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Restriction of hepatic competence by Fgf signaling

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SUMMARY

Hepatic competence, or the ability to respond to hepatic-inducing signals, is regulated by a number of transcription factors broadly expressed in the endoderm. However, extrinsic signals might also regulate hepatic competence, as suggested by tissue explant studies. Here, we present genetic evidence that Fgf signaling regulates hepatic competence in zebrafish. We first show that the endoderm posterior to the liver-forming region retains hepatic competence: using transgenic lines that overexpress hepatic inducing signals following heat-shock, we found that at late somitogenesis stages Wnt8a, but not Bmp2b, overexpression could induce liver gene expression in pancreatic and intestinal bulb cells. These manipulations resulted in the appearance of ectopic hepatocytes in the intestinal bulb. Second, by overexpressing Wnt8a at various stages, we found that as embryos develop, the extent of the endodermal region retaining hepatic competence is gradually reduced. Most significantly, we found, using gain- and loss-of-function approaches, that Fgf10a signaling regulates this gradual reduction of the hepatic-competent domain. These data provide in vivo evidence that endodermal cells outside the liver-forming region retain hepatic competence and show that an extrinsic signal, Fgf10a, negatively regulates hepatic competence.

KEY WORDS: Wnt signaling, Liver development, Developmental competence, Hepatic induction, Endoderm, Zebrafish

INTRODUCTION

The liver develops from the foregut endoderm in response to hepatic inducing signals such as Fgf (Jung et al., 1999; Zhang et al., 2004; Calmont et al., 2006; Shin et al., 2007; Wandzioch and Zaret, 2009), Bmp (Rossi et al., 2001; Zhang et al., 2004; Shin et al., 2007; Chung et al., 2008; Wandzioch and Zaret, 2009) and Wnt (Ober et al., 2006; Goessling et al., 2008) from adjacent mesodermal tissues. Hepatic competence, the ability to respond to hepatic inducing signals, has been principally analyzed at the transcriptional level (Zaret, 2002; Zaret et al., 2008). Foxa (Cirillo et al., 1998; Cirillo et al., 2002) and Gata (Bossard and Zaret, 1998; Cirillo et al., 2002) transcription factors have been defined as hepatic competence factors owing to their occupancy of the *albumin* gene enhancer prior to hepatic specification. Binding of Foxa transcription factors on the *albumin* gene enhancer opens the compacted chromatin structure, which enables the subsequent binding of Gata transcription factors at adjacent sites (Cirillo et al., 2002; Sekiya et al., 2009). Genetic studies in mouse support a role for Foxa transcription factors in hepatic competence. In *Foxa1;Foxa2* double-knockout mice, hepatic specification fails to occur, and the foregut endoderm dissected from these embryos fails to initiate expression of hepatic markers after FGF2 treatment (Lee et al., 2005).

The Foxa and Gata hepatic competence factors are expressed not only in the ventral foregut endoderm but also in the dorsal endoderm (Ang et al., 1993; Monaghan et al., 1993; Laverriere et al., 1994), which is posterior to the foregut endoderm and normally does not give rise to the liver. This expression pattern and data from mouse explant culture studies (Gualdi et al., 1996; Bossard and Zaret, 2000) suggest that the dorsal endoderm also possesses hepatic competence. When mouse dorsal endoderm was isolated between embryonic days (E) 8.5 and E11.5, and cultured free from its surrounding mesoderm, it could be induced to express *albumin* (Gualdi et al., 1996; Bossard and Zaret, 2000). This tissue eventually appears to lose hepatic competence as it could not be induced to express *albumin* when isolated at E13.5 and beyond (Bossard and Zaret, 2000). Models drawn from these in vitro data need to be tested in vivo, and the factors responsible for the loss of hepatic competence of the dorsal endoderm need to be identified.

In addition to the intrinsic control of hepatic competence, an extrinsic control is also suggested from explant studies. *Gata4* expression in mouse foregut endoderm explants was greatly reduced by the Bmp inhibitor Noggin (Rossi et al., 2001), suggesting that Bmp signaling plays a positive role in hepatic competence. Additional data from mouse explant culture studies suggest that the mesoderm adjacent to the dorsal endoderm causes the latter to lose hepatic competence (Gualdi et al., 1996; Bossard and Zaret, 2000): E13.5 mesoderm inhibited the expression of *Gata4* and *Foxa1* in E11.5 dorsal endodermal explants (Bossard and Zaret, 2000). However, to date, genetic data supporting the hypothesis that extrinsic signals regulate hepatic competence are lacking.

In this study, we provide genetic evidence for hepatic competence in the dorsal/posterior endoderm and for the regulation of hepatic competence by extrinsic signals. In zebrafish, Bmp (Shin et al., 2007; Chung et al., 2008), Fgf (Shin et al., 2007) and Wnt (Ober et al., 2006; Goessling et al., 2008) signaling are required for hepatic specification, and transgenic lines, which overexpress these factors or their constitutively active receptors upon heat-shock, allow a temporal manipulation of these signaling pathways. Specifically, using transgenic lines that ubiquitously overexpress *wnt8a* (Weidinger et al., 2005), *bmp2b* (Chocron et al., 2007) or constitutively active *Fgfr1* (*XIFgfr1*) (Marques et al., 2008) under the regulation of the *heat shock cognate 70 kd protein* (*hsp70l*)

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promoter (Halloran et al., 2000), we found that endodermal regions posterior to the liver-forming region, which are equivalent to the murine dorsal endoderm, retain hepatic competence. In addition, we discovered that this hepatic competence is regulated by Fgf10a, an extrinsic factor expressed by the surrounding mesenchyme.

MATERIALS AND METHODS

Zebrafish strains

Embryos and adult fish were raised and maintained under standard laboratory conditions (Westerfield, 2000). We used the following mutant and transgenic lines: *wnt2bb*^{s403} (Ober et al., 2006), *fgf10a*^{tbvbo} (Norton et al., 2005), *Tg(hsp70l:wnt8a-GFP)*^{w34} (Weidinger et al., 2005), *Tg(hsp70l:bmp2b)*^{fl3} (Chocron et al., 2007), *Tg(hsp70l:XlFgfr1)*^{pd3} (Marques et al., 2008), *Tg(hsp70l:dkk1-GFP)*^{w32} (Stoick-Cooper et al., 2007), *Tg(ins:dsRed)*^{m1081} (a gift from W. Driever, University of Freiburg, Germany), *Tg(fabp10:dsRed,ela3l:EGFP)*^{gz12} (Korzh et al., 2008), *Tg(ptf1a:eGFP)*^{jh1} (Godinho et al., 2005), *Tg(gutGFP)*^{s854} [also known as *Tg(XlEef1a1:GFP)*^{s854}] (Field et al., 2003) and *Tg(hsp70l:dnRas)*^{pd7} (Lee et al., 2009).

Heat-shock conditions

Embryos were heat-shocked at various stages by transferring them into egg water pre-warmed to 37°C [*Tg(hsp70l:dkk1-GFP)*] or 38°C [*Tg(hsp70l:wnt8a-GFP)*, *Tg(hsp70l:bmp2b)*, *Tg(hsp70l:XlFgfr1)* and *Tg(hsp70l:dnRas)*] and kept at this temperature for 25 minutes. After heat-shock, the plate containing the embryos was transferred back into a 28°C incubator.

