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Induction and differentiation of the zebrafish heart requires fibroblast growth factor 8 (*fgf8/acerebellar*)

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SUMMARY

Vertebrate heart development is initiated from bilateral lateral plate mesoderm that expresses the *Nkx2.5* and *GATA4* transcription factors, but the extracellular signals specifying heart precursor gene expression are not known. We describe here that the secreted signaling factor Fgf8 is expressed in and required for development of the zebrafish heart precursors, particularly during initiation of cardiac gene expression. *fgf8* is mutated in *acerebellar* (*ace*) mutants, and homozygous mutant embryos do not establish normal circulation, although vessel formation is only mildly affected. In contrast, heart development, in particular of the ventricle, is severely abnormal in *acerebellar* mutants. Several findings argue that Fgf8 has a direct function in development of cardiac precursor cells: *fgf8* is expressed in cardiac precursors and later in the heart ventricle. Fgf8 is required for the earliest stages of *nkx2.5* and *gata4*, but not *gata6*, expression in cardiac precursors. Cardiac gene expression is restored in *acerebellar* mutant embryos by injecting *fgf8* RNA, or by implanting a Fgf8-coated bead into the heart primordium. Pharmacological inhibition of Fgf signalling during formation of the heart primordium phenocopies the *acerebellar* heart phenotype, confirming that Fgf signaling is required independently of earlier functions during gastrulation. These findings show that *fgf8/acerebellar* is required for induction and patterning of myocardial precursors.

Key words: Cardiogenesis, Heart, Ventricle, *fgf8*, *acerebellar*, *nkx2.5*, *gata4*, BMP, Zebrafish (*Danio rerio*)

INTRODUCTION

The heart is the first organ to form and function during vertebrate embryogenesis. The fish heart can be viewed as a prototypical vertebrate heart, since the process of heart formation occurs in a similar way in all vertebrates (DeHaan, 1965; DeRuiter et al., 1992; Stainier and Fishman, 1992; Stainier et al., 1993; Fishman and Chien, 1997). In zebrafish, cardiogenic precursors involute shortly after the onset of gastrulation and migrate medially as part of the lateral plate mesoderm. The myocardial precursor cells form two tubular primordia on each side of the midline at early somitogenesis. These primitive tubes then fuse by the 20-somite stage and enclose the endocardial precursor cells to give rise to the definitive heart tube consisting of an inner, endocardial layer and an outer, myocardial layer (Stainier and Fishman, 1992; Stainier et al., 1993; Fishman and Chien, 1997). Around 24 hours post-fertilization (hpf), the heart starts beating and circulation begins. The definitive heart tube is subsequently divided into different chambers, with the atrium and the ventricle being the most prominent heart structures. The atrium can be distinguished from the ventricle by differential expression of myosin heavy chains already at 24 hpf, and a morphological distinction becomes apparent soon thereafter (Stainier and Fishman, 1992; Stainier et al., 1993).

Although heart development has been well described morphologically, the molecular events underlying this process are only beginning to be understood (for reviews see Lyons, 1996; Olson and Srivastava, 1996; Fishman and Chien, 1997; Mohun and Sparrow, 1997). Several families of transcription factors have been implicated in heart development. A key family are the vertebrate *Nkx2.5* genes, which are homologues of the *Drosophila* gene *tinman*. In *Drosophila*, *tinman* is expressed in the heart (or dorsal vessel) and visceral mesoderm, and is required for heart formation (Azpiazu and Frasch, 1993; Bodmer, 1993; Harvey, 1996). In vertebrates, *Nkx2.5* is the earliest marker for heart precursors in the lateral plate mesoderm (Lints et al., 1993; Komuro and Izumo, 1993; Chen and Fishman, 1996). Overexpression of *nkx2.5* in *Xenopus* or zebrafish causes a general increase in heart size (Chen and Fishman, 1996; Cleaver et al., 1996). Targeted disruption of *Nkx2.5* in the mouse does not prevent formation of the heart tube, but cardiac looping is absent and the expression of downstream cardiac transcription factors is disturbed (Lyons et al., 1995; Tanaka et al., 1999). At least two more *Nk2* class homeobox genes, *nkx2.7* and *Nkx2.8*, are expressed early in the developing heart, with *nkx2.7* being expressed even before *nkx2.5* in the zebrafish (Lee et al., 1996; Brand et al., 1997).

