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in the Zebrafish by *Shh* and *BMP4*

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Left–right (LR) asymmetry of the heart in vertebrates is regulated by early asymmetric signals in the embryo, including the secreted signal *Sonic hedgehog* (*Shh*), but less is known about LR asymmetries of visceral organs. Here we show that *Shh* also specifies asymmetries in visceral precursors in the zebrafish and that cardiac and visceral sidedness are independent. The transcription factors *fli-1* and *Nkx-2.5* are expressed asymmetrically in the precardiac mesoderm and subsequently in the heart; an *Eph* receptor, *rtk2*, and an adhesion protein, DM-GRASP, mark early asymmetries in visceral endoderm. Misexpression of *shh* mRNA, or a dominant negative form of protein kinase A, on the right side reverses the expression of these asymmetries in precursors of both the heart and the viscera. Reversals in the heart and gut are uncoordinated, suggesting that each organ interprets the signal independently. Misexpression of *Bone Morphogenetic Protein* (*BMP4*) on the right side reverses the heart, but visceral organs are unaffected, consistent with a function for *BMPs* locally in the heart field. Zebrafish mutants with midline defects show independent reversals of cardiac and visceral laterality. Thus, *hh* signals influence the development of multiple organ asymmetries in zebrafish and different organs appear to respond to a central cascade of midline signaling independently, which in the heart involves *BMP4*.

Key Words: **heart; *hedgehog; BMP;* left–right asymmetry.**

INTRODUCTION

In addition to anterior–posterior (AP) and dorsal–ventral (DV) body axes, vertebrates develop left–right (LR) asymmetries. The heart lies on the left side and nearly all of the visceral organs, such as the stomach, liver, spleen, and pancreas, are primarily one-sided. A molecular cascade of asymmetrically expressed genes that alter heart asymmetry when misexpressed have been defined in the avian embryo, including several secreted signals (Levin *et al.*, 1995; review Levin, 1998). However, it remains unclear how these transient asymmetries in gene expression during gastrulation translate into individual organ asymmetries.

LR asymmetries arise later than DV asymmetries, around the time that the dorsal organizer is established in the blastula, and are dependent on mesodermal derivatives of the organizer such as the notochord (Brown and Wolpert, 1990; Danos and Yost, 1995). *Vg1* localization along the LR axis is required to specify heart asymmetry in *Xenopus* in the early gastrula (Hyatt and Yost, 1998). In chick, *Shh*, *nodal related-1* (*cNR-1*), *activin βB* (*cAct βB*), and *activin type IIa receptor* (*cAct-RIIa*) are all expressed asymmetrically around the organizer, with all but *cActβB* and *cAct-RIIa* expressed on the left side (Levin *et al.*, 1995, 1997). Local misexpression of *Shh* on the right or *cAct-RIIa* on the left is sufficient to "randomize" heart asymmetry (i.e., the heart loops with equal probability to either the left or the right), revealing a genetic pathway that determines cardiac laterality. Expression of *nodal*-related and subsequently cardiac LR orientation are randomized following organizer removal in *Xenopus* and in *notail* (*ntl*) and *floating head* (*flh*) mutants in zebrafish that lack a differentiated noto-

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chord (Danos and Yost, 1995, 1996; Lohr *et al.*, 1997; Halpern *et al.*, 1993; Talbot *et al.*, 1995). Thus, some of the mechanisms of asymmetry are evolutionarily conserved, including *nodal*-related and the transcription factor *Pitx2* (Sampath *et al.*, 1997; Ryan *et al.*, 1998; reviews: Goldstein *et al.*, 1998; Yost, 1998).

Despite these insights into the cellular and molecular basis of heart asymmetry, it remains unclear how LR asymmetries in different organs are coordinated. In mice carrying an insertional mutation in the gene *inversion of embryonic turning* (*inv*), every organ lies on the wrong side of the body (Mochizuki *et al.*, 1998). In contrast, laterality defects occur independently in each organ in *inversus viscerum* mutants, which carry a defect in axonemal dynein (Hummel and Chapman, 1959; Yokoyama *et al.*, 1993; Supp *et al.*, 1997). Less is known about the development of endodermally derived visceral organs than about the heart. The viscera arise from evaginations of the posterior foregut, such as the hepatic diverticulum (Tahara and Nakamura, 1961). In *Xenopus*, the stomach and intestine twist into a gastrointestinal loop, and removal of the organizer disrupts looping and organ sidedness showing a requirement for axial mesoderm in asymmetry of the viscera (Danos and Yost, 1995). Of the many molecules implicated in LR asymmetry, only *nodal*-related expression in the lateral plate mesoderm correlates with visceral situs, and this asymmetric expression is conserved in all vertebrates examined (Collignon *et al.*, 1996; Heymer *et al.*, 1997; Lowe *et al.*, 1996; Rebagliati *et al.*, 1998). Thus, some mechanisms of LR asymmetry are shared between organs, while others may be separate.

One candidate for a conserved signal involved in LR asymmetries of all internal organs is a secreted protein of the *hh* family. *Shh* is expressed in the organizer at gastrula stages, and misexpression alters heart asymmetry, as well as other tissue polarities (Levin *et al.*, 1995; Chen *et al.*, 1997; Sampath *et al.*, 1997). In several zebrafish mutants, defects in heart asymmetry correlate with a reduction in expression of *hh*s in the midline (Danos and Yost, 1997; Chen *et al.*, 1997). Recent evidence has shown that expression of *Shh* on the right side in chick results in heterotaxia, independent randomization of sidedness of the heart, stomach, and embryonic turning (Levin *et al.*, 1997). One hypothesis is that the midline produces *shh* or some other *hh* asymmetrically during gastrulation to specify all of these asymmetries or, alternatively, that it acts both early on the heart and again later on the viscera.

