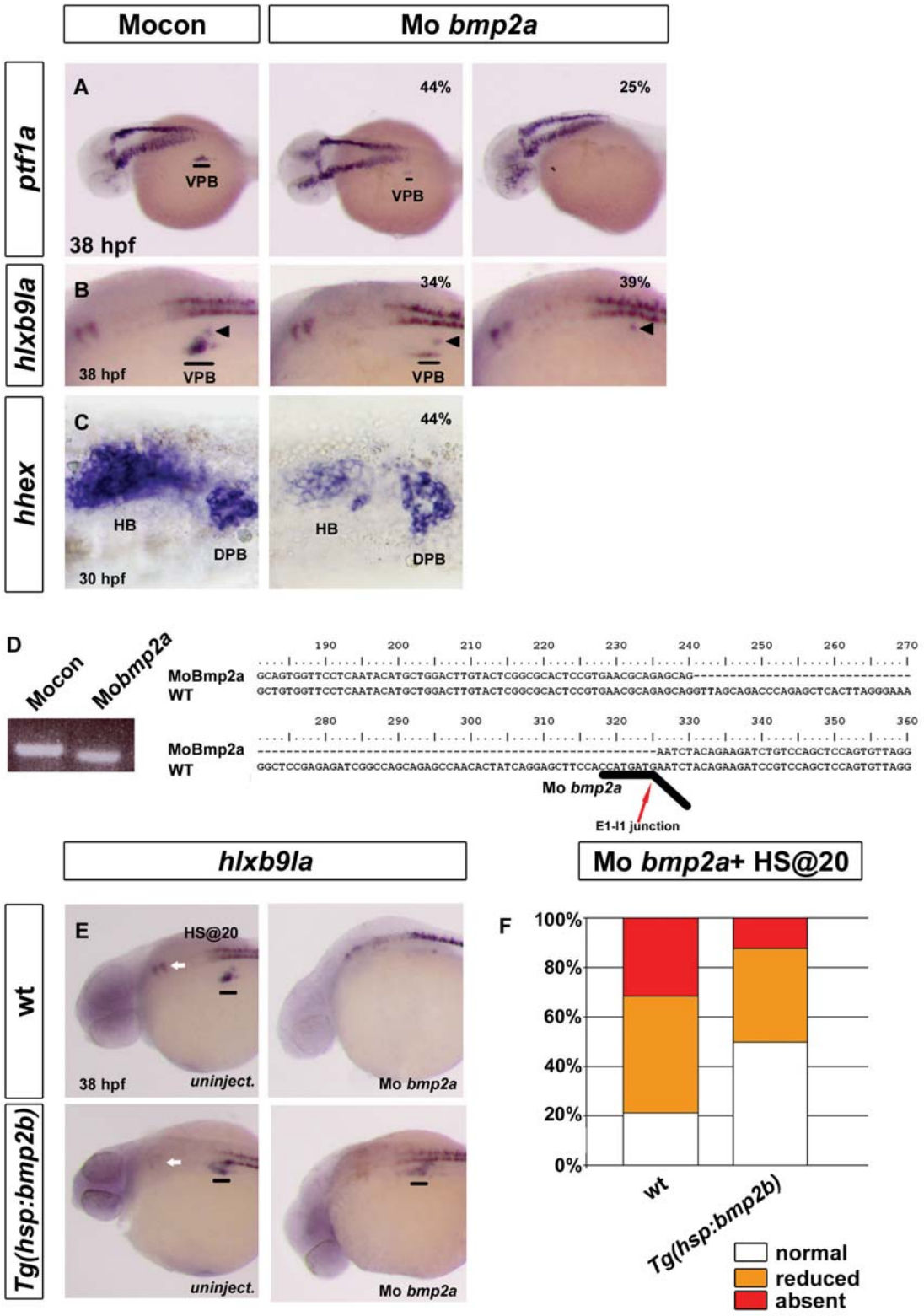

Figure 4

Knockdown *of bmp2a* represses VPB specification

(A-C) Analysis of ventral pancreatic and hepatic markers in control morphants (left column) and in *bmp2a* morphants (right columns) by *in situ* hybridization. (A) Laterial view of *ptf1a* expression at 36-38hpf. (B) Laterial view of *hlxb9la* expression at 36-38hpf.. (C)Ventral view