A direct target gene regulated by *tinman* is the myocyte enhancer binding factor-2 (*D-mef2*) of the MADS box-

containing proteins, and loss of D-MEF2 function prevents formation of cardiac muscle (Lilly et al., 1994; Bour et al., 1995; Lilly et al., 1995; Olson et al., 1995; Gajeweski et al., 1997). Likewise, the four *mef2* genes in vertebrates are expressed in precardiac mesoderm and mouse mutants for *MEF2C* exhibit heart abnormalities (Edmondson et al., 1994; Molkentin et al., 1996; Ticho et al., 1996; Lin et al., 1997). In addition, several members of the GATA family of zinc finger domain transcription factors are involved in cardiac development. *GATA4* is expressed in the presumptive heart mesoderm and overexpression of *GATA4* in mouse embryonic stem cells enhances cardiogenesis (Kelley et al., 1993; Heikinheimo et al., 1994; Laverriere et al., 1994; Jiang and Evans, 1996; Grepin et al., 1997). In contrast, the failure of the bilateral heart primordia to migrate medially and to fuse in *GATA4* knock-out mice is thought to be a secondary consequence of a defective endoderm (Kuo et al., 1997; Molkentin et al., 1997; Narita et al., 1997a,b). Finally, basic region helix-loop-helix (bHLH) domain proteins play important roles at later stages of cardiac morphogenesis, e.g. in chamber-specific gene expression (Srivastava et al., 1995).

Although localized expression of transcription factors is important for heart development, little is known about the signaling molecules that control this expression. Endoderm, ectoderm and Spemann's organizer have been implicated as sources for cardiac mesoderm inducing signals (Lyons, 1996; Fishman and Chien, 1997; Mohun and Sparrow, 1997). Misexpression of *BMPs* in anterior mesoderm of chick embryos suggested that BMPs may induce expression of the cardiac transcription factors *Nkx2.5* and *GATA4*, and *BMPs* are expressed close to the heart primordium (Schultheiss et al., 1997; Andrée et al., 1998). In *Drosophila*, *tinman* is indeed regulated by the *BMP* homologue *decapentaplegic* (*dpp*) (Frasch, 1995). In addition, tissue culture studies have demonstrated cardiogenic effects for Activin-A and FGF2, and treatment of non-precardiac mesodermal explants from early