In situ hybridization and immunohistochemistry

Whole-mount in situ hybridization was performed as previously described (Alexander et al., 1998), using probes for the following genes: *hhex* (Ho et al., 1999), *foxa1/2/3* (Odenthal and Nüsslein-Volhard, 1998), *gata4/6* (Reiter et al., 1999), *fgf10a* (Norton et al., 2005), *cp* (Korzh et al., 2001), *sepp1b* (Kudoh et al., 2001), *cdx1b* (Flores et al., 2008), *ptf1a* (Lin et al., 2004), *sox9b* (Yan et al., 2005), *anxa5b* (Teoh et al., 2010), *nkx2.1a* (Rohr and Concha, 2000) and *myca* (Yamaguchi et al., 2005). Whole-mount immunohistochemistry was performed as previously described (Dong et al., 2007), using the following antibodies: chicken polyclonal anti-GFP (1:1000; Aves Labs, Tigard, OR, USA), rabbit polyclonal anti-Prox1 (1:1000; Chemicon, Billerica, MA, USA), mouse monoclonal 2F11 (1:100; Abcam, Cambridge, MA, USA), rabbit polyclonal anti-dsRed (1:200; Clontech, Mountain View, CA, USA), guinea pig polyclonal anti-Pdx1 (1:200; a generous gift from C. Wright, Vanderbilt University, Nashville, TN, USA), mouse monoclonal anti-phospho-Histone 3 (1:100; Cell Signaling Technology, Danvers, MA, USA) and fluorescently conjugated Alexa antibodies (1:250; Molecular Probes, Carlsbad, CA, USA).

TUNEL assay

The TUNEL assay was performed as described in the product protocol using the In Situ Cell Death Detection Kit, Fluorescein (Roche, Basel, Switzerland). Whole-mount immunostaining was performed and followed by TUNEL labeling.

Morpholino injections

Embryos were injected at the one- or two-cell stage with 4 ng of an *fgf10a* splice MO (5′-AACAAGCTTTACTCACTGTACGGAT-3′).

Genotyping of *wnt2bb* and *fgf10a* mutants and *Tg(hsp70l:XlFgfr1)* embryos

For *wnt2bb*^{s403} genotyping, genomic DNA was amplified with the forward (5′-ACCAAACACTCAGGGTCCAGTC-3′) and reverse (5′-CCACA-GCGATTGTTATGAAGGTTC-3′) primers, followed by digestion with *Msp*I. The wild-type allele generates a band of 417 bp, and the *wnt2bb* mutant allele generates two bands of 182 and 235 bp. *fgf10a*^{tbvbo} genotyping was performed as previously described (Norton et al., 2005). For genotyping of *Tg(hsp70l:XlFgfr1)* embryos younger than 48 hpf, genomic DNA was amplified with the forward (5′-CACTCATCAGAG-CGATGTGTGGTC-3′) and reverse primers (5′-GCATGGCATGCCAAC-AGTCCTTC-3′), generating a band of 191 bp.

Chemical treatment

A 1 mM stock of the Fgfr inhibitor SU5402 (EMD Chemicals, Gibbstown, NJ, USA) was prepared in 100% DMSO and diluted to 1 μM with egg water. As a control, we used a 0.1% DMSO solution in egg water.

RESULTS

Wnt signaling is necessary and sufficient for liver induction in zebrafish

In zebrafish, liver specification, as assessed by the appearance of the hepatoblast markers *hhex* and *prox1* in the liver-forming region, occurs at approximately 22 hours post-fertilization (hpf) (Field et al., 2003; Wallace and Pack, 2003; Ober et al., 2006; Noel et al., 2008). A recent genetic study has implicated Wnt/β-catenin signaling in liver specification in zebrafish: *wnt2bb*, which is expressed in anterior lateral plate mesodermal cells, is required for the expression of the hepatoblast markers *hhex* and Prox1 (Ober et al., 2006). However, some *wnt2bb* mutants eventually form a liver, indicating a recovery of early liver defects in these animals (Ober

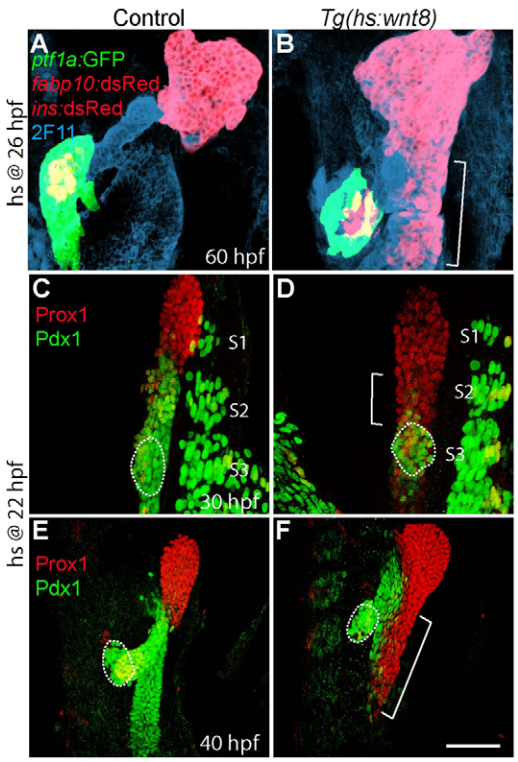

Fig. 1. Wnt8a overexpression induces liver cells in the endoderm posterior to the liver. Embryos obtained from outcrossing *Tg(hs:wnt8a)* zebrafish were heat-shocked at 26 (A,B) or 22 (C-F) hpf, and processed for whole-mount immunostaining. (**A**,**B**) The *Tg(fabp10:dsRed,ela3l:EGFP);Tg(ptf1a:eGFP);Tg(ins:dsRed)* line was used to reveal hepatocytes (red), pancreatic acinar cells (green) and pancreatic β cells (red); mAb 2F11 staining reveals the hepatopancreatic duct (blue). The bracket marks the region with ectopic hepatocytes (*n*=7 out of 7). (**C-F**) Anti-Prox1 and anti-Pdx1 staining at 30 hpf (D; *n*=4 out of 4) reveals that Wnt8a overexpression induced Prox1 expression in endodermal cells located at the level of the second somite (S2) and blocked Pdx1 expression in these cells (the Pdx1 staining in the somites is due to non-reactivity as *pdx1* is not expressed there). At 40 hpf, the endoderm posterior to the liver-forming region expressed Prox1 but not Pdx1 (F, bracket, *n*=4 out of 4). Dotted lines outline the dorsal pancreas, and S1-S3 represent the first, second and third somite, respectively. Ventral views, anterior up. Scale bar: 50 μm.

et al., 2006). We hypothesized that this recovery was due to compensation by other Wnt ligand(s). Thus, we further blocked Wnt signaling in *wnt2bb* mutants by using the *Tg(hsp70l:dkk1-GFP)*^{w32} line that overexpresses Dkk1, a secreted inhibitor of the Wnt/β-catenin signaling pathway (Glinka et al., 1998), upon heat-shock (Stoick-Cooper et al., 2007). The liver completely failed to form in *wnt2bb* mutants that overexpressed Dkk1 upon heat-shock at 18 hpf (see Fig. S1D in the supplementary material), providing further evidence that Wnt/β-catenin signaling is necessary for liver specification in zebrafish.