The heart tube forms during somitogenesis. In zebrafish, bilateral heart fields that originate in the dorsolateral mesoderm migrate into the midline, composed of separate myocardial and endocardial lineages (Stainier *et al.*, 1993; Lee *et al.*, 1994). The heart tube then breaks symmetry. First the heart primordium "jogs" to the left of the midline and later loops into an S-shaped organ with a single posterior ventricle lying to the right (Chen *et al.*, 1997). Zebrafish mutants with DV and midline defects show disruption of LR asymmetry of the heart, and this correlates with a disruption in *hh* expression in midline tissues and asymmetric expression of *BMP4* in the primitive heart tube (Chen *et al.*, 1997). The extent of laterality defects in other internal organs in these mutants has not been examined (Chen *et al.*, 1996; Pack *et al.*, 1996).

We describe early molecular asymmetries in the heart and foregut and present evidence that *hh* and *BMP* signals regulate laterality of organ precursors. The heart field expresses two transcription factors asymmetrically, *fli-1* (Brown *et al.*, 1998) and *Nkx-2.5* (Chen and Fishman, 1996), and the secreted protein, *BMP4* (Nikaido *et al.*, 1997; Chen *et al.*, 1997). We also found two cell surface molecules that mark asymmetry of the gut. One is a tyrosine kinase receptor, *rtk2*, expressed on the right side that may be involved in setting up visceral asymmetry. The other is an adhesion protein, DM-GRASP, that marks the hepatic diverticulum. Misexpression of *shh* on the right side, by mRNA injection, reverses expression of these markers and eventually the LR orientation of both the heart and the viscera, while *BMP4* injection only alters the heart. Zebrafish mutations that affect the developing midline disrupt the initial molecular shifts in organ asymmetry and do so in different organs independently, showing a requirement for midline development in the local control of organ asymmetries. These results suggest that *hh* signaling regulates the determination of LR axis development in different organs.

METHODS

Embryos and Mutant Strains

Embryonic zebrafish (*Danio rerio*) were collected in pair matings of wild-type (AB) or heterozygous mutant adults and raised at 28.5°C in the zebrafish facility at the ICRF in London. Embryos were staged in hours postfertilization (h), according to Kimmel *et al.* (1995), and fixed at 18–20 h for *fli-1*, *Nkx-2.5*, and *BMP4* expression in heart precursors, 24 h to examine heart morphology, or 36–40 h to examine *rtk2* and DM-GRASP expression in the viscera. Embryos homozygous for the *golden* (*gol*^{b1}) mutation were used for immunostaining at stages later than 36 h, because of their reduced pigmentation. Control stainings showed that LR asymmetries in homozygous and heterozygous *gol*^{b1} mutants are not significantly different than in wild type. We identified homozygous embryos carrying the recessive lethal mutations *notail* (*ntl*^{b160}) (Halpern *et al.*, 1993), *floating head* (*flh*^{n1}) (Talbot *et al.*, 1995), *cyclops* (*cyc*^{b16}) (Hatta *et al.*, 1991), *one-eyed pinhead* (*oep*^{il,zl}) (Strahle *et al.*, Schier *et al.*, 1997), *spadetail* (*spt*^{b104}) (Kimmel *et al.*, 1989), and *you* (*you*^{ty97}) (Van Eeden *et al.*, 1996), by their morphologies prior to visible organ asymmetries.

In Situ Hybridization and Immunostaining

After fixation in 4% paraformaldehyde from 2 to 12 h, zebrafish embryos were processed for whole-mount *in situ* hybridization as described in Thisse *et al.* (1994). cDNA clones were used to generate antisense RNA probes for *fli-1* (Brown *et al.*, 1998), *Nkx2.5* (Chen and Fishman, 1996), *rtk2* (Xu *et al.*, 1994), and *BMP2* and *BMP4* expression (Nikaido *et al.*, 1997). Whole-mount immunohistochemistry followed the protocol of Trevarrow *et al.* (1990).