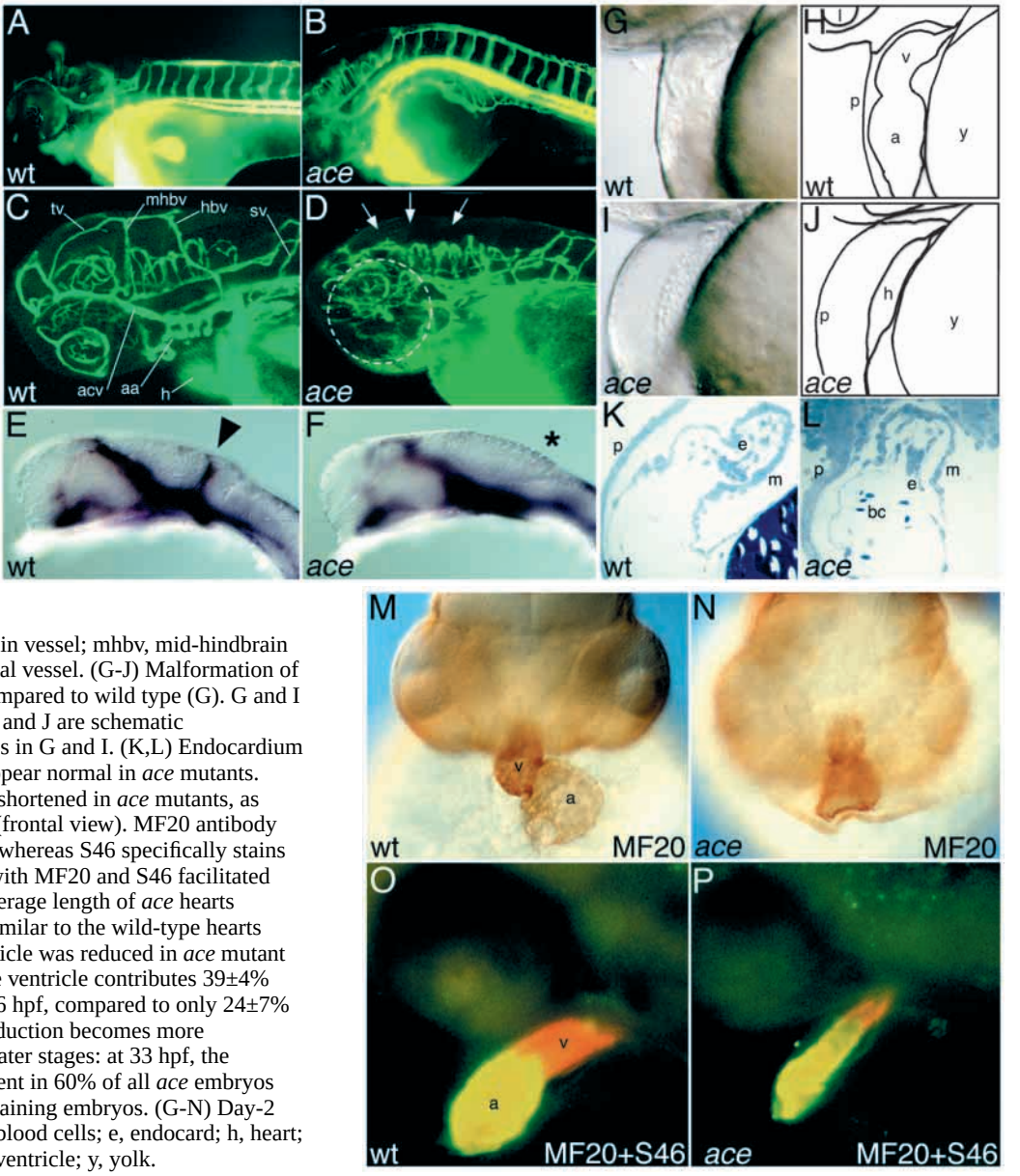

Fig. 1. Circulation, blood vessel formation and abnormal heart morphology in *acerebellar* mutants. (A,B) Overview of blood vessel system in a day-2 wild-type (A) and an *ace* (B) larva. (C,D) Confocal close-up of the blood vessels in the head in a wild-type (C) and an *ace* (D) larva on day 2, with the main vessels in the tectum, at the mid-hindbrain boundary and in the hindbrain being affected in *ace* mutants (arrows). The dashed circle in D marks the position of the eye. (E,F) The vessel at the mid-hindbrain boundary (E, arrowhead) is missing in *ace* mutant embryos (F, asterisk) at 24 hpf, detected by in situ hybridization with *flk-1*. aa, aortic arches; acv, anterior cardinal vein; h, heart; hbv, hindbrain vessel; mhbv, mid-hindbrain vessel; tv, tectal vessel; sv, segmental vessel. (G-J) Malformation of the heart in *acerebellar* larva (I) compared to wild type (G). G and I are lateral views of living larvae, H and J are schematic representation of the main structures in G and I. (K,L) Endocardium and myocardium are present and appear normal in *ace* mutants. (M,N) The heart is malformed and shortened in *ace* mutants, as shown by MF20 antibody staining (frontal view). MF20 antibody reacts with both cardiac chambers, whereas S46 specifically stains the atrium. (O,P) Double staining with MF20 and S46 facilitated measuring the chambers and the average length of *ace* hearts (83±16%, *n*=19) was found to be similar to the wild-type hearts (100±8%, *n*=10), whereas the ventricle was reduced in *ace* mutant embryos. In wild-type embryos, the ventricle contributes 39±4% (*n*=10) to the total heart length at 26 hpf, compared to only 24±7% (*n*=19) in *ace* mutants. Ventricle reduction becomes more pronounced, but is still variable at later stages: at 33 hpf, the ventricle is severely reduced or absent in 60% of all *ace* embryos (*n*=55), and less affected in the remaining embryos. (G-N) Day-2 larvae, (O,P) 26 hpf. a, atrium; bc, blood cells; e, endocard; h, heart; l, lens; m, myocard; p, pericard; v, ventricle; y, yolk.

