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Left-right pattern of cardiac *BMP4* may drive asymmetry of the heart in zebrafish

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SUMMARY

The first evident break in left-right symmetry of the primitive zebrafish heart tube is the shift in pattern of *BMP4* expression from radially symmetric to left-predominant. The midline heart tube then 'jogs' to the left and subsequently loops to the right. We examined 279 mutations, affecting more than 200 genes, and found 21 mutations that perturb this process. Some cause *BMP4* to remain radially symmetric. Others randomize the asymmetric *BMP4* pattern. Retention of *BMP4* symmetry is associated with failure to jog: right-predominance of the *BMP4* pattern is associated with reversal of the direction of jogging and looping. Raising BMP4 diffusely throughout the heart, via *sonic hedgehog* injection, or the blocking of its action by injection of a dominant negative BMP4 receptor, prevent directional jogging or looping. The genes crucial to directing cardiac asymmetry include a subset of those needed for patterning the dorsoventral axis and for notochord and ventral spinal cord development. Thus, the pattern of cardiac *BMP4* appears to be in the pathway by which the heart interprets lateralizing signals from the midline.

Key words: laterality, looping, genetic screen, *BMP4*, heart, cardiac development, symmetry, zebrafish

INTRODUCTION

Most vertebrate organs are asymmetric along the left-right axis. During development, this asymmetry is evident first in the heart. Most obvious is the bend of the midline primitive heart tube to the right, which, in air breathing animals, begins the separation of right from left chambers. This process, termed cardiac looping, is conserved in all vertebrates (Brown and Wolpert, 1990) and its perturbation believed related to a variety of congenital structural abnormalities (Hagler and O'Leary, 1989). Looping has been used traditionally as the standard assay for left-right asymmetry of the heart.

The mechanism of the original break in left-right symmetry is not known, but appears to depend in an important way upon proper establishment of the dorsoventral axis (Danos and Yost, 1995; Danos and Yost, 1996) and is reflected in asymmetric patterns of gene expression from the earliest stages, including *sonic hedgehog* (*shh*), *activin receptor IIa* (*ActRIIa*), *cNR*, *Xnr*, *cSnR*, *lefty*, and *flectin* (Levin et al., 1995; Hyatt et al., 1996; Isaac et al., 1997; Meno et al., 1996; Tsuda et al., 1996). Ectopic expression of *shh*, *activin* and *vg1*, 'randomize' cardiac looping and, therefore, are believed to be in the pathway influencing cardiac laterality (Levin et al., 1995; Hyatt et al., 1996). Since these genes are not expressed in the heart or in its progenitor field, the question remains open how the heart interprets lateralizing embryonic signals during generation of asymmetric form.

In principle, there are at least two different ways of perturbing the left-right asymmetry of the heart: loss of asymmetry, by removal or homogenization of the asymmetric information (either the signals or their reception), or inversion of the signals or the responses. The former would lead to randomization of heart asymmetry, as occurs in the *situs inversus viscerum* (*iv*) mutation in the mouse (Layton, 1976) and in all reported embryonic manipulations that affect cardiac asymmetry (Danos and Yost, 1995; Danos and Yost, 1996; Hyatt et al., 1996; Levin et al., 1995). Inversion of signals would be predicted to reverse looping and has been reported only in one situation, the *inversion of embryonic turning* (*inv*) mutation in the mouse (Yokoyama et al., 1993). The asymmetric expression pattern of one embryonic marker, *lefty*, is randomized in *iv* and reversed in *inv* homozygous mice (Meno et al., 1996). The *inv* and *iv* genes have not been cloned.

The systematic isolation and analysis of mutants in *Drosophila* and other invertebrates has led to the identification of molecular mechanisms underlying pattern formation during development. Most features of global vertebrate heart form, including asymmetry, appear to have arisen as vertebrates

branched from lower chordates during evolution (Fishman and Chien, 1997), so require analysis in vertebrates. Hence, we have turned for genetic analysis of heart development to the zebrafish (Fishman and Stainier, 1994; Chen et al., 1996; Stainier et al., 1996). The transparency and accessibility of the early zebrafish embryo are particularly useful for the study of early development of heart form and function (Chen et al., 1996; Stainier et al., 1996). We find a predictable progression of asymmetric development of the heart, in which a radially symmetric *BMP4* pattern converts to one which is a left-predominant, a change that precedes morphological evidence of asymmetry. We screened a collection of 279 zebrafish mutations for mutations that affect left-right symmetry of the heart. Mutations in 21 genes disrupt the normal break in morphological symmetry of the heart tube. In all of these, the pattern of *BMP4* in the heart is perturbed in a manner that suggests that *BMP4* drives the direction of break in symmetry.

MATERIALS AND METHODS

Zebrafish maintenance and embryo analysis

All zebrafish mutants used in this study were from the large-scale Tübingen screen (Haffter et al., 1996), except for the cardiovascular mutants, *cloche*, *bonnie and clyde*, *heart of glass*, *pandora* and *valentine*, which were isolated from the Boston screen (Stainier et al., 1996). Cardiac jogging was analyzed at 24 hours postfertilization (hpf), and designated as left (normal), midline or right, using the neural tube as a midline guide. Hearts remaining within the border of the neural tube are considered as midline (no jog). Embryos were then separated and scored between 36 and 48 hpf for cardiac looping. If the ventricle is on the right of the atrium, it is a right-loop (R loop), if the ventricle is on the left of the atrium, it is considered as a left-loop (L loop). If the ventricle fails to bend, it is considered as no-loop or straight. Rarely (4 out of 630 embryos, 0.6%), the heart itself swings back over the midline, after the jog, but this position does not affect looping and is not part of the quantitation. The incidence of abnormal cardiac laterality in the mutant embryos of each mutation was compared to that in the wild-type siblings. In most cases, multiple crosses of each mutation were scored for cardiac laterality. During the screen for morphological asymmetry, at least 60 embryos per clutch were scored in order to assess a minimum of 10-15 mutant embryos. For each affected mutation, at least 5 separate crosses were examined.