We next addressed whether Wnt/β-catenin signaling was sufficient for liver induction, using the *Tg(hsp70l:wnt8a-GFP)*^{w34} line that expresses Wnt8a under the heat-shock promoter (Weidinger et al., 2005). When Wnt8a was overexpressed via heat-shock at 26 hpf, ectopic hepatocytes appeared in the endoderm posterior to the liver (Fig. 1B, bracket). There are at least two possible explanations for the presence of these ectopic hepatocytes: migration from the original liver, or induction from non-hepatic endoderm. To distinguish between these possibilities, we analyzed embryos that overexpressed Wnt8a from heat-shock at 22 hpf with the hepatoblast marker Prox1 and the non-hepatic marker Pdx1 at 30 and 40 hpf. Eight hours after heat-shock, the Prox1$^+$ domain was expanded into the level of the second somite and the Pdx1$^+$ domain was restricted to the level of the third somite in the Wnt8a-overexpressing embryos (Fig. 1D). By contrast, in control embryos at this stage, the Prox1$^+$ domain is at the level of the first somite, whereas the Pdx1$^+$ domain is at the level of the second and third somites (Fig. 1C). At later stages, the expansion of the Prox1$^+$ domain and the restriction of the Pdx1$^+$ domain were much more pronounced (Fig. 1F). It is also possible that in Wnt8a-overexpressing embryos, the increase of Prox1$^+$ cells and the concomitant decrease of Pdx1$^+$ cells was caused by the proliferation of Prox1$^+$ cells and death of Pdx1$^+$ cells. Thus, we examined cell proliferation by whole-mount immunostaining with anti-phospho-Histone 3 (pH3) and cell death using TUNEL assay in control and Wnt8a-overexpressing embryos 10 hours after heat-shock. The number of pH3$^+$ endodermal cells also positive for Prox1 or Pdx1 was similar in controls and Wnt8a-overexpressing embryos (six versus 6.5; Fig. 2F); and when the numbers of Prox1$^+$;pH3$^+$ or Pdx1$^+$;pH3$^+$ cells were normalized relative to the area of the Prox1$^+$ or Pdx1$^+$ regions, respectively, again there were no significant differences. These data indicate that there is no clear increase in cell proliferation in these endodermal regions of Wnt8a-overexpressing embryos at least at 10 hours after heat-shock. In addition, there were no TUNEL-positive cells in the endoderm of both control or Wnt8-overexpressing embryos (Fig. 2D,E), although there were some TUNEL$^+$ cells outside of the endoderm (data not shown). Altogether, these results suggest that Wnt8a overexpression induces ectopic hepatoblasts from Pdx1$^+$, i.e. non-hepatic, endodermal cells.

In order to identify a direct Wnt target gene that could be used as a liver induction marker upon Wnt8a overexpression, we examined the expression of several known Wnt target genes, including *myca*, *ccnd1* and *axin2*, and found that *myca* was specifically expressed in the liver-forming region at 26 hpf (Fig. 3B) and clearly expressed in the liver at 48 hpf (Fig. 3D). Ectopic *myca* expression was evident in the posterior endoderm of Wnt8a-overexpressing embryos 6 hours after heat-shock (Fig. 3F). In fact, *myca* expression was clearly seen in the posterior endoderm as early as 3 hours after heat-shock (Fig. 3H). The extent of *myca* expression in the posterior endoderm and time interval between the heat-shock treatment and the appearance of ectopic *myca* expression further suggest that Wnt8a overexpression induces ectopic hepatoblasts from non-hepatic endodermal cells.

We also examined whether Wnt8a overexpression could induce other hepatic markers and repress other non-hepatic endodermal markers. Wnt8a overexpression at 26 hpf induced the expression of the hepatoblast marker *hhex* (Fig. 4B, bracket), the hepatocyte markers *cp* (Fig. 4D, bracket) and *sepp1b* (Fig. 4F, bracket), and the hepatic ductal marker *sox9b* (Fig. 4H, bracket) in the endoderm posterior to the liver. By contrast, Wnt8a overexpression initially repressed the expression of the early intestinal marker *cdx1b* (Fig. 4J) and the early ventral pancreatic marker *ptf1a* (Fig. 4O), but later this repression was reduced (Fig. 4L; Fig. 4P, arrow) except for *cdx1b* expression in the intestinal

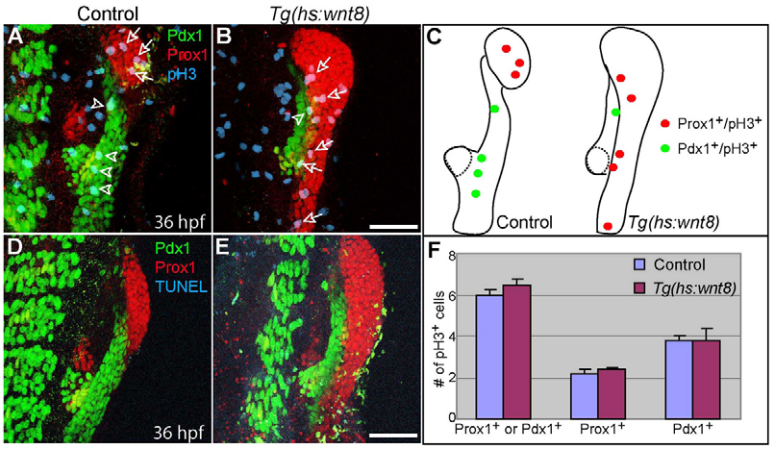

Fig. 2. Wnt8a overexpression does not significantly affect cell proliferation or cell death in the endoderm 10 hours after heat-shock. *Tg(hs:wnt8a)* embryos were heat-shocked at 26 hpf and harvested at 36 hpf for whole-mount immunostaining. (**A**,**B**) Anti-Prox1 (red), anti-Pdx1 (green) and anti-pH3 (blue) triple labeling reveals that there was no significant difference in proliferation in Prox1$^+$ or Pdx1$^+$ endodermal cells between control (n=5 out of 5) and Wnt8a-overexpressing (n=4 out of 4) embryos 10 hours after heat-shock. Arrows point to Prox1$^+$;pH3$^+$ cells and arrowheads point to Pdx1$^+$;pH3$^+$ cells. (**C**) The distribution of pH3$^+$ cells in A and B. Dotted lines outline the dorsal pancreas. (**D**,**E**) Anti-Prox1 (red), anti-Pdx1 (green) and TUNEL (blue) triple labeling reveals that no apoptosis was observed in Prox1$^+$ or Pdx1$^+$ endodermal cells of control or Wnt8-overexpressing embryos. (**F**) The number of pH3$^+$ cells normalized over the size of the Prox1$^+$ or Pdx1$^+$ area are shown as a graph. Data are mean+s.e.m. Ventral views, anterior up. Scale bars: 50 μm.