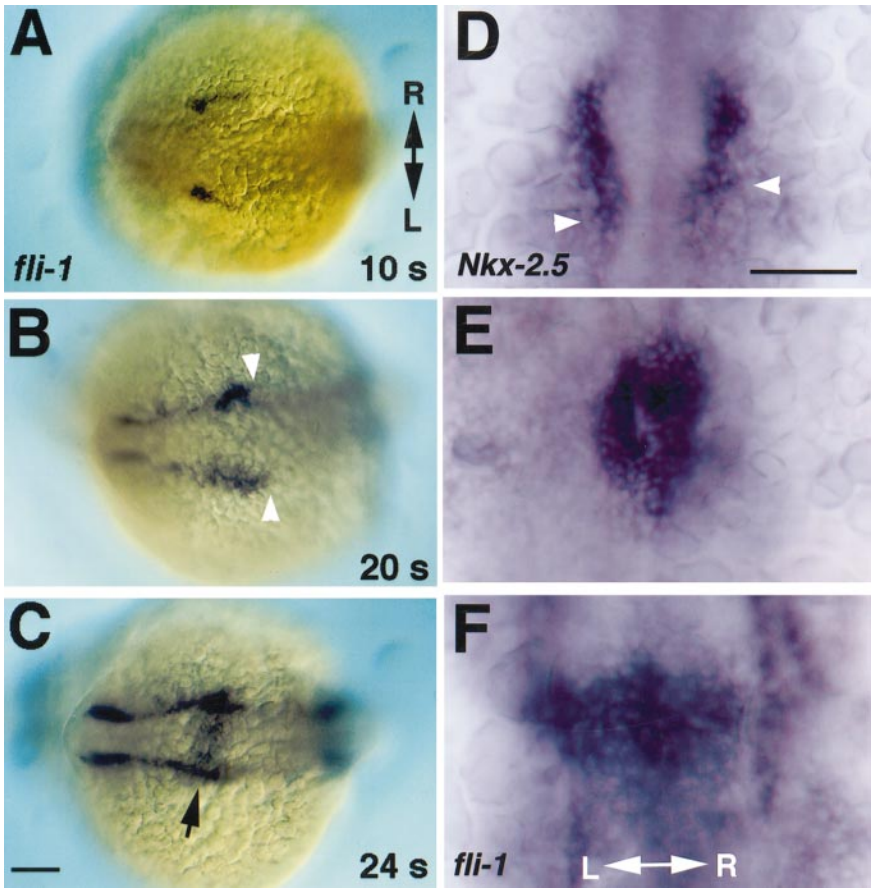


FIG. 1. *fli-1* and *Nkx-2.5* expression in heart precursors. Dorsal views of whole-mounted zebrafish embryos after *in situ* hybridization, anterior to the left in A–C and to the top in D–F. (A–C) *fli-1* expression in precardiac cells at 10 somites (10 s), in bilateral condensations lateral to the midbrain. Expression becomes progressively expanded along the AP axis, extending more posteriorly on the left side (compare arrowheads in B) in the forming heart, flanked anteriorly and posteriorly by narrow bands of expression in the head mesoderm. (D–F) *Nkx-2.5* is expressed in the heart at the same stage as *fli-1* in bilateral heart fields. (D) *Nkx-2.5* expression becomes broader on the left side of the midline at 20 s, as does *fli-1* (compare arrowheads in B and D). (E, F) Expression of *Nkx-2.5* (E), largely confined to the heart tube, and *fli-1* (F) asymmetrically in the tube and in surrounding mesoderm, beneath the pharyngeal arches. Scale bars, 100 μm.

Antibodies included zn-5, which recognizes a DM-GRASP/BEN/SC-1-related cell adhesion molecule (Trevarrow *et al.*, 1990; Kanki *et al.*, 1994), and anti-Isl-1 (Korzh *et al.*, 1993). All whole-mount embryo photomicrographs were performed with a Zeiss Axioplan microscope, scanned from slides, and processed using Adobe Photoshop.

mRNA Injections

To express genes ectopically, mRNA was injected into embryos at the one–two cell stage (Westerfield, 1995). Synthetic capped mRNA (cRNA) was transcribed *in vitro* from linearized pSP64T plasmids containing cDNA encoding zebrafish *shh* (Krauss *et al.*, 1993) *BMP2* or *BMP4* (Nikaido *et al.*, 1997), using the Ambion Message Machine kit. Synthesized RNA was resuspended in water and injected at approximately 5 pl/embryo at concentrations from 10 to 50 ng/μl. cRNAs encoding green fluorescent protein (GFP) or β-galactosidase were coinjected to monitor injection efficiencies and distributions of injected messages. The *BMP* expression constructs were kindly provided by Dr. Masazumi Tada.

RESULTS

Asymmetries in fli-1 and Nkx2.5 Expression in the Heart

Heart precursors in the zebrafish form bilaterally, fuse to form a linear heart tube, and then break symmetry and move to the left side. We compared the expression of three genes in precardiac cells during this process: *fli-1*, broadly expressed in hematopoietic mesoderm (Figs. 1A–1C and 1F); *Nkx2.5*, in a more restricted pattern that roughly defines the heart field (Figs. 1D and 1E); and *BMP4*, strongly on the left side of the heart (Fig. 4C). At 14 h, *fli-1* and *Nkx2.5* transcripts are distributed symmetrically in longitudinal

bands of the precardiac mesoderm (Fig. 1A), while *BMP4* remains expressed diffusely throughout the ventrolateral mesoderm (Nikaido *et al.*, 1997). Expression of *fli-1* and *Nkx-2.5* becomes asymmetric at 19 h, extending further posteriorly on the left than on the right (Figs. 1B and 1D). The two expression domains diverge at 21 h, *fli-1* expression forming a diagonal band that is broader on the left side (Figs. 1C and 1F), while *Nkx-2.5*-expressing cells form a symmetrical ring (Fig. 1E). *fli-1* is expressed in some cells outside the heart tube and eventually marks the presumptive endocardium and the developing vasculature throughout the body (Brown *et al.*, 1998). *Nkx-2.5* marks the primitive heart tube. *BMP4* expression increases in the heart field and decreases in the ventral and lateral mesoderm at 19 h, becoming predominant on the left side at 21 h (Fig. 3A). All three genes mark the heart later and are expressed in cells of the ventricle as it begins to beat (Figs. 3A and 5A). These data indicate a critical period between 19 and 21 h, when shifts in gene expression mark the first break in LR asymmetry of the heart tube.

Expression of rtk2 and DM-GRASP in the Developing Foregut

To compare the development of visceral asymmetries with the heart, we sought early markers of visceral organ precursors. In contrast to the heart, which arises from embryonic mesoderm, precursors of the viscera develop as evaginations of the endoderm. A subset of foregut cells express the Eph-receptor *rtk2* on the right side at 36 h, at the level of somite 3 (Fig. 2C). Expression is transient and cannot be detected at 48 h. Soon after this initial asymmetry, cells evaginate to form an hepatic diverticulum on the left side at 40 h, as revealed by the monoclonal antibody zn-5 which recognizes DM-GRASP, a member of the L2 family of cell surface molecules (Fig. 2A; Kanki *et al.*, 1994). This protein is also found on the atrium and ventricle of the heart, the endodermal lining of the pharyngeal pouches, and in many differentiating neurons. Labeled cells in the foregut form a tight cluster of ~30 cells that remain connected to the gut tube (Fig. 2B). Expression of DM-GRASP persists in the developing liver at 4–5 days of development and overlaps with expression of HNF3β and Islet-1, suggesting that these asymmetric precursors give rise to several types of organ progenitors (data not shown; Korzh *et al.*, 1993; Pack *et al.*, 1996). The earliest visible asymmetry in the viscera, that of *rtk2*, develops 12–16 h later than molecular LR asymmetries in the heart.