For *BMP4* expression analysis, embryos were fixed at the 22-somite stage. Mutant embryos of *curly up*, *heart of glass*^{m552}, *locke*^{to237b}, *momo*^{th211}, *pandora*^{m313}, *santa*^{ty219c}, *schmalhans*^{tn222a}, *scotch tape*^{te382}, *valentine*^{m201}, *tg238a*, *tj2a*, *tm243b*, *tm317b*, *tn20b* and *tw29b* cannot be distinguished from the wild-type siblings prior to 22-somite stage. Mutant and wild-type embryos of *cyclops*^{b16}, *cyclops*^{f219}, *dino*^{tm84}, *doc*^{tt202}, *floating head*^{n1}, *iguana*^{ts294e}, *notail*^{tc41}, *schmalspur*^{ty68b}, *silberblick*^{tz216}, *sleepy*^{ti263a}, *snailhouse*^{ty68a}, *sonic-you* and *spadetail*^{tm41} can be distinguished prior to 22-somite stage, and so were separated and independently scored for cardiac expression of *BMP4*.

Whole-mount in situ hybridization and immunostaining

Nkx2.5 and *BMP4* antisense RNA were used as probes for whole-mount in situ hybridization as described previously (Chen and Fishman, 1996). Anti-myosin heavy chain antibody, MF20, was used to visualize the heart at 48 hour postfertilization (Stainier and Fishman, 1992; Chen and Fishman, 1996).

shh and XBMP4tr injection

The RNA injection procedure was performed as described previously (Chen and Fishman, 1996). In brief, *shh*, XBMP4tr and *β-gal* mRNA were synthesized in vitro and mixed with phenol red in KCl with final concentration as 100 ng/μl for *β-gal*, 60 ng/μl or 30 ng/μl for *shh*, and 100 ng/μl, 50 ng/μl or 25 ng/μl for XBMP4tR. Approximately 1 nl of the RNA/phenol red mixture was injected to zebrafish embryo at 1- to 2-cell stage. These embryos were raised at 28.5°C and fixed at the 22-somite stage for cardiac *BMP4* expression or at 24 hours post-fertilization for cardiac jogging. The *shh* expression vector and XBMP4tR vector are kind gifts of Dr M. Hammerschmidt and Dr D. Melton, respectively.

RESULTS

Leftward 'jogging' precedes rightward 'looping'

In the zebrafish, cardiac precursors migrate through the lateral plate and form bilateral heart tubes, flanking the posterior prechordal plate and anterior notochord (Fig. 1A). The bilateral heart tubes fuse at the midline, anterior to the notochord, at about 19 hpf (the 20-somite stage) (Fig. 1B). This midline primitive heart tube first forms a short cone-shape structure, which subsequently elongates in the anteroposterior direction (Stainier and Fishman, 1992) before looping to the right at 36 hpf. This cardiac looping is the first organotypic left-right asymmetry reported in vertebrates. However, it occurs relatively late, well after the tube is formed and after the circulation starts, and may be dependent upon cellular differentiation (Taber et al., 1995). In addition, looping is not stopped, but rather is 'randomized' in all reported manipulations that perturb embryonic left-right asymmetry. Randomization is a difficult assay to follow in genetic screens for recessive defects, which affect at most 25% of the offspring so that only 12.5% of the offspring

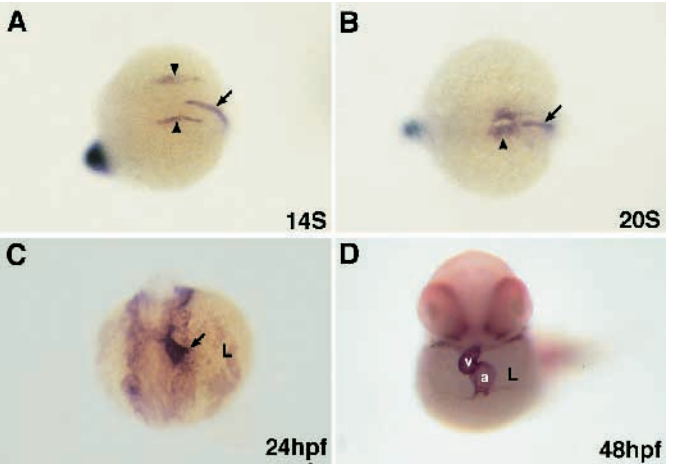

Fig. 1. Cardiac morphogenesis in zebrafish. The cardiac precursors are in the ventral margin at the onset of gastrulation in zebrafish. (A) These ventral-derived cardiac precursors migrate toward the animal axis and form bilateral heart primordia on either side of the embryo (arrowhead), flanking the prechordal plate and notochord (arrow) at the 14-somite stage. (B) The bilateral heart tubes then fuse at the midline (arrowhead), anterior to the notochord (arrow). (C) At 24 hours post fertilization (hpf), the primitive heart 'jogs' to the left (arrow points to the prospective atrium). The heart then gradually returns to the midline. (D) At 48 hpf, the ventricle of the midline heart loops to the right. The bilateral heart tubes and primitive heart are labeled with *Nkx2.5* in A, B and C, and MF20 in D. Notochord precursors are labeled with *Brachyury* in A and B. (A,B) Dorsal-lateral view, anterior to the left. (C) Dorsal view, anterior to the bottom of the panel. (D) A ventral view. a, atrium; v, ventricle; L, embryo left.

from each heterozygous cross might have abnormal looping. This is close to background levels in zebrafish (see below). Hence, we were concerned that cardiac looping might be an insensitive guide to the heart's break in symmetry.