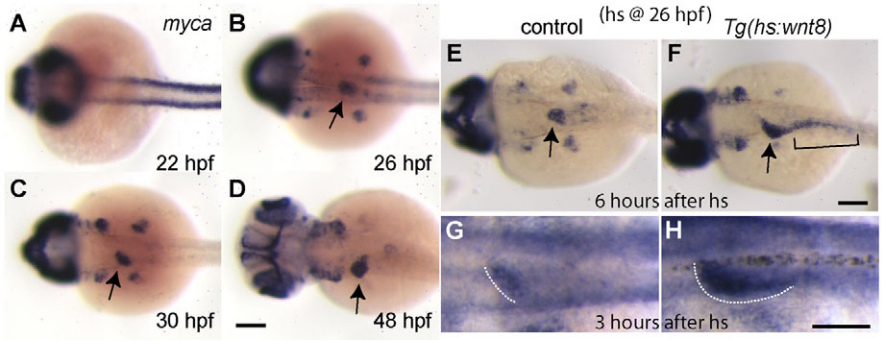

Fig. 3. *myca* is specifically expressed in the liver-forming region, and is rapidly induced in the posterior endoderm upon Wnt8a overexpression. (**A-D**) *myca* starts to be expressed in the liver-forming region around 26 hpf (B, arrow) and is specifically expressed in the liver at least up to 48 hpf (C,D; arrows). (**E-H**) Wnt8a overexpression via heat-shock at 26 hpf induced ectopic *myca* expression in the posterior endoderm by 6 hours after heat-shock (F, bracket, *n*=10 out of 10) and ectopic expression can often be seen even 3 hours after heat-shock (H, *n*=13 out of 18). Dotted lines (G,H) delineate the endodermal region in which *myca* is expressed. Arrows in E,F point to the liver. Dorsal views, anterior to the left. Scale bars: 100 μm.

bulb region (Fig. 4L, bracket). However, Wnt8a overexpression did not appear to affect the expression of other endodermal markers, such as the swim bladder marker *anxa5b* (Fig. 4R, arrow) or the thyroid marker *nkx2.1a* (Fig. 4T, arrow). These data suggest that Wnt8a overexpression can induce ectopic liver formation and subsequent differentiation of hepatocytes and hepatic ducts at the expense of intestinal bulb and ventral pancreatic tissues, but that it does not appear to affect the formation of the swim bladder or thyroid tissues.

The extent of the endodermal region retaining hepatic competence is gradually reduced with time

Data from mouse explant culture studies indicate that the endoderm posterior to the foregut endoderm retains hepatic competence (Gualdi et al., 1996; Bossard and Zaret, 2000), but in vivo evidence for this hypothesis has been lacking. Our findings that Wnt8a overexpression can induce ectopic hepatocytes and expand the Prox1^{+} domain at the expense of the Pdx1^{+} domain in the endoderm posterior to the liver

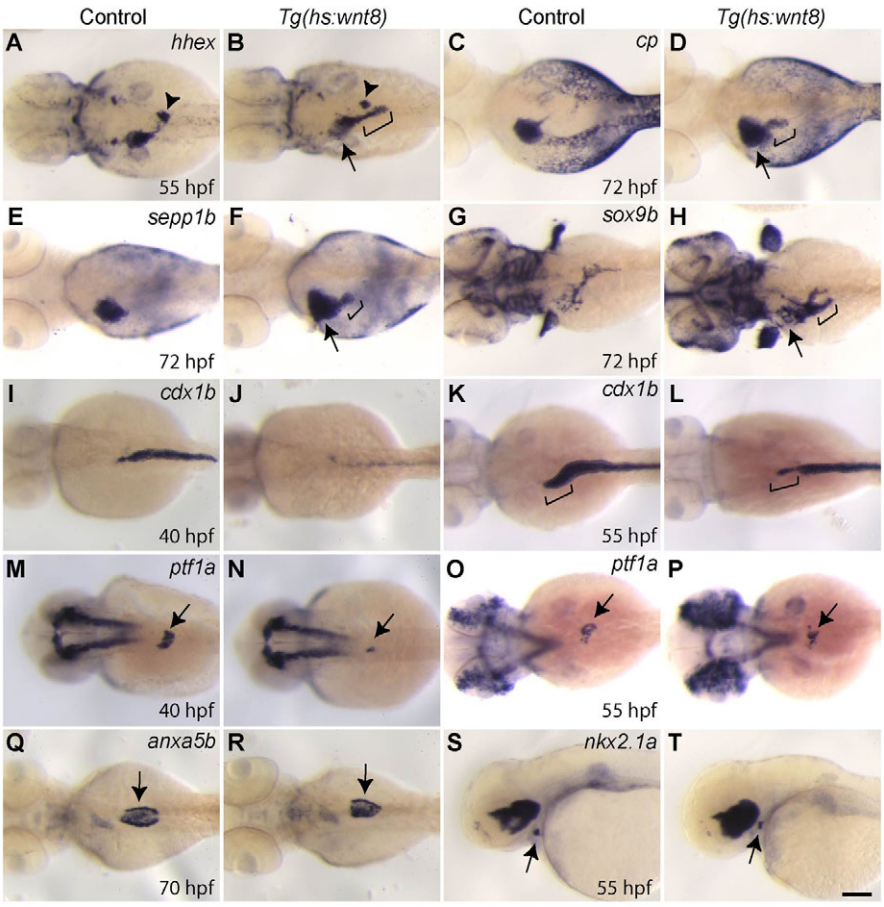

Fig. 4. Wnt8a overexpression induces the expression of various hepatic markers and represses the expression of an intestinal and a ventral pancreatic marker. Embryos obtained from outcrossing *Tg(hs:wnt8a)* zebrafish were heat-shocked at 26 hpf and harvested at 40 (I,J,M,N), 55 (A,B,K,L,O,P,S,T), 70 (Q,R) or 72 (C-H) hpf for in situ hybridization. (**A-H**) Wnt8a overexpression induced the expression of the hepatoblast marker *hhex* (B, *n*=10 out of 10), the hepatocyte markers *cp* (D, *n*=14 out of 14) and *sepp1b* (F, *n*=12 out of 14), and the hepatic ductal marker *sox9b* (H, *n*=13 out of 13) in the endoderm (brackets) posterior to the liver (arrows). Arrowheads indicate the dorsal pancreas (A,B). (**I-P**) By contrast, Wnt8a overexpression initially repressed the expression of the intestinal marker *cdx1b* (J, *n*=17 out of 17) and the early ventral pancreatic marker *ptf1a* (N, arrow, *n*=18 out of 18), but later their expression recovered (L,P), except for *cdx1b* expression in the intestinal bulb region (L, bracket, *n*=10 out of 10). Arrows in M-P point to the ventral pancreas or its progenitor cells. (**Q-T**) However, Wnt8a overexpression did not appear to affect the expression of other endodermal markers such as the swim bladder marker *anxa5b* (R, arrow, *n*=13 out of 13) or the thyroid marker *nkx2.1a* (T, arrow, *n*=10 out of 10). Arrows point to the swim bladder (Q,R) or thyroid (S,T). Dorsal (A-R) or side (S,T) views, anterior to the left. Scale bar: 100 μm.

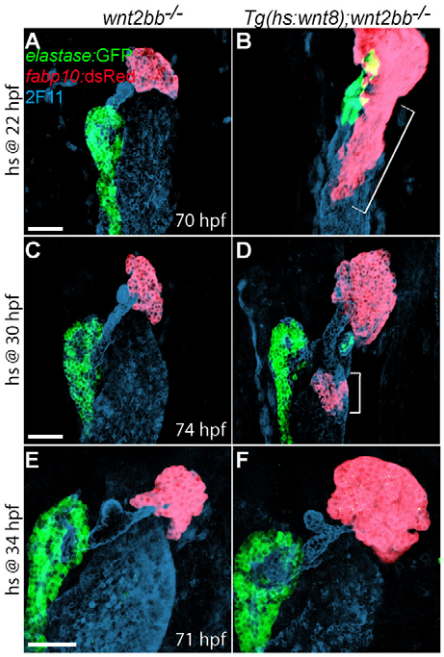

Fig. 5. The extent of the endodermal region retaining hepatic competence is gradually reduced with time. (**A-F**) Embryos obtained from crossing *wnt2bb*$^{-/-}$ with *Tg(hs:wnt8a);wnt2bb*$^{-/-}$ zebrafish were heat-shocked at 22 (A,B), 30 (C,D) or 34 (E,F) hpf, and harvested at 70-74 hpf for whole-mount immunostaining. The *Tg(fabp10:dsRed,ela3l:EGFP)* line was used to reveal hepatocytes (red) and pancreatic acinar cells (green); mAb 2F11 staining reveals the hepatopancreatic duct (blue). Wnt8a overexpression at 22 hpf (B, bracket, *n*=6 out of 6) induced more ectopic hepatocytes than at 30 hpf (D, brackets, *n*=14 out of 14); Wnt8a overexpression at 34 hpf failed to induce ectopic hepatocytes (F, *n*=6 out of 6). However, the liver size in *wnt2bb* mutants increased even when Wnt8a was overexpressed at 34 hpf. Ventral views, anterior up. Scale bars: 50 μm.