Shh Induces Reversed LR Asymmetries of Heart and Viscera

To address the role of *shh* in LR organ asymmetries, *shh* mRNA was coinjected with βgal or GFP mRNA into one cell at the two-cell stage, resulting in expression that was often restricted to left or right sides. Heart asymmetry was assayed using Nomarski optics of living embryos at 24 h, as

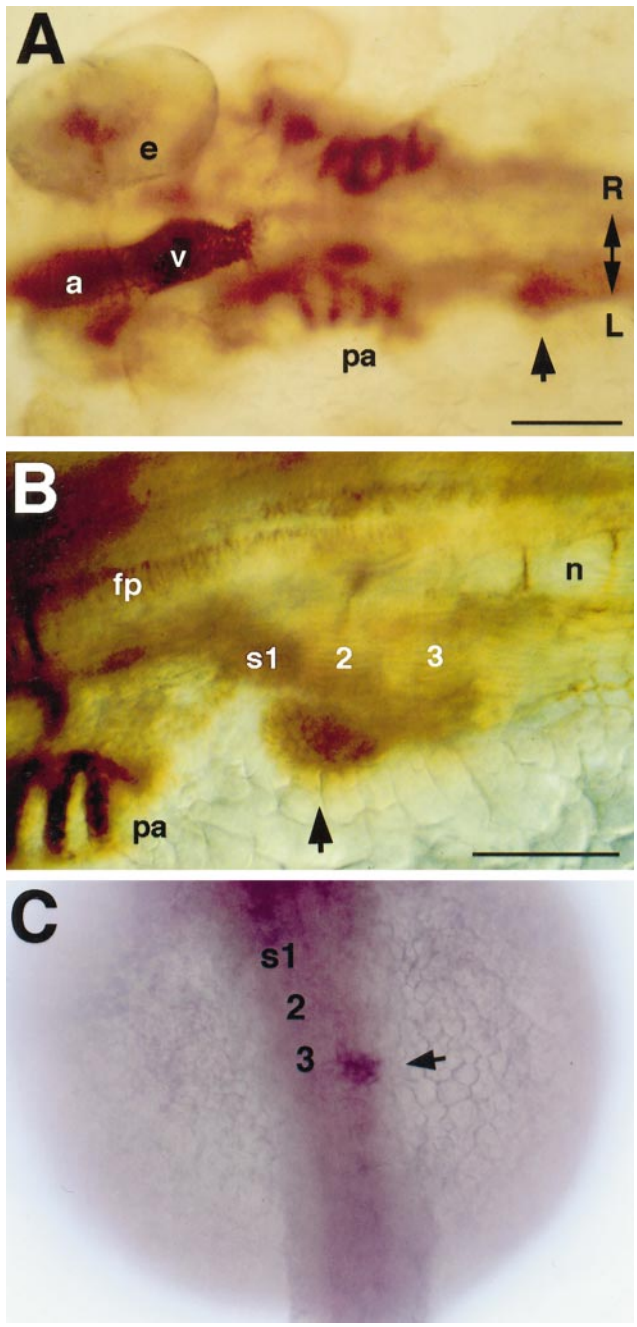


FIG. 2. Expression of DM-GRASP protein on the left and *rtk2* mRNA on the right in endodermal precursors of the visceral organs. Dorsal views of whole-mounted embryos, anterior to the left. (A) zn-5 immunoreactive cells (brown) are found in both the atrium and ventricle of the heart tube, the endodermal lining of the pharyngeal arches, as well as a cluster of 15–20 cells lying to the left of the notochord (arrow). (B) Higher magnification view of zn-5-labeled cells on the left side just posterior to the pharyngeal arches at the level of the second somite (arrow). (C) *rtk2* expression (arrow) is found in a cluster of 5–10 cells lying ventrally and to the right of the midline at the level of somite 3. a, atrium; e, eye; fp, floor plate; n, notochord; pa, pharyngeal arch; s1–s3, somites 1–3; v, ventricle. Scale bars, 100 μm.