We find in the zebrafish that there is morphological left-right asymmetry of the heart prior to looping. At 24 hpf, just as the heart tube forms, the prospective atrial end of the heart abruptly moves to the left (Fig. 1C). We term this process cardiac jogging. The heart tube subsequently returns to the midline, and then loops by 36 hpf, with rightward bending of the ventricle (Fig. 1D). Therefore, in zebrafish, the leftward movement of the heart (jogging) precedes the rightward bending of the ventricle (looping).

The direction of jogging predicts the direction of looping (Figs 1,2). Normally, jogging to the left is followed by looping to the right (Fig. 1C,D). However, in 2-10% of the individual embryos in wild-type strains (AB, Tü, TüAB or TL), the heart jogs to the right. As shown in Fig. 2A, this rightward jogging is always followed by leftward looping. This linkage is true in mutant embryos as well (see below).

BMP4 is asymmetrically expressed in the zebrafish heart

We sought a molecular marker of left-right asymmetry in the early heart tube. *BMP4* is a vertebrate homolog of *Drosophila dpp*. It is expressed in zebrafish heart primordia from the bilateral heart tube stage and its expression persists in the heart up to 3 days of development (Chin et al., 1997). *BMP4* expression is uniform in the developing heart at the time of fusion (20-somite stage) (Chin et al., 1997). However, at the 22-somite stage, just prior to jogging, the pattern of *BMP4* expression becomes markedly asymmetric, with far more on the left than on the right side of the heart tube (Fig. 3A,C). This left-predominant asymmetry persists through the stages of jogging (Fig. 4A,C). Other genes, such as *Nkx2.5* and *MEF2*, are expressed symmetrically in the heart throughout these stages (Fig. 3B) (Chen and Fishman, 1996; Ticho et al., 1996). The sidedness of *BMP4* expression in the heart is linked to the direction of jogging and looping. In all cases examined in which there is reversed jogging and looping (i.e. the 2-10% of background, and see below), *BMP4* is on the right side of the heart tube (Fig. 4B,D).

BMP4 is involved in the asymmetric signaling pathway

If the asymmetric pattern of cardiac *BMP4* regulates asymmetric morphogenesis, disturbance of the pattern of *BMP4* expression, or its signaling, should interfere with jogging and looping of the heart. *shh* has been shown to be upstream of *dpp* and *BMP4* in several systems (Hammerschmidt et al., 1997). Rendering *shh* symmetric around the chick node randomizes looping in the chick (Levin et al., 1995). We have found that zebrafish embryos injected with *shh* mRNA at the 1- to 2-cell stage have increased *BMP4* expression in normal expression sites (data not shown). *shh* injection causes more embryos to

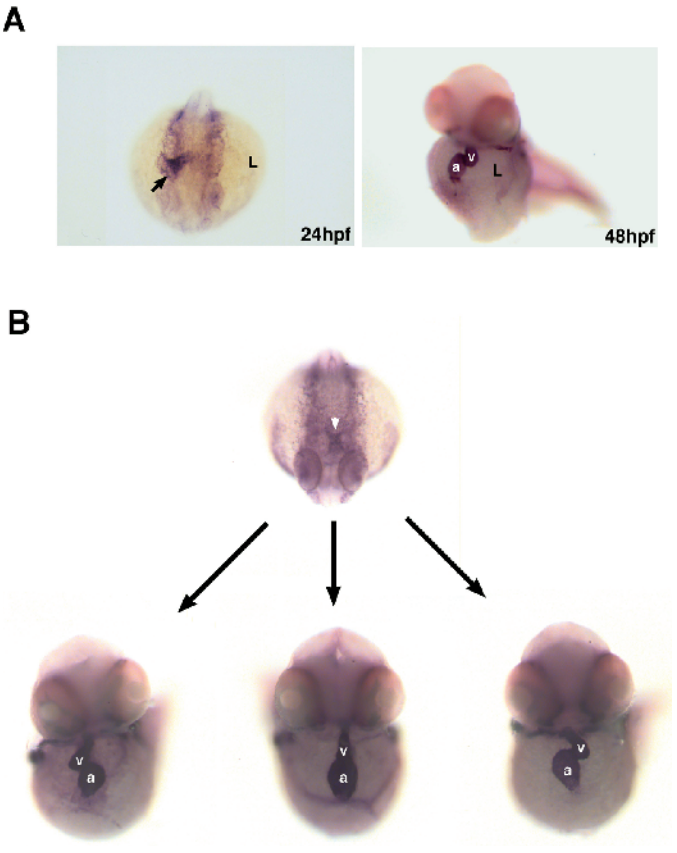

Fig. 2. Abnormal jogging and looping. (A) Jog reversal. Hearts that jog to the right (left panel, arrow points to the prospective atrium) always loop to the left (right panel). (B) Directionless jog. In *no tail* mutant embryos, the hearts fail to jog (arrowhead points to the midline) and may loop to left, right or remain straight. The heart is labeled with *Nkx2.5* in 24hpf embryos and MF20 in 48hpf embryos. a, atrium; v, ventricle; L, embryo left.