provide such in vivo evidence. Furthermore, these findings suggest that Pdx1^{+} cells retain hepatic competence. By overexpressing Wnt8a via heat-shock at various stages, we further investigated which endodermal regions retain hepatic competence and how long endodermal cells retain this competence. As the reduced liver size in *wnt2bb* mutants helps in identifying ectopic hepatocytes, we decided to overexpress *wnt8a* in the *wnt2bb* mutant background. Wnt8a overexpression at 22 hpf greatly induced ectopic hepatocytes in the endoderm posterior to the liver (Fig. 5B, bracket); overexpression at 30 hpf still induced ectopic hepatocytes but noticeably fewer than at 22 hpf. In addition, Wnt8a overexpression at 34 hpf did not induce any ectopic hepatocytes, although it still increased liver size in *wnt2bb* mutants (Fig. 5E versus Fig. 4F). These data reveal that hepatic competence is gradually lost with time. Moreover, the data from Wnt8a overexpression at 30 hpf show that ectopic hepatocytes are always located in the intestinal bulb (Fig. 5D, bracket), suggesting that this tissue retains hepatic competence longer than other endodermal tissues.

Fgf signaling greatly reduces the effect of Wnt8a overexpression on ectopic hepatocyte formation

Not only Wnt (Ober et al., 2006; Goessling et al., 2008) but also Bmp (Rossi et al., 2001; Zhang et al., 2004; Shin et al., 2007; Chung et al., 2008; Wandzioch and Zaret, 2009) and Fgf (Jung et al., 1999; Zhang et al., 2004; Calmont et al., 2006; Shin et al., 2007; Wandzioch and Zaret, 2009) signaling are essential for liver specification, and these signals have been considered to be hepatic-inducing signals. Thus, we investigated whether enhancing Bmp or Fgf signaling in all tissues could also induce ectopic hepatocytes, as observed following enhanced Wnt signaling. To answer these questions, we used the *Tg(hsp70l:bmp2b)*^{fi13} (Chocron et al., 2007) and the *Tg(hsp70l:XlFgfr1)*^{pd3} (Marques et al., 2008) lines that express Bmp2b and constitutively active Fgfr1 (caFgfr1), respectively, upon heat-shock. In contrast to Wnt8a overexpression, Bmp2b overexpression with heat-shock at 22 or 26 hpf did not induce ectopic hepatocytes or lead to the recovery of liver size in *wnt2bb* mutants (see Fig. S2 in the supplementary material). caFgfr1 overexpression at 26 hpf also failed to induce ectopic hepatocytes (Fig. 6B).

As it is possible that two different hepatic-inducing signals can synergize to induce hepatocytes, we next examined whether overexpression of Bmp2b or caFgfr1 together with Wnt8a could induce more ectopic hepatocytes than overexpression of Wnt8a alone. Overexpression of Bmp2b and Wnt8a had no additional or synergistic effect on ectopic hepatocyte formation (data not shown). Surprisingly, overexpression of caFgfr1 and Wnt8a blocked the effect of Wnt8a on ectopic hepatocyte formation, although it did not block the effect of Wnt8a on the recovery of liver size in *wnt2bb* mutants (compare Fig. 6D with 6C and Fig. 6H with 6G). This finding prompted us to investigate whether reducing Fgf signaling could enhance ectopic hepatocyte formation induced by Wnt8a overexpression. The Fgfr inhibitor SU5402 (Mohammadi et al., 1997) was used to reduce Fgf signaling. Treatment with SU5402 from 24 to 38 hpf greatly enhanced ectopic hepatocyte formation induced by Wnt8a overexpression at 30 hpf (Fig. 6L, bracket). These gain- and loss-of-function results suggest that Fgf signaling negatively regulates hepatic competence.

fgf10a knockdown greatly enhances ectopic hepatocyte formation induced by Wnt8a overexpression

We next sought to identify the Fgf ligand(s) that negatively regulate hepatic competence. We first tested *fgf10a* because it is expressed in the mesenchyme surrounding the endoderm that gives rise to the pancreas and intestinal bulb (Dong et al., 2007; Manfroid et al., 2007), and *fgf10a* mutants occasionally exhibit a few ectopic hepatocytes in the proximal pancreas (Dong et al., 2007). We examined the expression pattern of *fgf10a* in greater detail. *fgf10a* appears to be expressed around the Pdx1^{+} domain at 30 hpf (Fig. 7A-C), and this pattern appears to be maintained at 36 hpf (Fig. 7D-F). This expression pattern further suggests that *fgf10a* is a good candidate for the Fgf ligand(s) that negatively regulates hepatic competence.

We next performed loss-of-function experiments using *fgf10a* morpholino antisense oligonucleotides (MO). *fgf10a* MO-injected embryos completely failed to form pectoral fins (data not shown), validating MO efficacy (Norton et al., 2005). Moreover, as this *fgf10a* MO is a splicing blocking MO, its efficacy was further tested and confirmed by RT-PCR (see Fig. S3 in the supplementary material). Wnt8a overexpression following heat-shock at 30 hpf in the *wnt2bb* mutant background resulted in considerably more ectopic hepatocytes in *fgf10a*-knockdown embryos than in control (compare Fig. 8D with 8C, brackets). This phenotype was also confirmed in *fgf10a* single mutants (compare Fig. 8H with 8G, brackets). Ectopic hepatocytes were occasionally located in the extrapancreatic duct of these *fgf10a*-knockdown embryos (Fig. 8D, arrow). The hepatic marker Prox1 was also ectopically expressed