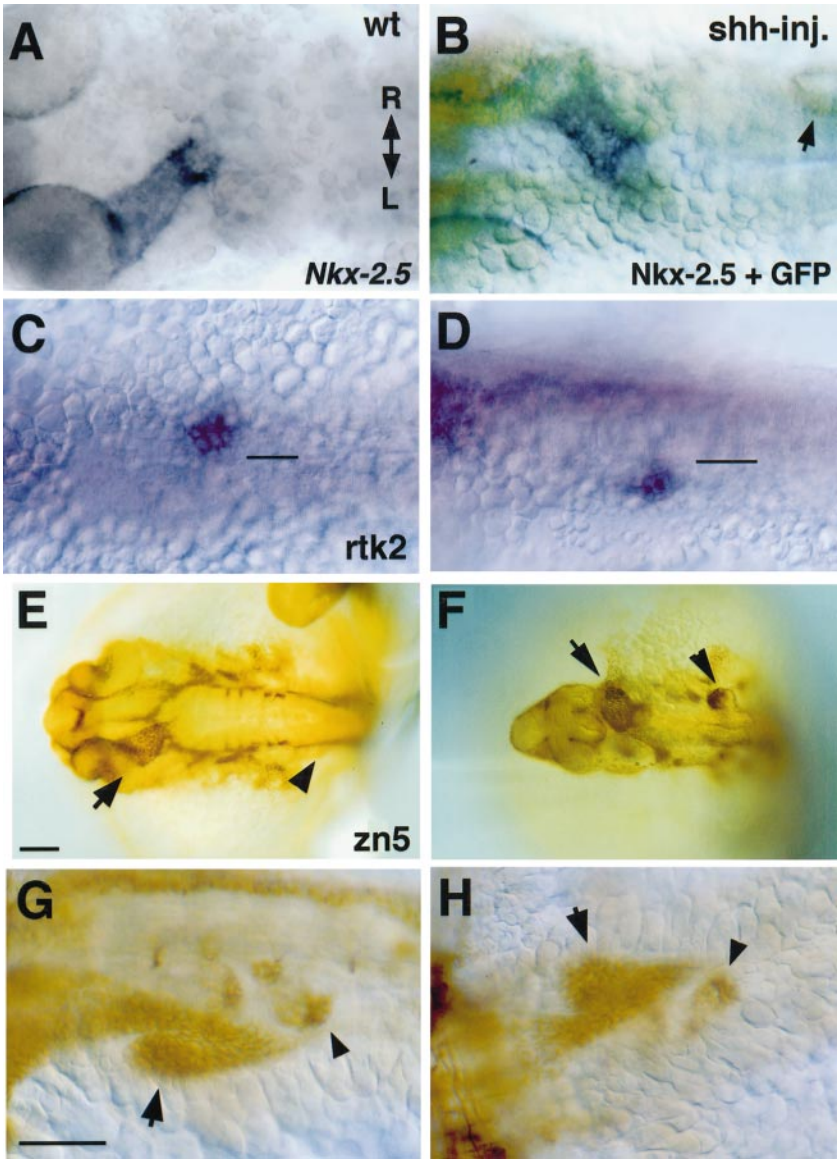


FIG. 3. Ectopic *shh* reverses or disrupts gene expression in cardiac precursors and visceral precursors. Dorsal views of whole-mounted embryos, anterior to the left. (A) Uninjected wild-type control showing *BMP4* expression on the left side of the midline in the heart tube. (B) Embryo injected with *shh* + GFP mRNA that localized to the right side, as determined by staining with anti-GFP (brown), which is easily visible in the right otic capsule (arrow) and right eye. (C) Uninjected control showing distribution of *rtk2* mRNA on the right side of the gut. (D) Reversal in *rtk2* expression following injection of *shh* + GFP on the right side. (E) DM-GRASP in the differentiated heart (arrow) and gut (arrowhead) on the left side. (F) Injected embryo with heart and gut on the right. (G, H) Higher magnification views of reversals in large (arrow) and small (arrowhead) clusters of DM-GRASP expressing cells in the gut. Scale bars, 100 μm.

well as molecular markers of the precardiac mesoderm (Fig. 3). Misexpression of *shh* RNA on the right side induced heart reversals as shown by double labeling for GFP and *Nkx-2.5* mRNA (Fig. 3B), while misexpression on the left, or GFP RNA injected alone on the right, did not (Table 1). Heart reversals in uninjected or sham-injected control embryos were less frequent than reported previously (Chen *et al.*, 1997). In 24% of embryos injected with 50 pg of *shh* mRNA on the right side, a beating heart was visible at 24 h on the right side (Fig. 3B; Table 1). Asymmetric expression of *fli-1* was reversed in a slightly smaller percentage of injected embryos (Table 1).

To determine if *shh* plays a role in the development of visceral asymmetries, we examined *rtk2* and DM-GRASP

TABLE 1
Organ Asymmetries Following mRNA Injection

RNA injected	Amt. (pg)	Left injected, *N* (% heart; % gut reversals)	Right injected, *N* (% heart; % gut reversals)	Bilateral injected, *N* (% heart; % gut reversals)	Right injected % heterotaxia
Uninjected	—	—	—	150 (1;0)	1
GFP alone	50	29 (0;0)	33 (0;0)	25 (0;0)	0
β-gal alone	50	19 (0;0)	28 (0;0)	—	0
shh+GFP	10	32 (2;1)	48 (10;8)	—	8
	50	18 (0;0)	32 (24;15)	25 (12;8)	15
PKAdn*	100	—	—	49 (30;[a])	[a]
BMP4+GFP	10	12 (0;0)	29 (17;0)	—	—
	50	24 (1;0)	21 (19;0)	18 (17;0)	19

[a] Only heart morphology was assayed in embryos injected bilaterally with PKAdn.

expression in the endoderm of *shh*-injected embryos at 40 h (Figs. 3C–3H). Heart reversals in uninjected, control embryos were not always accompanied by corresponding asymmetry shifts in the viscera, demonstrating that there are normal fluctuations in local asymmetry mechanisms (Table 1). In 15% of embryos injected on the right side the viscera developed on the right. The remainder showed heterotaxia; heart and visceral sidedness were not correlated. Though not included in the statistical analysis, it is of interest to note that at 40 h the most severely affected embryos also have a bifurcated gut. These results indicate that, in addition to heart situs, ectopic expression of *shh* disrupts visceral situs and suggest that both molecular cascades determining different organ asymmetries involve *hh* signaling.