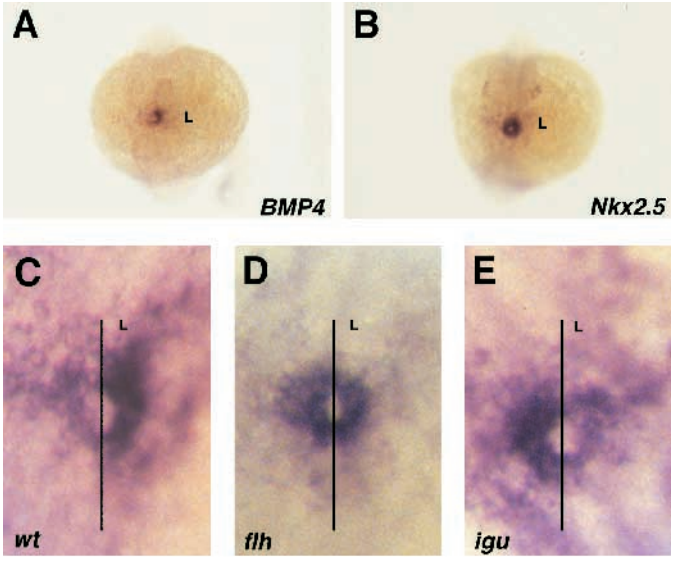

Fig. 3. Cardiac *BMP4* asymmetry is disturbed in laterality mutants. (A,C) Normally, *BMP4* transcripts accumulate predominantly on the left side of the heart tube at the 22-somite stage. (B) At the same stage, *Nkx2.5* expression in the heart is symmetric. In those mutant embryos in which the heart fails to jog, such as *floating head* (*flh*), *BMP4* is more evenly distributed in the heart (D). Mutants with a higher incidence of right-jogged heart, such as *iguana* (*igu*), have a higher incidence of right-dominant cardiac *BMP4* (E). All are dorsal view, anterior to the bottom of the panel. Lines mark the midline. L, embryo left.

Table 1. Injection of *shh* or XBMP4tR disrupt normal cardiac jogging

	No. of embryos	Left-predominant *BMP4*	Symmetric *BMP4*	Right-predominant *BMP4*	No. of embryos	Left jog	No jog	Right jog
Uninjected	40	94%	4%	2%	214	92%	4%	4%
β-gal					167	96%	0%	4%
30 pg *shh*	40	62%	30%	8%	72	68%	13%	19%
60 pg *shh*	45	40%	49%	11%	106	56%	24%	20%
25 pg XBMP4tR					50	94%	0%	6%
50 pg XBMP4tR					64	81%	13%	6%
100 pg XBMP4tR	40	92%	8%		109	67%	23%	10%

exhibit uniform *BMP4* expression (Table 1) and, concomitantly, increases the number without jog. In the heart tube, *BMP4* transcripts are at high levels all around the heart tube after *shh* injection (Fig. 5A; Table 1). Although the injected embryos appear grossly normal, there is a dose-dependent incidence of abnormal jogging and looping (Table 1).

In theory, BMP4 could be involved in one of several redundant pathways that regulate cardiac asymmetry. To examine this, we diminished BMP4 signalling by injection of RNA for the dominant negative *Xenopus* BMP4 truncated receptor (XBMP4tR) (Graff et al., 1994), which interferes with signalling by BMP4 and related molecules of the TGF-β family (Graff et al., 1994). The native *BMP4* expression pattern in the XBMP4tR-injected embryos is not disturbed (Fig. 5B) and, at this level of injection, the rest of the embryo is grossly normal. However, there is a high and dose-dependent incidence of abnormal jogging and looping (Table 1). Control embryos injected with *β-gal* have normal heart asymmetry (Table 1). This suggests that, if other pathways exist, they play a relatively minor role compared to those that work through *BMP4* or related molecules.

Screen for mutations affecting left-right asymmetry of the heart

We examined cardiac left-right symmetry in 279 mutant lines, affecting more than 200 genes identified in the Tübingen screen (Haffter et al., 1996). Of these, 215 are in 201 complementation groups. Complementation has not yet been resolved for the other 64 mutations. These mutations can be classified into 20 phenotypic groups, as shown in Table 2. This classification is by visible phenotype (Haffter et al., 1996) and does not imply a unity of mechanism within each class. Mutations in 21 of these genes have cardiac asymmetry defects (Table 2). All perturb both jogging and looping. Interestingly, mutants with abnormal cardiac left-right asymmetry fall into only four phenotypic groups: those classified as affecting gastrulation, notochord or ventral spinal cord, or causing a curly tail.

All gastrulation mutants that develop long enough to be assayed for heart morphogenesis perturb jogging and looping (Table 2). *piggytail* (*pgy*), *lost-a-fin* (*laf*) and *snailhouse* (*snh*) are mutants with expansion of dorsolateral structures (Mullins et al., 1996). *dino* (*din*) is ventralized, with tail enlarged at the expense of dorsoanterior structures (Hammerschmidt et al., 1996b). The most obvious phenotype of *spadetail* (*spt*) is the abnormal convergence of muscle precursors, which end up as a bulge in the tail, but *spt* also causes a morphologically abnormal notochord and floor plate (Kimmel et al., 1989) (Table 2).

The asymmetry mutations that affect the notochord or ventral spinal cord do not evidence gross ventralization or dorsalization. The mutant embryos of *momo* (*mom*) and *floating head* (*flh*) lack notochord and floor plate in the trunk, although prechordal plate and anterior floor plate appear to be normal (Halpern et al., 1995; Odenthal et al., 1996a; Talbot et al., 1995). *no tail* (*ntl*) mutant embryos do not have a differentiated notochord, but do appear to have notochord precursors and to develop a floor plate (Halpern et al., 1993; Odenthal et al., 1996a). *ntl* and *flh* mutant embryos have been noted previously to have abnormal looping (Danos and Yost, 1996). Mutant embryos of *cyclops* (*cyc*), *schmalspur* (*sur*), *schmalhans* (*smh*) and *iguana* (*igu*) have ventral spinal cord defects in the presence of apparently normal notochord (Brand et al., 1996b; Hatta et al., 1991, 1994). Although these mutants appear to implicate the ventral spinal cord as a principle modulator of cardiac asymmetry, ventral spinal cord is known to be dependent upon notochord for normal differentiation, so it is equally plausible that *cyc*, *sur*, *smh* and *igu* have currently undefined molecular defects in the notochord. In addition, *monorail* (*mol*) and *detour* (*dtr*) have ventral spinal cord defects (Brand et al., 1996b) but have normal cardiac left-right asymmetry (Table 2).