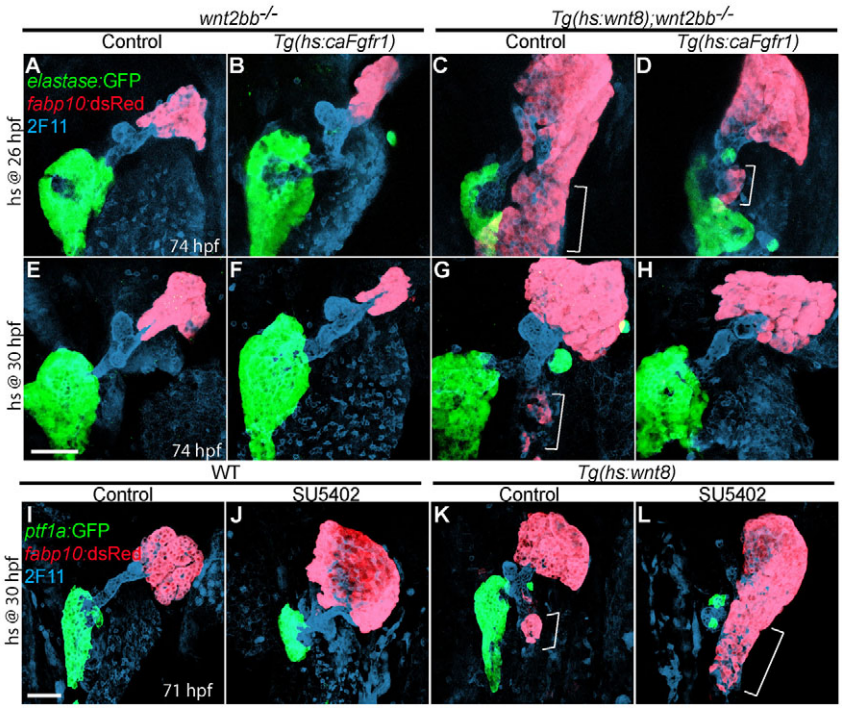

Fig. 6. Fgf signaling negatively regulates hepatic competence. (**A**-**H**) Enhancing Fgf signaling by overexpressing caFgfr1 via heat-shock at 26 (A-D) or 30 (E-H) hpf greatly reduced or abolished the effect of Wnt8a overexpression on ectopic hepatocyte formation, whereas it did not block its effect on the increase of liver size in *wnt2bb* mutants. Fewer ectopic hepatocytes appeared to be induced in embryos that overexpressed both Wnt8a and caFgfr1 than in ones that overexpressed Wnt8a only (a representative comparison is shown in D versus C and H versus G, brackets, *n*=6 out of 6), but liver size was comparable between these embryos. (**I**-**L**) Reducing Fgf signaling with the Fgfr inhibitor SU5402 greatly enhanced the effect of Wnt8a overexpression on ectopic hepatocyte formation. Embryos that overexpressed Wnt8a from a 30 hpf heat-shock exhibited significantly more ectopic hepatocytes when treated with SU5402 from 24 to 38 hpf [L (*n*=6 out of 6) versus K (*n*=5 out of 5), brackets]. SU5402 treatment in the absence of Wnt8a overexpression did not induce ectopic hepatocytes, but increased liver size and reduced pancreatic size (J, *n*=5 out of 5). Ventral views, anterior up. Scale bars: 50 μm.

in a larger region in *fgf10a*-knockdown embryos than in controls (compare Fig. 8L with 8K, brackets). Altogether, these data suggest that the gradual loss of hepatic competence with time is mediated at least in part by Fgf10a signaling.

Expression of the *foxa1/2/3* and *gata4/6* transcription factor genes in the endoderm does not appear to be reduced with time or by enhanced Fgf signaling

Data from mouse explant culture studies have shown that the *Foxa1* and *Gata4* transcription factor genes are expressed in gut endodermal explants isolated from E11.5, but not E13.5, embryos, and that the E11.5 endodermal explants lose *Foxa1* and *Gata4* expression when they are cultured with surrounding mesoderm isolated from E13.5 embryos (Bossard and Zaret, 2000). These data suggest that signals from the surrounding mesoderm negatively regulate hepatic competence by repressing at least *Foxa1* and *Gata4* expression in the endoderm. This hypothesis led us to investigate whether Fgf signaling also negatively regulates hepatic competence by repressing expression of *foxa* and *gata* transcription factor genes. First, we examined *foxa1/2/3* and *gata4/6* expression in wild-type embryos from 26 to 48 hpf, as the extent of the endodermal region retaining hepatic competence is gradually reduced with time (Fig. 5). At least during these stages, *foxa1*, *foxa3*, *gata4* and *gata6* are continuously expressed in the endoderm posterior to the liver-forming region (see Fig. S4 in the supplementary material, arrows), where ectopic hepatocytes can form upon Wnt8a overexpression. *foxa2* expression is relatively restricted to the anterior endoderm and is barely detected in the liver-forming region (see Fig. S4B in the supplementary material, arrows). These data suggest that the loss of hepatic competence with time is not due to the absence of expression of these transcription factor genes. We also examined *foxa1/2/3* and *gata4/6* expression in embryos that overexpress caFgfr1, in which ectopic hepatocyte formation upon Wnt8a overexpression is greatly reduced. Expression of these genes in the endoderm appeared to be unaffected in embryos with enhanced Fgf signaling compared with controls (see Fig. S5 in the supplementary material). Altogether, these data suggest that Fgf signaling does not regulate hepatic competence by modulating *foxa1/2/3* or *gata4/6* expression.

The Ras intracellular signaling pathway mediates the effect of Fgf signaling on hepatic competence

Fgf signaling is generally mediated by three intracellular signaling pathways: the Ras/MAP kinase, PI3 kinase/AKT and PLCγ/PKC pathways (Bottcher and Niehrs, 2005). A recent mouse explant

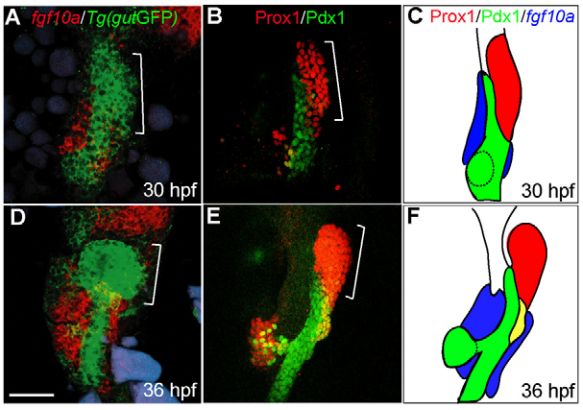

Fig. 7. *fgf10a* is expressed around the Pdx1⁺ domain. (**A**,**D**) *fgf10a* in situ hybridization combined with anti-GFP immunostaining in *Tg(gutGFP)* embryos reveals *fgf10a* expression around the endoderm directly caudal to the liver-forming region at 30 (A) and 36 (D) hpf. (**B**,**E**) Comparison with the Prox1 and Pdx1 expression pattern at these stages suggests that *fgf10a* is expressed around the Pdx1⁺ domain. (**C**,**F**) The expression patterns of Prox1 (red), Pdx1 (green) and *fgf10a* (blue) at these stages. Broken lines outline the dorsal pancreas; brackets mark the liver-forming region. Ventral views, anterior up. Scale bar: 50 μm.