In *Drosophila,* protein kinase A (PKA) represses downstream activators of *hh* target genes, and *hh* relieves this repression. Such inhibitory functions of PKA in the *hh* pathway are conserved in vertebrates. Thus, inhibition of cAMP-dependent PKA activation is predicted to mimic overexpression of *shh*. To test this, we misexpressed a dominant negative form of PKA (pkaDN) by injection of mRNA encoding a mutated PKA regulatory subunit. pkaDN was injected at the one–two cell stage and heart symmetry was monitored by Nomarski optics at 24 h. This resulted in a significant increase in heart reversals or bifurcations, compared with uninjected or sham-injected embryos (Table 1). Visceral sidedness was not assayed in these experiments. Together with the *shh* ectopic expression data, this result indicates that downstream components of *hh* signaling are also sufficient to modulate LR asymmetry of the heart.

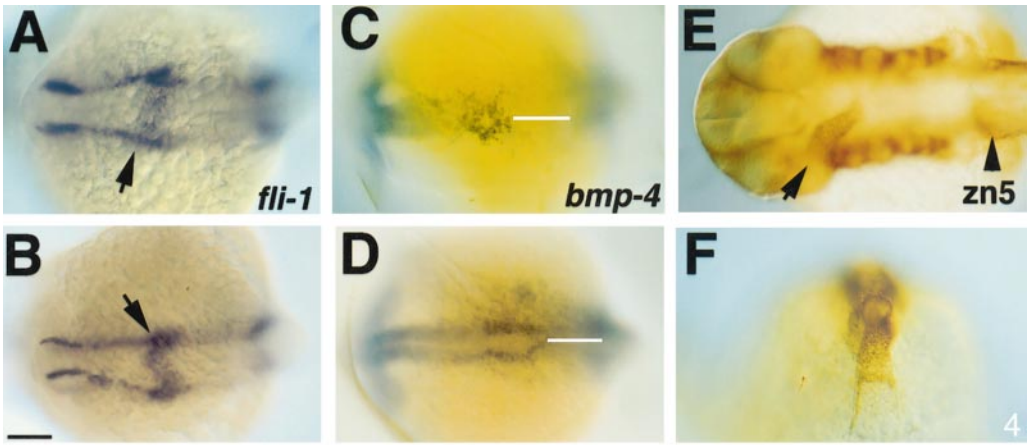
Exogenous BMP4 Induces Reversals in Heart but Not Gut Asymmetry

To test the role of *BMP* signaling in organ asymmetries, we increased the levels of *BMP2* or *BMP4* expression on either the left or right sides of the embryo by RNA injection. Injection on the right side resulted in heart asymmetry defects, including reversals in *fli-1* (Figs. 4A and 4B) and *BMP4* (Figs. 4C and 4D) and subsequent looping (Figs. 4E and 4F), but had no effects on *rtk2* or DM-GRASP expression in the viscera (Table 1). Variability in the effects of *BMP*-induced ventralization may reflect the DV localization of the injected RNA, as well as its concentration or retention. Initially exogenous *BMP* expands the heart field, as determined by expression of *fli-1* or of *BMP4* in *BMP2*-injected embryos. However, this expanded field regulates to form a heart of normal size either on the left or on the right.

Midline Defects in Mutants Correlate with Reversed Heart and Gut Asymmetries

Cardiac LR orientation and *BMP4* expression are randomized in notochord-defective mutants, ntl^{b160} and flh^{n1}, and in floor plate-defective mutants such as *cyclops* (cyc^{b16}), indicating a requirement for midline cells in asymmetry (Danos and Yost, 1996; Chen *et al.,* 1997). All three of these mutants have variably reduced expression of *hh* genes in the midline. To determine if midline cells are required for visceral organ asymmetries, we analyzed *rtk2* and DM-GRASP expression in six midline mutants (Fig. 5; Table 2). There are two classes of organ phenotypes among these mutants, randomized asymmetry and organ bifurcation. Asymmetry is randomized in *flh* and *ntl* in which heart and visceral sidedness do not correlate (Figs. 5C and 5F; Table 2). Likewise, heart and gut sidedness are randomized in homozygous *cyc* mutants (Table 2). At 21 h, *fli-1* and *BMP4* expression patterns in the heart are reversed in *flh* and *ntl* and *cyc* mutant embryos, indicating that organ laterality defects in these mutants result from early specification defects in heart precursors rather than later growth or differentiation.

In $oep^{il,zl}$ mutants the notochord develops but early morphogenesis of more anterior axial mesoderm is disrupted, including the prechordal plate, as well as the endoderm and ventral neuroectoderm (Strahle *et al.,* Schier *et al.,* 1997). In *oep* mutants heart precursors accumulate in a symmetrical central field, as determined by *BMP4* expres-