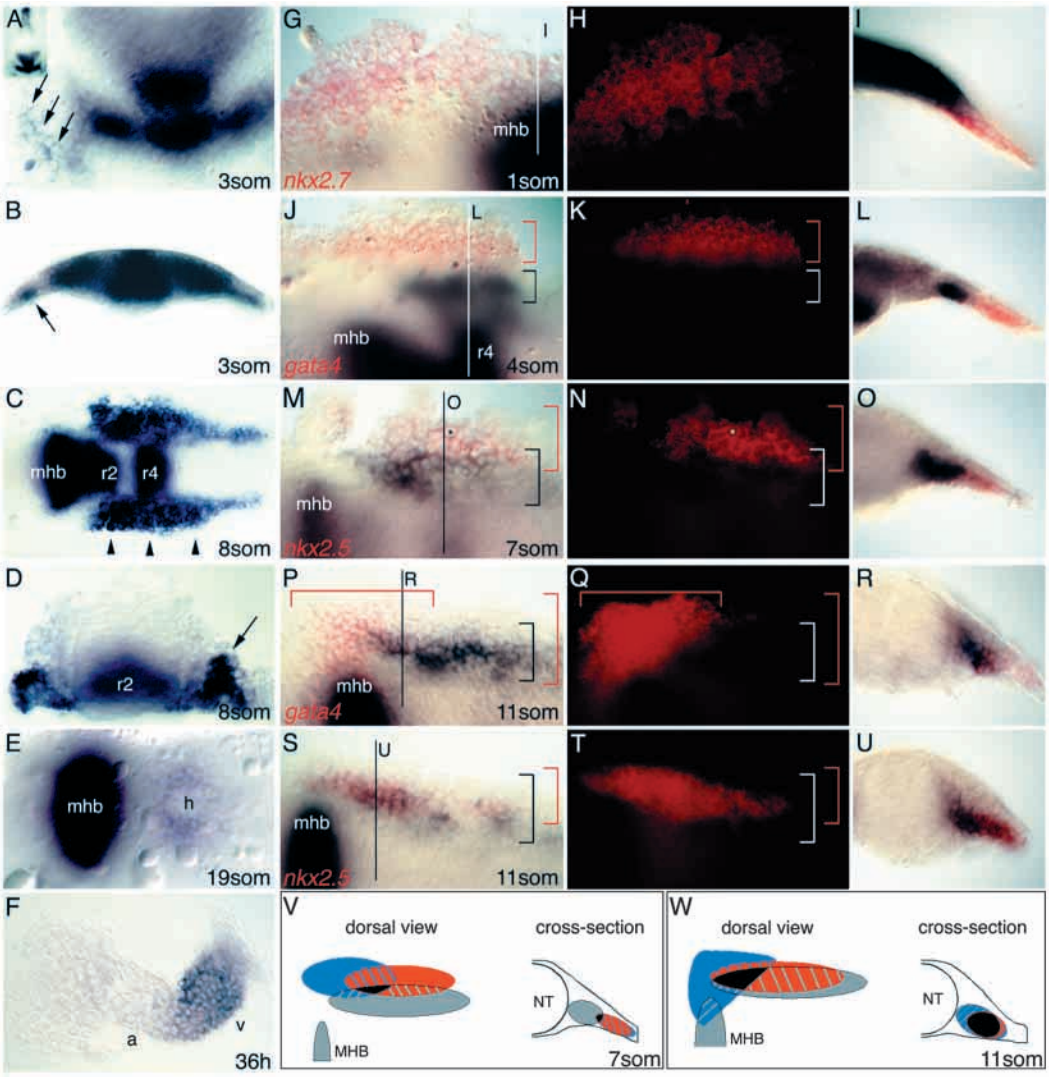

Fig. 2. Cardiac expression of *fgf8* in relation to heart marker genes in wild-type embryos. (A-F) *fgf8* expression at various stages of development, as indicated. B and D are cross sections of A and C, respectively. (A,B) Cells in lateral plate mesoderm express *fgf8* (arrows) at the 3-somite stage. (C,D) Expression bilaterally to the neural tube including cardiogenic fields (arrowheads in C and arrow in D) at the 8-somite stage. (E) Ring-shaped expression in the heart at 19-somite stage. (F) Expression is predominantly in the ventricle at 36 hpf. (F) Dissected heart. (A) Anterior to the top; (C,E) anterior to the left; a, atrium; h, heart; mhb, mid-hindbrain boundary; os, optic stalks; r2, rhombomere 2; r4, rhombomere 4; v, ventricle. (G-U) Double in situ hybridization with *fgf8* (black) and indicated heart markers (red fluorescence) of wild-type embryo at given stages. (G-I) *fgf8* is expressed in close proximity to *nkx2.7*. (J-L) *fgf8* is expressed close to *gata4*. (M-O) *fgf8* expression partially overlaps *nkx2.5* expression (star marks the same cell in M and N). (P-R) *fgf8* expression partially overlaps *gata4* expression. (S-U) *fgf8* expression strongly overlaps *nkx2.5* expression. Brackets indicate the two expression domains. (V,W) Summary of *fgf8* expression relative to the described heart marker genes at given stages (grey, *fgf8*; red, *nkx2.5*; blue, *gata4*, black, triple overlap). Embryos in G,H,J,K,M,N,P,Q,S,T are flat mounted, anterior to the left; G,J,M,P,S are bright-field images, H,K,N,Q,T are fluorescent images of the same embryos; I,L,O,R,U are cross sections at the indicated levels, lateral is to the right. mhb, mid-hindbrain boundary; r4, rhombomere 4.