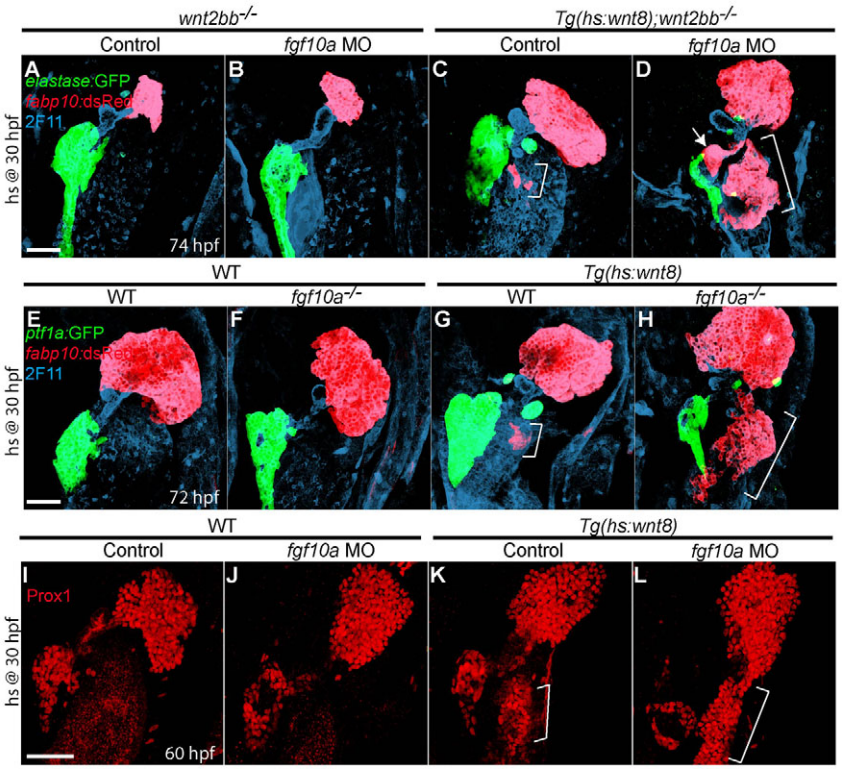

Fig. 8. *fgf10a* knockdown greatly enhances ectopic hepatocyte formation induced by Wnt8a overexpression. (**A-D**,**I-L**) Embryos obtained from crossing *wnt2bb*$^{-/-}$ with *Tg(hs:wnt8a);wnt2bb*$^{-/-}$ zebrafish (A-D) or outcrossing *Tg(hs:wnt8a)* zebrafish (I-L) were injected with *fgf10a* MO, heat-shocked at 30 hpf, and harvested at 60 (I-L) or 74 (A-D) hpf for whole-mount immunostaining. (**E-H**) Embryos obtained from crossing *fgf10a*$^{+/-}$ with *Tg(hs:wnt8a); fgf10a*$^{+/-}$ zebrafish were heat-shocked at 30 hpf and harvested at 72 hpf for whole-mount immunostaining. *fgf10a* knockdown in *wnt2bb* mutants did not induce ectopic hepatocytes or affect liver size (B, *n*=8 out of 8), but it greatly enhanced ectopic hepatocyte formation induced by Wnt8a overexpression [D (*n*=6 out of 6) versus C (*n*=7 out of 7), brackets]. The ectopic hepatocytes were located in the intestinal bulb (brackets) and occasionally in the extrapancreatic duct (D, arrow). Ectopic hepatocyte formation was also greatly enhanced upon Wnt8a overexpression in *fgf10a* mutants (H, *n*=5 out of 5). Wnt8a overexpression greatly induced Prox1 expression in *fgf10a* MO-injected embryos compared with controls [L (*n*=5 out of 5) versus K (*n*=3 out of 3), brackets]. Ventral views, anterior up. Scale bars: 50 μm.

culture study using signaling inhibitors has suggested that Fgf signaling positively regulates hepatic differentiation via the MAP kinase pathway and hepatic tissue growth via the PI3 kinase pathway (Calmont et al., 2006). To achieve a mechanistic understanding of how Fgf signaling represses the effect of Wnt8a overexpression on hepatic induction, we investigated the Fgf intracellular signaling pathways. The use of small molecule inhibitors did not lead to consistently significant data. Thus, we tested the *Tg(hsp70l:dnRas)*^{pd7} line (Lee et al., 2009), which expresses a dominant-negative version of Ras upon heat-shock. Overexpression of both Wnt8a and dnRas via heat-shock at 30 hpf greatly enhanced ectopic hepatocyte formation compared with overexpression of Wnt8a alone (Fig. 9D versus 9C, brackets), whereas overexpression of dnRas alone appeared to have no effect on liver formation (Fig. 9B). These data suggest that Ras signaling mediates the effect of Fgf signaling on hepatic competence.

DISCUSSION

In this study, we provide two new lines of evidence for the role of Wnt/β-catenin signaling in liver specification. We show that further suppression of Wnt/β-catenin signaling in *wnt2bb* mutants completely blocks liver formation and that Wnt8a overexpression can induce ectopic hepatoblasts. Furthermore, by taking advantage of the effect of Wnt8a overexpression on liver induction, we investigated hepatic competence in vivo, leading to three novel findings. First, endodermal cells posterior to the liver-forming region retain hepatic competence. Second, these cells gradually lose hepatic competence with time. Finally, this gradual loss of hepatic competence is mediated at least in part by Fgf10a signaling.

We have previously reported that mesodermal Wnt2bb signaling positively regulates liver specification through the identification and analysis of *wnt2bb* mutants (Ober et al., 2006). However, some of these mutants eventually form a liver and develop into fertile

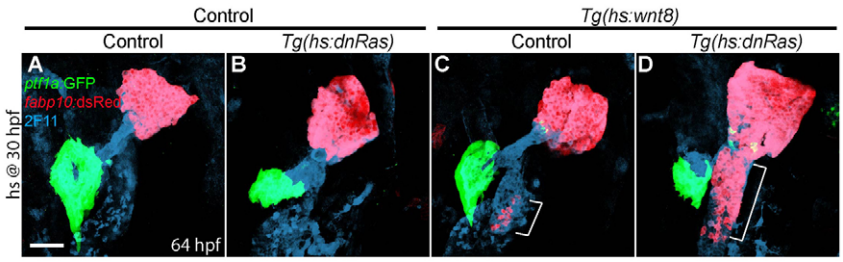

Fig. 9. Blocking Ras function greatly enhances ectopic hepatocyte formation induced by Wnt8a overexpression. (**A-D**) Embryos obtained from crossing *Tg(hs:wnt8a)* with *Tg(hs:dnRas)* zebrafish were heat-shocked at 30 hpf, and harvested at 64 hpf for whole-mount immunostaining. Overexpression of dnRas did not induce ectopic hepatocytes or affect liver size in controls (B, *n*=5 out of 5), but it greatly enhanced ectopic hepatocyte formation induced by Wnt8a overexpression [D (*n*=7 out of 7) versus C (*n*=8 out of 8), brackets]. Ventral views, anterior upwards. Scale bar: 50 μm.

adults (Ober et al., 2006), indicating that the adult mutant fish contain a fully functional liver. This recovery of early liver defects in *wnt2bb* mutants weakened the claim for a role for Wnt/β-catenin signaling in liver specification. Here, we provide additional evidence to support such a role: additional suppression of Wnt/β-catenin signaling in *wnt2bb* mutants completely blocks liver formation, and Wnt8a overexpression can induce ectopic hepatoblasts. Furthermore, we found that Wnt8a overexpression could induce the expression of hepatocyte and biliary differentiation markers, indicating that not only liver specification but also its differentiation can be induced by Wnt8a overexpression. Interestingly, recent data from genetic studies in mouse show that activation of Wnt/β-catenin signaling in Shh^+ endodermal cells could induce lung specification but not its differentiation (Goss et al., 2009; Harris-Johnson et al., 2009). Wnt8a was temporarily expressed in our study, whereas Wnt/β-catenin signaling was continuously activated by the Cre-loxP system in the mouse studies, and we speculate that continuous activation of Wnt/β-catenin signaling is not compatible with differentiation. However, it is also possible that the difference between the data sets might result from the requirement of additional signals for lung differentiation that are not present in the environment of the ectopic lung progenitors.