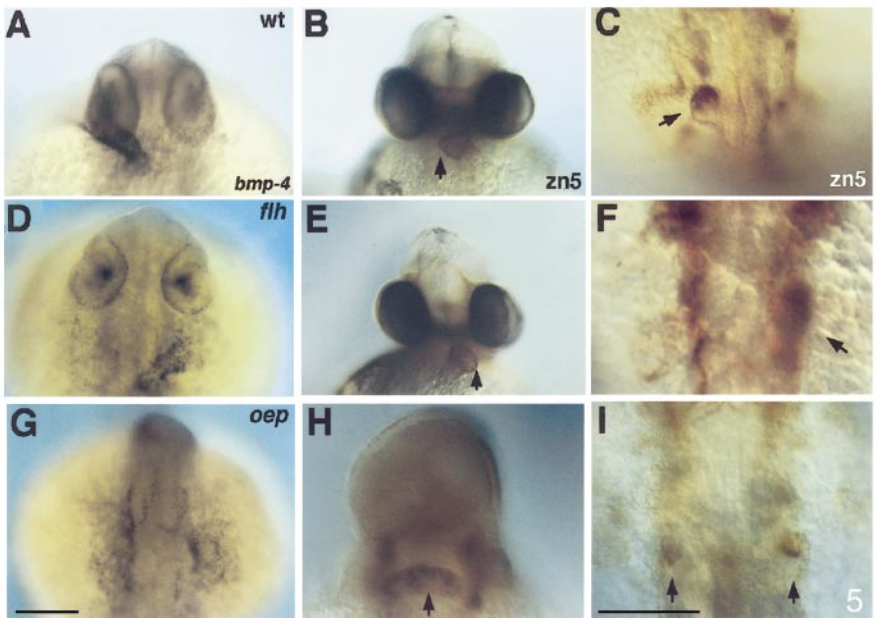


FIG. 4. Ectopic *BMP* signaling reverses LR asymmetry in heart precursors. Dorsal view of whole-mounted embryos, anterior to the left, except F which is an anterior view with dorsal to the top. (A, B) *In situ* hybridization showing assymetric expression of *fli-1* in the putative endocardium (arrow) in uninjected control and reversed in a *BMP4*-injected embryo. (C, D) *BMP4* expression itself is expressed with reversed asymmetry relative to the midline (white line) following *BMP2* injection. (E, F) DM-GRASP expression in an uninjected control (E) and a *BMP4*-injected embryo (F). Scale bar, 100 μm.

FIG. 5. Midline mutants disrupt heart and gut asymmetries. Dorsal views of whole-mounted wild-type (A–C), flh (D–F), and oep (G–I) mutant embryos, anterior to the top, except middle panels which are anterior views with dorsal to the top. (A, D, G) *BMP4* expression in the primitive heart tube. (B, E, H) DM-GRASP expression in the ventricle (arrows). (C, F, I) DM-GRASP expression in the hepatic diverticulum (arrows). Scale bars, 100 μm.

sion at 21 h and DM-GRASP expression at 40 h (Figs. 5G and 5H; Table 2). DM-GRASP-expressing cells of the viscera are reduced in *oep* mutants and invariably form two small clusters on either side of the midline (Fig. 5I). These results show that in the absence of prechordal plate and endoderm in *oep*, both heart and gut precursors fail to fuse in the midline.

We also found a significant percentage of heart and viscera reversals in spt^{b104} mutants (Table 2). In *spt*, cells fail to converge in the paraxial mesoderm, and somites are absent in the trunk region of the embryo (Kimmel *et al.*, 1989; Griffin *et al.*, 1998). The hepatic diverticulum of the gut in *spt* forms on the right side at a significantly higher frequency than in wild type (Table 2). We also looked at asymmetries in a midline mutant called *you* (you^{ty97}) that develops somite defects, such as *flh* and *ntl*, and a curved

TABLE 2
LR Organ Asymmetries in the Cardiac Laterality Mutants

Mutant	Genotype	*N*	% Wildtype	% Situs inversus	% Heterotaxia
Wild type	+/mutant or +/+	218	99	1[a]	1
notail	*ntl/ntl*	76	20	50	30
floating head	*flh/flh*	100	50	45	5
cyclops	*cyc/cyc*	60	76	21	3
spadetail	*spt/spt*	120	87	8	5
one-eyed pinhead	*oep/oep*	48	100	0	0
you	*you/you*	36	100	0	0

[a] The wild-type siblings of homozygous recessive mutants included both heterozygotes and homozygous wild type. The rate of organ reversal was similar in the siblings of all of the different mutations.

axis, such as *cyc*, but does not show cardiac laterality defects (vanEeden *et al.*, 1996; Chen *et al.*, 1997). In this mutant, the number of reversals of LR asymmetry in the viscera does not differ significantly from wild type (Table 2).

DISCUSSION

Shh Expression Biases Cardiac and Visceral Situs

We provide evidence that *hh* signaling in the zebrafish regulates LR asymmetries of the heart and viscera and that this role is conserved among the vertebrates. Thus, *hh* can be included among the few mechanisms currently known to be conserved in determining visceral situs, in addition to *nodal*-related (Collignon *et al.*, 1996; Lowe *et al.*, 1996; Rebagliati *et al.*, 1998) and *Pitx2* (Ryan *et al.*, 1998). Misexpression of *shh* on the right side disrupts organ asymmetries, and this effect is mimicked by inhibition of PKA, which represses downstream targets of *hh* signaling. Organ reversals only result from misexpression on the right side, consistent with a normal role in biasing heart or visceral sidedness to the left (Levin *et al.*, 1995). No asymmetries have been reported in expression of any of the four known *hh* genes in zebrafish (*shh, twhh, ehh, hhc*), though a subtle or transient asymmetry may not have been detected (Krauss *et al.*, 1993). Furthermore, a mutation in *shh* called *sonic you* has no laterality defects (Schuaerte *et al.*, Chen *et al.*, 1997). *Shh* itself is not asymmetrically expressed in the mouse node and mice carrying a deletion of *Shh* have no clear laterality defects (Chiang *et al.*, 1996). Thus, in some vertebrates another *hh* may serve the key role (Collignon *et al.*, 1996).

Following bilateral expression of *shh*, asymmetric expression of *fli-1* and *BMP4* in the heart, as well as *rtk2* in the gut, resolves randomly to one side or the other, suggesting that symmetry is determined by a stochastic process and that subsequent signals inevitably follow. Our results are consistent with *shh* acting early in the molecular cascade on the left side to initiate or maintain asymmetric *fli-1* and *BMP4* in the heart field, while it may repress *rtk2* on the left in the gut (Levin *et al.*, 1995). At least some of these effects may occur through a *nodal*-related intermediate. Misexpression of *Xshh* alters the asymmetric expression of *Xnr-1* which is normally expressed in the left lateral plate mesoderm, and this reverses laterality of both heart and viscera, in most cases (Sampath *et al.*, 1997). Thus, *nodal*-related could form the link between asymmetric signals in the gastrula and the subsequent asymmetric specification of internal organs.