chick embryos with a combination of FGF4 and BMP2 is able to induce cardiogenesis in vitro (Sugi and Lough, 1995; Lough et al., 1996).

Here we show that the secreted signaling factor Fgf8 is required in vivo for heart development of the zebrafish. Although expression of several *Fgfs* has been described in the developing heart (Parlow et al., 1991; Spirito et al., 1991; Engelmann et al., 1993; Mason et al., 1994; Crossley and Martin, 1995; Zhu et al., 1996; Hartung et al., 1997; Miyake et al., 1998), studying the function of Fgfs in development of the vertebrate heart has been difficult, often due to early lethality of the mutants or possible functional redundancy. Analysis of the available loss-of-function mutations has not revealed a specific function for Fgf signaling in the developing heart so far (Feldman et al., 1995; Dono et al., 1998; Meyers et al., 1998). Similarly, 3 out of 4 vertebrate Fgf receptors are expressed during heart development, but their inactivation in mouse embryos has not been informative with respect to cardiac development (Orr-Urtreger et al., 1991; Yamaguchi et al., 1991; Peters et al., 1992; Deng et al., 1994; Arman et al., 1995; Sugi et al., 1995; Thisse et al., 1995; Colvin et al., 1996; Weinstein et al., 1998).

In contrast, functional studies in *Drosophila* do suggest a role for Fgf signaling in cardiac development (Beiman et al., 1996; Gisselbrecht et al., 1996; Michelson et al., 1998).

The isolation of many mutants affecting zebrafish heart development in the systematic screens for embryonic zebrafish mutants opens up the possibility to study the genetic control of vertebrate heart development in great detail (Haffter et al., 1996). The zebrafish mutant *acerebellar (ace)* was orginally classified as a brain mutant (Brand et al., 1996) in which the *fgf8* gene is inactivated (Reifers et al., 1998). Here we show that analysis of this mutant unexpectedly reveals a pivotal role for Fgf8 in myocardial induction. We examine in detail the expression and function of Fgf8 in the developing zebrafish heart, and propose that Fgf8 functions together with Bmps in

induction of heart-specific gene expression upstream of *nkx2.5* and *gata4*.

MATERIALS AND METHODS

Zebrafish were raised and kept under standard laboratory conditions at about 27°C (Westerfield, 1994; Brand and Granato, 1999). Mutant carriers were identified by random intercrosses. To obtain embryos showing the mutant phenotype, two heterozygous carriers for a mutation were crossed to one another. Typically, the eggs were spawned synchronously at dawn of the next morning, and embryos were collected, sorted, observed and fixed at different times of development at 28.5°C. In addition, morphological features were used to determine the stage of the embryos, as described by Kimmel et al. (1995). In some cases, 0.2 mM phenylthiourea (PTU) was added to prevent melanization. Isolation and characterization of *acerebellar* is described by Brand et al. (1996) and Reifers et al. (1998).

Microangiography

Microangiography was essentially done as described by Weinstein et al. (1995). Briefly, embryos were injected with 0.01 mm diameter fluorescent latex beads (Molecular Probes). Bead suspension was diluted 1:1 with 2% BSA in deionized distilled water, sonicated and subjected to centrifugation for 2 minutes in an Eppendorf microcentrifuge. Dechorionated embryos were anaesthetized with tricaine as described and placed on an injection platform (Westerfield, 1994). A large bolus of bead suspension was injected into the sinus venosus. The fluorescent beads were uniformly distributed throughout the vasculature of the embryo within minutes. Specimens were either photographed on a Zeiss Axioskop microscope or scanned using the confocal microscope (Leica TCS4D) as rapidly as possible. Images were assembled using Adobe Photoshop.