Some mutants with abnormal cardiac left-right asymmetry fall into the ‘curly tail’ group (Table 2), which display a curved body shape but have no obvious defects in notochord, ventral spinal cord or prechordal plate (Brand et al., 1996b). It is nevertheless notable that a similar curly tail phenotype is characteristic of all mutants with ventral spinal cord defects, suggesting that more subtle abnormalities in midline structures could be present.

It appears that the asymmetry defect is dependent on the strength of the allele. For example, cyc^{b16} is stronger than cyc^{tf219} (Brand et al., 1996b). 90% of the cyc^{b16} embryos, and only 20% of the cyc^{tf219} have abnormal jogging (Table 4).

Among the mutant embryos, there are two abnormal patterns of jogging. In one, there is a reversed jog (i.e. to the right). This resembles a pattern sometimes noted in the background and is consistently associated with reversed looping (i.e. to the left). Some mutants, however, have a high rate of ‘no jogging’. In these mutants, there is no predictable direction to subsequent looping, as shown in Table 3 for three mutants (*flh*, *ntl* and *cyc*). In these mutants, there are rare instances of right or leftward jogging, followed predictably by left or rightward looping, respectively. In the vast majority of mutant embryos, there is no jog, followed by an apparently randomized direction of looping.

Mutants of other groups, some of which may be grossly deformed with pronounced defects in somites (van Eeden et al., 1996a), brain (Brand et al., 1996a; Furutani-Seiki et al.,

Table 2. The cardiac laterality screen

Phenotypic group	Subgroup	Laterality affected	Laterality unaffected (or #unscorable)
Gastrulation and tail formation		*dino, lost-a-fin, piggytail, spadetail, snailhouse*	*banshee#, biber#, ghoul#, harpy#, kasper#, kugelig, mercedes, nirvana, ogre#, pipe tail, speed bump#, spector, trilobite, troll#, zombie#*
Mesoderm	Notochord	*floating head, momo, no tail*	*bashful, crash test dummy, doc, dopey, grumpy, happy, kinks, korken, lucky, quasimodo, sleepy, sneezy, wavy tail, zickzack, tc265b, tn21, tv214a*
	somite		*after eight, beamter, chameleon, choker, deadly seven, fused somites, sonic-you, you, you-too, u-boot*
CNS	Forebrain and prechordal plate		*dirty nose, knollnase, masterblind, silberblick*
	Midbrain-hindbrain		*acerebellar*, no isthmus*
	Hindbrain		*eisspalte, fullbrain, parachute, natter#, viper, white tail**
	Brain degeneration		*ta53b, tc1, tc234e, tc294a, tc31, tg279, tj20c, tm42f, tu13, ty19a, tf216c, tj250b*
	Floor plate	*cyclops, iguana, schmalspur, schmalhans*	*detour, monorail, one-eye-pinhead#*
Body shape	Curly tail	*curly up, locke, tg238a, tj2a, tm243b, tm317b, tn20b, tw29b*	*cosinus, pirueta, saltarin, schnitter, sense, sickle, sinus, vicious cycle, wirbel, tg292c, th242d, th269, tl55, tp49d, tt209a, tw17b, tz288*
Organs	Blood		*chardonnay, frascati, moonshine, retsina, riesling, sauternes, thunderbird, weißherbst, yquem*
	Heart morphology		*cloche*, bonnie and clyde#, heart of glass*, miles apart#, overlooped, santa*, scotch tape, superglue, valentine**
	Heart beat		*breakdance*, hiphop*, polka*, pipe heart*, silent heart*, slowmo, still heart*, stretched*, tango*, tremblor*, weak atrium*, weak beat**
	Liver		*tippelbruder, tramp*
	Eye		*bumper, helderziend, leprechaun, sunrise, tj266c, tq262a, tu235b, ty118a, tz284*
	Ear		*ear ache, einstein, half stoned, headphones, hypersensitive, keinstein, little ears, microtic, stein und bein, what's up?*
	Fin and skin		*blasen, boxer, dackel, dandruff, fransen, frayed, frilly fins, ikarus, krom, microwaved, mini fin, nagel, rafels, tutu*
Pigment cells			*blanched, bleached, bleich, blurred, brassy, choco, colourless, cookie, edison, esrom, fading vision, fade out, ivory, kefir, matt, melancholic, obscure, pech, pepita, pistachio, shady, sahne, sparse, sparse-like, submarine, tartar,tinte, touch-down, weiss, yobo, zwart, te374b, tg306, tj266c, to253, tq262a, tx216, tu235b, tw212c, tz284, tz298*
Jaw and gills			*flathead, hammerhead, geist, lockjaw, schmerle, screamer, sucker, tc4, tg18, th9, tm42d, to274, tu259, tx224, ty22e, ty118a*
Motility			*accordion, backflip, bandoneon, diwanka, expander, fusili, herzschlag, macho, quetschkommode, sapje, sloth, slop, slow motion, softy, runzel, turtle, steifftier, unplugged, slinky, space cadet, spaced out, sputnik, techno trousers, twitch once, twitch twice, wavy, ziehharmonika, tb204, tc326c, tg248c, tm90d, tm271b, tm276d, ts299a, tu205, tz272*
Retinotectal	Pathfinding		*belladonna, blowout, umleitung*

#Mutations in which the heart fails to form or the development is arrested before the formation of the heart and so are unscorable.
*Mutations with normal cardiac jogging but with abnormal looping.