BMP4 Regulates Heart but Not Visceral Asymmetry

BMPs have a signaling role in the heart field. Ectopic *BMP4* reverses or disrupts heart looping in zebrafish, but we have shown there are no defects in visceral situs. Furthermore, we found that heart reversals only occur when *BMP4* is misexpressed on the right side, consistent with the hypothesis that normally higher levels on the left side promote heart looping to the left (Chen *et al.*, 1997). Misexpression of *BMP4* also reverses the early asymmetry in *fli-1* expression, suggesting that *BMP4* normally maintains *fli-1* asymmetry. There are at least two alternative ways that *BMPs* might exert their control on the heart, either early in the formation of ventral mesoderm during gastrulation (Danos and Yost, 1995) or later during cardiac myogenesis (Schultheiss *et al.*, 1997). Both *BMP2* and *BMP4* ventralize embryos when ectopically expressed on the dorsal side (Nikaido *et al.*, 1997; Neave *et al.*, 1996), and cardiac LR asymmetry is typically randomized in such embryos (Danos and Yost, 1995; Chen *et al.*, 1997). In *Xenopus*, midline cells of the pharyngeal endoderm initially promote heart tube formation and myogenesis through both *BMP2* and *BMP4* (Nascone and Mercola, 1995; Schultheiss *et al.*, 1997). However, because of the one sidedness and organ specificity of its effects, our results suggest a polarizing function of *BMP* signaling in the heart field asymmetry itself.

Genetic Pathways Underlying LR Asymmetry

Zebrafish mutants that have defects in heart asymmetry belong to a class that affects the midline, and we have shown that these mutants develop asymmetry defects in other organs. Two transcription factors required in the notochord, *flh* and *ntl,* are also required for asymmetry of the viscera, and the loss of midline tissues disrupts organ asymmetries independently (Danos and Yost, 1995). Dorsoanterior mesoderm, particularly the notochord, has been implicated in LR heart asymmetry either as a source of an asymmetric signal or as a barrier to signals that are expressed on one side or the other. From our results, it seems unlikely that the notochord forms a barrier to an asymmetric signal, since heart and gut asymmetries are disrupted in *cyc* and *spt,* which have normal notochords. Furthermore, asymmetry changes in mutants are not simply defective due to body shape, such as curvature in *cyc* or *oep,* since *you* mutants with similar curvature form a normal pattern of organs. The organizer interacts with dorsolateral mesoderm to allow it to develop heart-forming potential and may also specify its orientation (Sater and Jacobson, 1990; Nascone and Mercola, 1997). *ntl* and *flh* mutants are required in the axial mesoderm (Halpern *et al.,* 1993; Talbot *et al.,* 1995). *spt,* a transcription factor of the Tbox family, is required in paraxial mesodermal cells that form somites (Kimmel *et al.,* 1989; Griffin *et al.,* 1998). *cyc* is required in the floor plate of the central nervous system (Hatta *et al.,* 1991). Thus, as for the heart, genes required for several different aspects of dorsoanterior development are necessary for appropriate LR asymmetries of visceral organs.

Coordinating Different Organ Asymmetries

Genetic studies in the mouse have indicated that there are mechanisms that globally specify the LR axis of the body as well as more local controls of individual organ asymmetries (Hummel and Chapman, 1959; Layton, 1976; Yokoyama *et al.,* 1993; Supp *et al.,* 1997). Because different organs are affected independently following *shh* or PKAdn injection, our results place *hh* signaling as a global signal interpreted independently by different organs. A similar independence of organ sidedness in response to *shh* also occurs in chick embryos (Levin *et al.,* 1997). That the same signaling pathway is involved may reflect the fact that *shh* is expressed throughout the length of the body in the axial mesoderm, floor plate, and foregut and in close proximity to the heart and all of the visceral organs. The results in zebrafish have identified potential organ-specific signals such as *BMP4* in the heart and *rtk2* in the gut.

A clear distinction has been made in *Xenopus* between the generation of the heart field, which requires a normal distribution of proteoglycans and actin, and the determination of its LR orientation (Yost, 1992). Our mutational analysis shows that these are not easily distinguished genetically, since formation of a field of precursors and the shift of those precursors into an LR asymmetric position can be affected in the same mutants or following *shh* or *BMP* injection. Thus, the same signals may play a dual role, both in organ development and in laterality, and these two possibilities could eventually be dissected using genetic analysis in zebrafish.

Evolutionary Conservation of LR Patterning Mechanisms

Despite considerable differences in organ morphologies among vertebrates, there are striking molecular similarities in the development of LR asymmetry between zebrafish and tetrapods. The simple heart tube of the zebrafish, with its single atrium and ventricle, shifts to one side in a manner reminiscent of heart looping in the four-chambered heart of the chick and this shift responds to at least one of the same molecules (Goldstein *et al.,* 1998). We suspect that the similarities between cellular responses in different species are not coincidental, but reflect a similar underlying mechanism of LR asymmetry. We found that in zebrafish embryos *shh* misexpression alters the first asymmetric cell specification events in the future organs and randomizes heart and visceral asymmetries independently, as it does in the chick (Levin *et al.,* 1997). Our mutant analyses show that LR asymmetry of internal organs in zebrafish requires the axial mesoderm, as in *Xenopus* (Danos and Yost, 1995). Expression of at least one component of the known asymmetry pathway, *nodal*-related, is also asymmetrical in the fish (Rebagliati *et al.,* 1998). Apparent differences between the molecular control of LR patterning in zebrafish and the chick may be resolved with further studies of the developmental mechanisms controlling asymmetry.

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