In situ hybridization and immunohistochemistry

In situ hybridizations (ISH) were done as described by Reifers et al. (1998), and histology is described by Kuwada et al. (1990). Immunohistochemistry is described by Stainier and Gilbert (1990). MF20, anti-myosin heavy chain, was obtained from the Developmental Studies Hybridoma Bank. MF20 stains all cardiac chambers. S46 was a kind gift from Frank Stockdale, Stanford University. S46 specifically reacts with the atrium and the sinus venosus. Double-labeled embryos were stained with monoclonal antibodies S46 (IgG1) and MF20 (IgG2b) followed by fluoresceinated goat anti-mouse IgG1 and rhodaminated goat anti-mouse IgG2b. Heart length was measured on photographs of individually mounted wild-type and *acerebellar* embryos, heart length is given as percentage of wild-type length.

RNA injections

fgf8, subcloned into pCS2+ (Rupp et al., 1994), and XFD (Amaya et al., 1991) were linearized and transcribed using the SP6 message machine kit (Ambion). The amount of RNA injected was estimated from the concentration and volume of a sphere of RNA injected into oil at the same pressure settings. Typically, about 25 pg of *fgf8* RNA or XFD RNA were injected; RNA was dissolved in 0.25 M KCl with 0.2% phenol red and backloaded into borosilicate capillaries prepared on a Sutter puller. During injection, RNA was deposited into the cytoplasm of 2-cell stage embryos; in embryos after the first cleavage, the RNA usually stays in the progeny of the injected blastomere, as judged from the often unilateral distribution of control *lacZ* RNA, as detected with anti-β-gal antibody (Promega, 1:500) after ISH (Dornseifer et al., 1997). The embryos were fixed at the 6- to 8-somite stage prior to ISH and antibody staining.

Bead implantations and inhibitor treatment

Heparin acrylic beads (Sigma) were washed in ethanol for 30 minutes, then washed twice in PBS for 5 minutes and finally coated with recombinant mouse FGF8 protein (R&D Systems; 0.25 mg/ml). Protein-coated beads were stored for several weeks at 4°C without detectable loss of activity. FGF8 or PBS control beads were implanted in the lateral plate mesoderm of 5- to 7-somite stage *acerebellar* mutant embryos, the embryos were fixed at the 10- to 12-somite stage prior to ISH. For pharmacological inhibition of Fgfr activity, wild-type embryos were treated with SU5402 (Calbiochem; Mohammadi et al., 1997) at 8 μm in embryo medium at 28.5°C in the dark for the indicated time periods.

RESULTS

Circulatory system shows only minor defects in *acerebellar*

Wild-type embryos examined at 36 and 48 hpf (*n*=107) have successfully established normal circulation, whereas *ace* embryos fail to do so, with 23% having no circulation, and 77% of the *acerebellar* mutants (*n*=159) having variably reduced circulation. To examine whether the defect in the circulatory system of *acerebellar* mutant embryos is due to a malformation of the heart itself or the blood vessel system, we analysed vessel formation by microangiography, i.e. visualization of circulation by injecting fluorescent latex beads into the sinus venosus, and by staining for blood vessel marker gene expression. Microangiography at 48 hpf revealed an overall normal organization of the blood vessels in those *acerebellar* embryos that established circulation (Fig. 1A-D). Likewise, the expression pattern of *flk-1*, a receptor tyrosine kinase that is a marker for blood vessel endothelial cells (Fouquet et al., 1997; Liao et al., 1997) appears largely normal in all *acerebellar* mutants, except for an absence of vessels in the dorsal brain (Fig. 1E,F), which is most likely a secondary effect due to the brain defects in mutant embryos (Reifers et al., 1998, Picker et al., 1999). Taken together, these results suggest that blood vessel formation in *acerebellar* mutants is overall normal; in contrast heart development is severely abnormal.