1996; Heisenberg et al., 1996; Jiang et al., 1996), jaw (Piotrowski et al., 1996; Schilling et al., 1996) or other organ systems (Chen et al., 1996; Granato et al., 1996; Hammerschmidt et al., 1996a; Kane et al., 1996a,b, Kelsh et al., 1996; Odenthal et al., 1996b; Ransom et al., 1996; Trowe et al., 1996; van Eeden et al., 1996b; Whitfield et al., 1996), all develop normal cardiac asymmetry (Table 2). Most interestingly, jogging is normal in cardiovascular mutants, including those that primarily disrupt the heart's form and those that perturb its function. This indicates that the reception of the asymmetric signals and their morphogenetic transformation are normal in these mutants (also see below for similar observation with regard to *BMP4*). Later placement of the heart, after jogging, when it normally swings back to the midline, may be abnormal

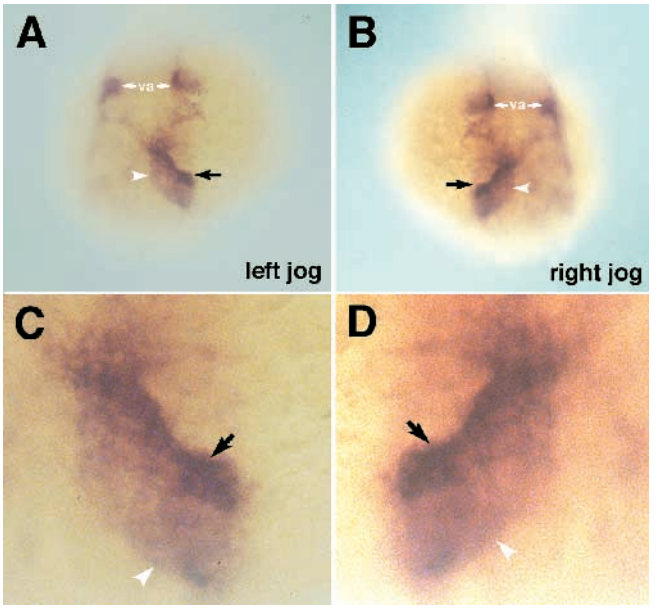

Fig. 4. *BMP4* asymmetry persists through the jogging phase. (A) Left-predominant *BMP4* and left jog. (B) Right-predominant *BMP4* and right jog. C is enlargement of the heart region of A and D is the enlargement of the heart region of B. va, visceral arches. Black arrow and white arrow head point to the heart.

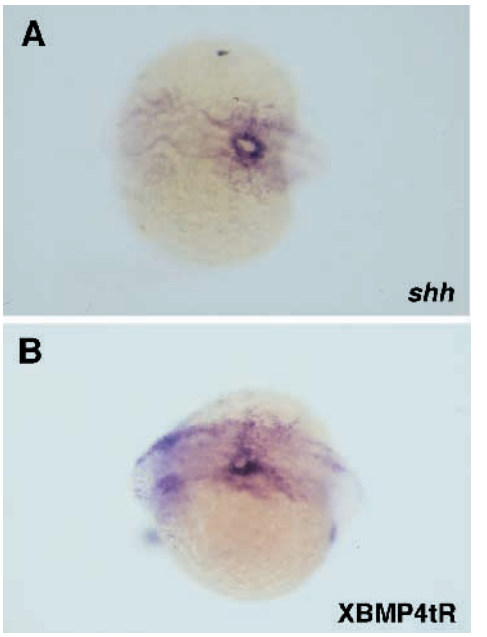

Fig. 5. Cardiac *BMP4* expression pattern in *shh* and XBMPtR injected embryos. (A) *BMP4* expression is more symmetric in the heart tube after injection of *shh* mRNA at the 1- to 2-cell stage. (B) Injection of the truncated *BMP4* receptor, XBMP4tR mRNA, which interferes with jogging and looping, has no effect on cardiac *BMP4* expression pattern. Dorsal-lateral views, anterior to the left.

in these cardiovascular mutants, but this placement is not a reliable criterion for asymmetry, because it is associated with distortion of the tube. Looping in these mutant embryos also frequently is abnormal, or impossible to define, because of the heart's deformation. Because looping appears to depend upon cellular differentiation (Taber et al., 1995), it may be a less reliable assay for heart asymmetry than is the direction of jogging.

BMP4 cardiac expression pattern is changed in heart left-right asymmetry mutants

Mutations that perturb jogging appear to fall into two classes (Table 4). In Class I, the heart of a mutant embryo does not jog. In Class II, there is a high incidence of lateral jogging in mutant embryos, but it is to the right as often as to the left, and there are many embryos in which the heart does not jog. The frequency of lateral jogging versus no jogging varies with the mutations.

The pattern of *BMP4* appears to correlate with the class and to predict the direction of jogging. We evaluated this relationship for all the mutations for which we could distinguish whether an embryo is mutant or not prior to the time of *BMP4* expression. For Class I mutants, which do not jog, it is clear that *BMP4* remains radially symmetric (Fig. 3D; Table 5). For Class II mutants, *BMP4* is more often lateralized, but may be to the right or to the left, or it may remain symmetric (Table 5). In individual mutant embryo of this class, right-predominant *BMP4* (Fig. 3E) is associated with right jogging, left-predominant with left jogging, and symmetric *BMP4* with an absence of jogging.

DISCUSSION

The focus of this study is how asymmetric information is transferred to the heart. There are a plenitude of asymmetrically expressed molecules in the early embryo (Hyatt et al., 1996; Isaac et al., 1997; Levin et al., 1995; Meno et al., 1996; Tsuda et al., 1996), although none with defined effects upon cardiac asymmetry have been identified in the heart or its progenitor field.

In order to identify genes that play a role in establishing left-right asymmetry of the heart, we screened 279 mutations from the Tübingen stock collection and identified 21 mutations with

Table 3. The relationship of jogging and looping

	Left jog			No jog			Right jog		
Mutant	R loop	No loop	L loop	R loop	No loop	L loop	R loop	No loop	L loop
no tail (*n*=166)	1%	0%	0%	48%	28%	23%	0%	0%	0%
floating head (*n*=393)	3%	0%	0%	59%	15%	23%	0%	0%	0%
cyclops (*n*=118)	10%	0%	0%	24%	33%	20%	0%	0%	13%

R loop: ventricle bends to the right.
L loop: ventricle bends to the left.