Wnt/β-catenin signaling has multiple roles during liver development. It regulates hepatoblast proliferation (Monga et al., 2003; Suksaweang et al., 2004; Tan et al., 2006; Goessling et al., 2008; Tan et al., 2008), biliary differentiation (Decaens et al., 2008), liver zonation (Benhamouche et al., 2006) and hepatocyte metabolism (Sekine et al., 2006). Studies from the conditional knockout of β-catenin in the mouse endoderm (Tan et al., 2008) or hepatocytes (Tan et al., 2006), from mouse embryonic liver cultures (Monga et al., 2003), and from chick in ovo microinjection (Suksaweang et al., 2004) reveal the role of Wnt/β-catenin in the regulation of hepatoblast and hepatocyte proliferation. In addition, recent zebrafish studies (Goessling et al., 2008) show that enhancing Wnt/β-catenin signaling at mid-somitogenesis stages increases liver size, further illustrating the role of Wnt/β-catenin signaling in liver growth. We also observed such an effect of Wnt/β-catenin signaling on liver growth (Fig. 5F). Furthermore, by enhancing Wnt/β-catenin signaling at later stages and examining the liver by confocal microscopy, we observed that ectopic hepatoblasts and hepatocytes appeared in the intestinal bulb, which together with other data suggests that non-hepatic endodermal cells can trans-fate into hepatic endodermal cells. Moreover, the finding that Fgf signaling did not block liver growth caused by Wnt8a overexpression but did block ectopic hepatocyte formation in Wnt8a-overexpressing embryos (Fig. 6) supports the concept that Wnt/β-catenin signaling plays a role in liver specification as well as in hepatocyte proliferation. Our findings add the regulation of liver specification to the list of roles that Wnt/β-catenin signaling plays during liver development. And, although our data suggest a fate-change of $Pdx1^+$ non-hepatic endodermal cells into $Prox1^+$ hepatic cells upon Wnt8a overexpression, detailed lineage tracing of $Pdx1^+$ endodermal cells is required to rigorously test this model.

Wnt8a overexpression induced ectopic hepatoblasts and hepatocytes only in the endoderm posterior to the liver. This region is equivalent to the region from which the stomach and duodenum are derived in *Xenopus* and mouse. Interestingly, a recent *Xenopus* study showed that Ptf1a misexpression could convert the stomach and duodenum into pancreatic tissues (Jarikji et al., 2007). Thus, our data in zebrafish and the *Xenopus* data suggest that the endoderm that gives rise to the stomach and duodenum is highly plastic.

Implantation of BMP2-soaked beads in chick embryos can induce ectopic *HHEX* expression in the endoderm just posterior or lateral to the endogenous *HHEX* expression domain (Zhang et al., 2004); the addition of BMP4 and FGF2 in culture media can induce hepatocytes from endodermal progenitors derived from mouse embryonic stem cells (Gouon-Evans et al., 2006). In addition, FGF2 can induce hepatic gene expression in mouse (Jung et al., 1999) and chick (Zhang et al., 2004) explant cultures. Based on these data, we anticipated that overexpression of Bmp2b or caFgfr1 would induce ectopic hepatocytes much like Wnt8a overexpression does. However, Bmp2b or caFgfr1 overexpression at 22 hpf, or later, did not induce ectopic hepatocytes, suggesting that Bmp or Fgf signaling is not sufficient to induce ectopic hepatocyte formation at these stages. In interpreting these data, we have to consider the developmental time windows when these signaling pathways normally function. We recently reported that *bmp2b* is expressed in the lateral plate mesoderm starting at around 14 hpf and that when Bmp2b was overexpressed at 13 hpf but not at 16 hpf, the $hhex^+$ liver domain was expanded at the expense of the $pdx1^+$ domain (Chung et al., 2008). These data indicate the importance of studying precise developmental stages in assessing the role of Bmp signaling in liver induction, as has been shown in mouse (Wandzioch and Zaret, 2009).

We have also previously reported that Fgf signaling positively regulates liver specification in zebrafish (Shin et al., 2007) as it does in mouse (Jung et al., 1999; Calmont et al., 2006) and chick (Zhang et al., 2004); however, here we show that Fgf signaling mediated by Fgf10a negatively regulates hepatic competence. As is the case with the aforementioned Bmp signaling pathway, developmental timing has to be taken into account when assessing the role of Fgf signaling in liver development. Although it is not clear when Fgf signaling is required for liver specification because the Fgf ligand that regulates this process has not yet been identified, Fgf10a signaling appears to regulate hepatic competence negatively in the posterior endoderm at around 30 hpf, a time after liver specification has occurred. There are other instances when the same signaling pathway plays opposite roles at different developmental stages. In the mouse ventral foregut endoderm, Bmp signaling represses *Hnf6* and *Pdx1* expression at the 3-4 somite stage, whereas it induces it at the 5-6 somite stage (Wandzioch and Zaret, 2009). In zebrafish, Bmp signaling is required for the budding of the ventral pancreas, but needs to be suppressed for the formation of ventral bud derived pancreatic β cells (Chung et al., 2010). Wnt/β-catenin signaling needs to be suppressed during early somitogenesis stages to form a liver in *Xenopus* (McLin et al., 2007) and zebrafish (Goessling et al., 2008), but is required at later stages for liver formation (Ober et al., 2006; Goessling et al., 2008). During heart development, Wnt/β-catenin signaling positively regulates cardiac induction, but later inhibits cardiac differentiation (Cohen et al., 2008). Therefore, our findings concerning the opposite roles of Fgf signaling in liver development re-emphasize the importance of developmental context, especially temporal context, in understanding the role of signaling pathways during organogenesis.

Data from mouse explant culture studies have shown that the dorsal endoderm at E11.5 or earlier retains hepatic competence and suggest that signals from the adjacent mesoderm repress this hepatic competence (Gualdi et al., 1996; Bossard and Zaret, 2000). Similarly, we found that, in zebrafish, the endodermal region

posterior to the liver-forming region retains hepatic competence at 30 hpf or earlier (Fig. 5). Furthermore, our results suggest that $Pdx1^+$ cells can differentiate into $Prox1^+$ liver cells upon Wnt8a overexpression. More interestingly, we discovered that Fgf signaling can negatively regulate hepatic competence, and propose that the gradual loss of hepatic competence with time is mediated at least in part by Fgf10a. Although *fgf10a* knockdown extends the duration during which endodermal cells retain hepatic competence, these cells eventually lose hepatic competence, suggesting that additional factors negatively regulate hepatic competence. These factors might be other extrinsic factors expressed by the surrounding mesenchyme such as Fgf10a, or intrinsic factors whose expression in the endoderm is initiated at later stages.

In zebrafish, the *foxa1/3* and *gata4/6* transcription factor genes are broadly expressed in the endoderm at least until 48 hpf (see Fig. S4 in the supplementary material), suggesting that the loss of hepatic competence with time is not due to the absence of expression of these genes. So far, the regulation of hepatic competence is understood only at the level of these transcription factors; however, hepatic competence could also be regulated at other levels: at the level of (1) the receptors for the hepatic inducing signals, (2) the intracellular components transferring hepatic inducing signals into the nucleus, (3) the chromatin structure of the regions containing hepatoblast genes, and (4) the co-repressors, co-activators and other transcription factors that can regulate hepatoblast gene expression. A recent report showing that the Grg3 co-repressor can repress hepatic competence through binding to Foxa transcription factors (Santisteban et al., 2010) supports this notion that hepatic competence can be, and is likely to be, regulated at multiple levels. Therefore, the result that expression of the *foxa1/2/3* and *gata4/6* transcription factor genes appears unaffected in embryos that overexpress caFgfr1 (see Fig. S5 in the supplementary material) is not overly surprising. Identification and functional analysis of Fgf10a target genes in the endoderm posterior to the liver-forming region should lead to a mechanistic understanding of how Fgf10a signaling negatively regulates hepatic competence.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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