of *hhex* expression at 30 hpf. Percentage represents the proportion of morphants exhibiting a reduction (middle column) or an absence (right column) of gene expression. (D) Gel electrophoresis after RT-PCR (left) and sequencing result (right) illustrating the RNA splicing defects caused by the *bmp2a* morpholino in injected embryos.. The sequence of the amplified *bmp2a* fragment was aligned with wt *bmp2a* cDNA and revealed a 86bp deletion in exon1. Black line underlines the binding site of the *bmp2a* morpholino. Red arrow shows the junction between exon 1 and intron 1. (E and F) Lateral view of *hlxb9la* expression at 38 hpf in wt and *Tg(hsp70l:bmp2b)* in uninjected embryos (E) or in *bmp2a* morphants (F). Embryos were heat shocked at 20 hpf. (G) *hlb9la* expression in wt and *Tg(hsp70l:bmp2b)* embryos injected with MO *bmp2a,* heat shocked at 20 hpf and fixed at 38 hpf. Data are presented as the percentage of embryos displaying normal (white), reduced (orange), or absent (red) expression of *hlxb9la.* Black brackets underline the VPB; black arrowheads indicate the dorsal pancreatic bud (DPB); white arrows indicate rhombomeres 5 and 6 localisation. HB, hepatic bud.