Table 4. The pattern of jogging in the cardiac laterality mutants

	No. of mutant embryos (*n*)	% mutant embryos			% abnormal jog in wild-type siblings (*n*)
		Left jog	No jog	Right jog	
Laterality affected					
Class I					
floating head	393	3%	97%		4% (442)
lost-a-fin	31		100%		6% (32)
no tail	166	1%	99%		3% (169)
snailhouse	31		100%		6% (64)
spadetail	42		100%		4% (75)
Class II					
curly up	34	35%	35%	30%	17% (34)
cyclops^{b16}	118	10%	77%	13%	2% (120)
cyclops^{tf219}	86	80%	19%	1%	9% (149)
dino	39	28%	44%	28%	6% (80)
iguana	45	31%	58%	11%	0% (45)
locke	85	68%	8%	24%	4% (105)
momo	22	9%	86%	5%	13% (84)
piggytail	37	11%	86%	3%	25% (78)
schmalspur	51	6%	90%	4%	10% (73)
schmalhans	28	43%	21%	36%	0% (31)
tj2a	82	43%	12%	45%	0% (48)
tm243b	74	45%	5%	50%	0% (51)
tm317b	58	45%	14%	41%	4% (25)
tn20b	37	70%	11%	19%	19% (110)
tw29b	43	40%	16%	44%	7% (98)
tg238a	92	83%	9%	9%	1% (92)
Laterality unaffected					
silberblick	18	100%			0% (18)
sonic-you	34	100%			0% (34)
doc	58	90%	10%		0% (58)
sleepy	30	90%	7%	3%	14% (30)
santa	56	100%			0% (42)
pandora	43	99%	1%		2% (52)
heart of glass	81	100%			0% (81)
scotch tape	52	100%			2% (64)
valentine	30	100%			0% (36)

Table 5. The pattern of cardiac *BMP4* expression in the cardiac laterality mutants

	No. of mutant embryos (*n*)	% of mutant embryos		
		BMP4 enriched on the left	*BMP4* radially symmetric	*BMP4* enriched on the right
Laterality affected				
Class I				
floating head	67	7%	88%	4%
no tail	71		97%	3%
snailhouse	23	9%	91%	
spadetail	24	8%	88%	4%
Class II				
cyclops^{b16}	46	9%	82%	9%
cyclops^{tf219}	28	79%	18%	3%
dino	18	28%	61%	11%
iguana	12	42%	33%	25%
momo	28	11%	75%	14%
piggytail	16	19%	75%	6%
schmalspur	18	11%	89%	
Laterality unaffected				
silberblick	14	100%		
sonic-you	15	80%	20%	
doc	14	100%		
sleepy	16	87%	13%	

abnormal cardiac left-right asymmetry. By the nature of the collection, all of the cardiac asymmetry mutants described here have at least one other identifiable defect. During the original Tübingen and Boston screens, we sought mutants with left-right abnormalities, but recovered none. This may in part be due to the use of looping as the assay at that time. In the case of looping randomization, only 12.5% of the progeny from each heterozygous cross would be abnormal, which is close to the backgound level in the zebrafish strains used. Although 24 candidates were identified with randomized cardiac left-right looping in the initial screen, none of them appeared to be a heritable mutation in subsequent generations (Haffter, Odenthal, van Eeden and Nüsslein-Volhard, unpublished data). We presume we could have identified mutations that reliably reversed the direction of looping, similar to the mouse *inv* mutant, but have found none.

Asymmetry of cardiac *BMP4* expression correlates with jogging and looping

We present several lines of evidence that cardiac *BMP4* is an integral component of the pathway by which the heart interprets left-right information from the embryo. First, *BMP4* expression in the heart is predominently on the left side of the heart tube, from just after its generation. This asymmetric expression precedes the transient leftward jogging of the heart tube, which is the first morphological evidence of the heart's asymmetry. Second, the cardiac expression pattern of *BMP4* is

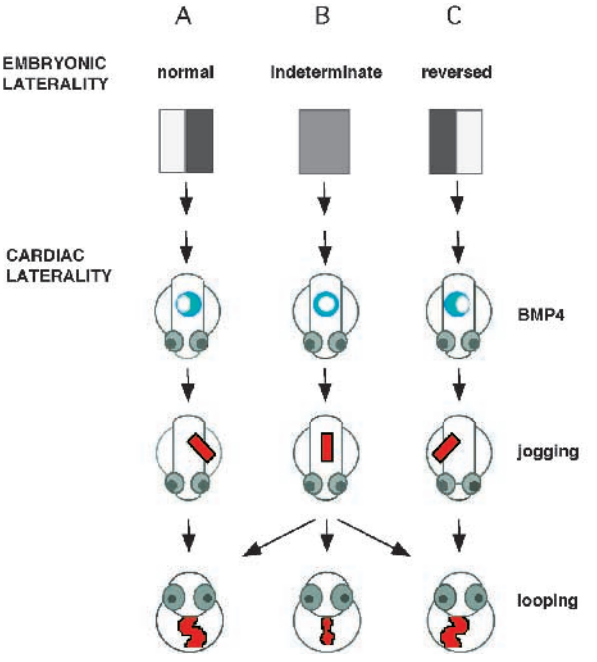

Fig. 6. Model of the role of *BMP4* in the decisions of cardiac laterality. (A) Normally, the primitive heart tube reads the embryonic left-right signals and places *BMP4* transcripts predominantly on the left. This leads to left-sided jogging and, later, right-sided looping. (B) If the embryonic left-right signals are lost or the heart fails to interpret embryonic left-right asymmetry, *BMP4* is evenly distributed in the heart. The heart then fails to jog. Looping will have no directionality and the heart may bend to the left, right or not at all. (C) If the embryonic left-right signals are reversed, cardiac *BMP4* is predominantly placed on the right. This causes right-sided jogging and left-sided looping.

perturbed in all cardiac asymmetry mutants. Third, rendering *BMP4* expression more symmetric by overexpression, or interrupting its signalling by injection of a truncated receptor, block leftward cardiac jogging and disrupt subsequent looping. Interestingly, one of the mutations affecting heart asymmetry, *dino*, is believed to cause increased BMP4 activity, in that its phenotype resembles that of embryos injected with *BMP4* and can be reversed by the dominant-negative BMP4 receptor (Hammerschmidt et al., 1996c).