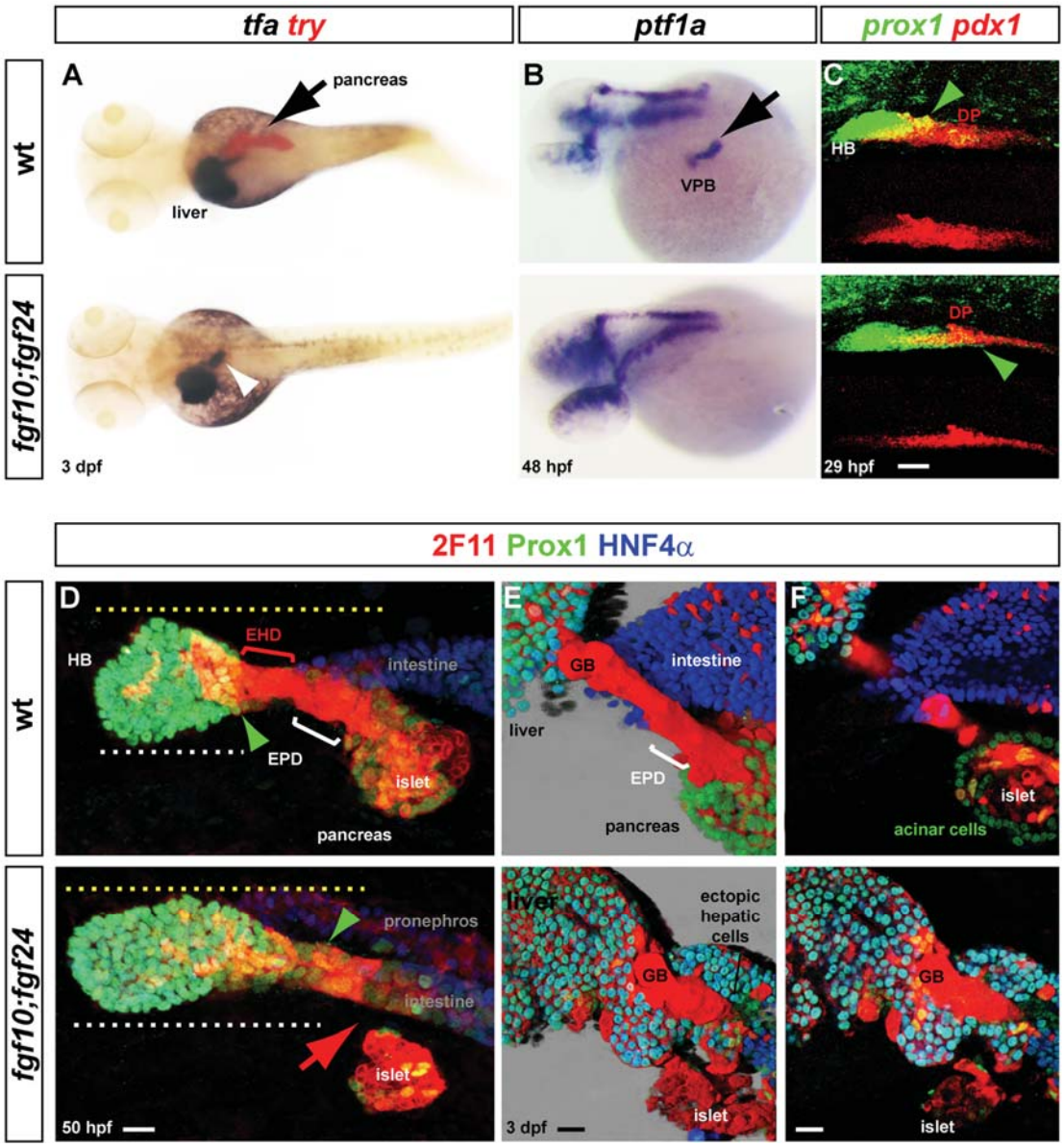

Figure 5

Absence of VPB cells and ectopic hepatic cells i*n fgf10; fgf24* compound mutants

(A) Dorsal view of *transferrin (tfa)* and *trypsin (try)* expression, comparing wild type (wt) and *fgf10; fgf24* mutants at 3dpf. (B) Dorso-lateral view of *ptf1a* expression comparing wt and double mutants at 48 hpf. (C) Confocal projection of the *pdx1* and p*rox1* expression domains at 29 hpf showing the severe decrease of *pdx1* in the anterior part of the pancreatic region and the extension of *prox1* in *fgf10; fgf24* mutants (green arrowheads). (D-F) Immunolabelling of the hepatopancreatic region with 2F11, Prox1 and HNF4α in wild type and *fgf10; fgf24* double mutants. (D) At 50 hpf, the ventral pancreas and the EPD are absent (red arrow) in *fgf10; fgf24* mutants (confocal projections). (E and F) Morphology and differentiation of the hepatopancreatic ducts connecting the pancreas and liver to the intestine in *fgf10; fgf24* double mutants at 3 dpf (E, 3D Blend projection and F, z-planes). Black arrows indicate pancreas and VPB; white arrow head indicates ectopic *tfa* expression; islet, endocrine islet; EHD, extrahepatic duct; EPD, extrapancreatic duct; GB, gall bladder. Scale bar = 20 µm.

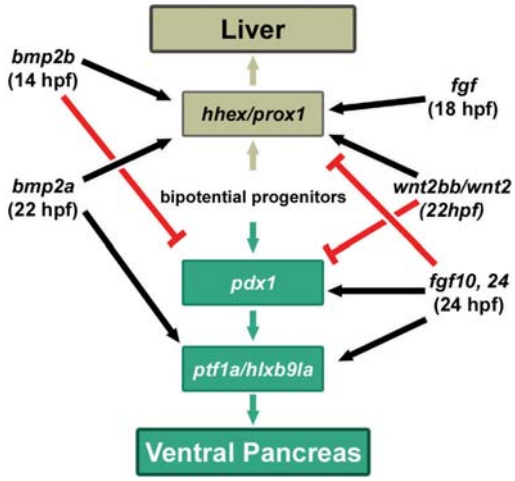

Figure 6
Model for the role of BMP and FGF pathways in hepatopancreatic patterning
Bipotential endodermal progenitors, shown in the center of the scheme, can give rise to liver and pancreatic cells through the consecutive action of extrinsic signals secreted from the lateral mesoderm. Hepatic specification is determined by *hhex* and *prox1* expression while *pdx1*, *ptf1a* and *hlxb9la* are markers of pancreatic cell commitment.