Our interpretation of the relationship of cardiac *BMP4* to jogging and looping is diagrammed in Fig. 6. Normally, as shown in Fig. 6A, *BMP4* is predominantly expressed on the left side of the forming heart tube, in response to a yet undefined embryonic signal, and this leads to proper jogging and looping. If the signal or its reception from the embryo is reversed (Fig. 6C), in a manner perhaps analogous to the *inv* mutation in mouse, *BMP4* expression is highest on the right side of the forming heart tube. As a consequence, the heart jogs right and the ventricle loops left (Fig. 6C). Jogging and looping in this case might well be accomplished by cell biological mechanisms identical to those operating under normal circumstances, but mirror-image reversed. If *BMP4* is symmetric, the heart does not jog (Fig. 6B). This could reflect the failure to generate, receive or transduce asymmetric information. It is plausible that the subsequent looping of the heart in such embryos is not mechanistically identical to normal looping and does not reflect any left-right vectorial decision by the embryos, but rather a bending to accommodate constraints on longitudinal growth, which normally are not evident when proper looping occurs (Taber et al., 1995).

The mutations that affect cardiac asymmetry fall into two classes with regard to effects on *BMP4*. Class I mutants retain symmetric *BMP4* and do not jog. This suggests that this class of mutations causes a failure to transmit or receive lateralizing signals, as evidenced by a symmetric *BMP4* pattern of expression. Class II mutants more often have asymmetric *BMP4*, but it may be on the right, in which cases it is associated with a proportional increase in rightward jogging. This suggests that, in Class II mutations, there is a randomization of signals to the heart.

Both classes of asymmetry mutations are associated with what appears to be 'randomized' looping, but the mechanisms of randomization may differ. In Class I, there may be no directionality to the looping because of a lack of symmetry-breaking information. The bending of the heart in this class may be due to continued longitudinal growth in the presence of external constraints, rather than due to the normal looping process. In Class II mutants, there is a randomization of the signal to the heart. Reversed looping in this class therefore might be by the normal mechanical process, but with reversed directionality.

How does jogging compare with looping as an assay for cardiac asymmetry? The pattern of the earliest marker of heart asymmetry, *BMP4*, is more accurately reflected in the direction of the jog. For example, in a mutant embryo with retained symmetric *BMP4*, there is no jog, but the direction of looping is not predictable. In addition, jogging occurs earlier than does looping, and unlike looping, is unaffected by defects in cardiac differentiation. In these regards, jogging is a more straightforward assay for asymmetry of the heart than is looping.

Midline structures and cardiac asymmetry

The patterning of the left-right axis has been postulated to be dependent on the patterning of the dorsoventral axis. Ventralization, by overexpression of *Xwnt8* or UV irradiation or extirpation of dorsal structures in *Xenopus*, disrupts normal looping (Danos and Yost, 1995, 1996; Yost, 1995). In zebrafish, mutants with a ventralized phenotype (*dino*) or dorsalized phenotype (*lost-a-fin*, *piggytail* and *snailhouse*) have abnormal jogging and looping.

The phenotype of the zebrafish mutants suggests that midline structures may be the source of signals that drive cardiac asymmetry, although the specific tissue is not defined. Clearly, combined absence of notochord and adjacent ventral spinal cord is associated consistently with defects in cardiac asymmetry. Some mutants with cardiac asymmetry defects have markedly abnormal ventral spinal cord, but have apparently normal notochord (e.g. *cyc*, *igu*, *sur* and *smh*). Although this might be taken to suggest that signals that are needed to establish the ventral spinal cord might also pattern left-right cardiac asymmetry, some mutants have markedly reduced ventral spinal cord (e.g. *detour* and *monorail*) but have normal cardiac asymmetry. This is made more complex by the recent observation that midline and lateral ventral spinal cord cells have different properties (Odenthal, van Eeden, Haffter and Nüsslein-Volhard, unpublished data). Similarly, there is no distinguishing visible characteristic between curly tail mutants with and without defects in cardiac asymmetry. There is a need, therefore, for additional phenotypic and molecular characterization to determine what defects may be shared among the mutations that perturb cardiac asymmetry.

One group of candidates to consider as midline signals are the products of the hedgehog genes. In chick, *sonic hedgehog* (*shh*) injection randomizes the direction of cardiac looping (Levin et al., 1995) and in zebrafish injection of *shh* mRNA upregulates *BMP4* expression, disrupting *BMP4* asymmetry in the heart and jogging and looping. *shh* is expressed in notochord and floor plate, which are affected in many of the zebrafish cardiac asymmetry mutants. However, *shh* is unlikely to be the asymmetric signal to the heart because *shh* loss-of-function mutations, *sonic you* (*syu*) (which include both a point mutation allele and a deletion allele), have normal cardiac laterality (Haffter, unpublished data). In *shh*-targeted mouse mutants, cardiac left-right asymmetry is normal (Chiang et al., 1996). Other *hedgehog* genes, of course, remain candidates, especially those expressed in midline structures close to the heart (Currie and Ingham, 1996; Ekker et al., 1995). These zebrafish mutations may provide a window onto the source and nature of these embryonic signals.

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