Ventricle is strongly reduced in *acerebellar*

The zebrafish heart is composed of four subdivisions: the sinus venosus, the posterior atrium, the anterior ventricle and the bulbus arteriosus (Stainier and Fishman, 1992). The prominent atrium can be readily distinguished from the adjacent ventricle in living wild-type embryos (Fig. 1G,H). Heartbeat frequency is approximately normal in the mutants at 28 hpf (106±7 beats/minute, *n*=5) versus the wild-type (111±6 beats/min, *n*=5), but the heart of *acerebellar* mutant embryos is severely dysmorphic (Fig. 1I,J). Histological sections show that subdivision into an inner endocardial and outer myocardial layer occurs normally (Fig. 1K,L). In contrast, immunohistochemical analysis with the myosin heavy chain antibodies MF20 and the ventricular-specific antibody S46 and measurements of heart length shows that the heart is overall shorter, and that in particular the ventricular part is reduced (Fig. 1M-P).

fgf8 is expressed in the developing heart

Since the heart is affected in *acerebellar* mutant embryos and since *fgf8* expression during zebrafish heart development has not been analyzed previously, we examined the expression pattern of *fgf8* in the developing heart in detail, relative to several markers for cardiac precursors by double in situ

hybridisation. Already at the blastula stage, *fgf8* is expressed all around the margin of the embryo (Fürthauer et al., 1997; Reifers et al., 1998), which includes the fate map position of cardiac precursors ventrolaterally (Warga and Kimmel, 1990; Stainier and Fishman, 1992). The expression of *fgf8* at the mid-hindbrain boundary at tailbud stage (Fig. 2G-I) is located close to the posterior part of the expression domain of *nkx2.7*, which includes cardiac precursors in zebrafish (Lee et al., 1996). From the 3-somite stage onwards *fgf8*-positive cells can be observed in the lateral plate mesoderm in the area of the incipient heart field (Fig. 2A,B). At the 4-somite stage *fgf8* is expressed in the lateral plate mesoderm adjacent to the posterior part of the *gata4* expression domain (Fig. 2J-L), which includes the heart precursors (Kelley et al., 1993; Jiang and Evans, 1996; Serbedzija et al., 1998). Mesodermal *fgf8* expression partially overlaps with the expression domain of *nkx2.5* at the 7-somite stage (Fig. 2M-O), for which it has been shown that the anterior cells contribute to the heart (Serbedzija et al., 1998). At the 8-somite stage a subset of the *fgf8* expression lateral to the embryonic axis includes the myocardial precursors (Fig. 2C,D), which arrive in this region at this timepoint in development (Stainier and Fishman, 1992). The *fgf8* expression overlaps partially with the expression domains of *gata4* and almost completely with *nkx2.5* at the 11-somite stage (Fig. 2P-U). At the 19-somite stage the bilateral myocardial primordia meet caudal to the mid-hindbrain boundary to form a mesodermal ring-like structure which expresses *fgf8* (Fig. 2E). At 36 hpf atrium and ventricle can be morphologically distinguished, and *fgf8* expression is stronger in the ventricle (Fig. 2F); expression persists at least until 48 hpf, the latest stage we examined. These data suggest that Fgf8 could function during the earliest stages of cardiac precursor development, and in the differentiated heart.

Expression of heart markers is affected in *acerebellar*

Since *fgf8* is expressed early on in close association with the heart precursors and *acerebellar* embryos fail to develop proper hearts, we analysed the expression of cardiac marker genes in *acerebellar* mutant embryos (Table 1 and Fig. 3). *nkx2.7*, *gata4* and *nkx2.5* are affected from the onset of expression. Importantly, *gata6*-positive cells are present in mutant embryos, demonstrating that the lateral plate mesoderm cells that are normally destined to become cardiogenic precursors are present (Fig. 3I,J). In addition, we could not detect an increase in apoptotic cell death in the lateral plate mesoderm of *acerebellar* embryos (data not shown). Expression of *nkx2.7* is strongly reduced as early as the 1-somite stage in *acerebellar* embryos (Fig. 3A,B), but recovers quickly. Expression of the zinc-finger transcription factor *gata4* is also clearly reduced in mutant embryos at the 3- to 6-somite stage (Fig. 3C,D). The most severe effect was detected for *nkx2.5* expression, which was strongly reduced or absent between the 3-somite and 15-somite stages in *acerebellar* embryos (Fig. 3E,F). At the 11-somite stage the *fgf8* expression domain partially overlaps the *nkx2.5* domain and reaches further posterior at least to the level of rhombomere 5. In *acerebellar* mutant embryos the anterior part of *nkx2.5* expression is severely reduced and the expression in the posterior subdomain is completely absent (Fig. 3G,H). At late somitogenesis stages, all heart markers tested (*nkx2.5*, *nkx2.7*, *gata4*, *gata6* and *bmp4*) recover in the intensity of their expression in the remaining heart precursors of *acerebellar* mutant embryos (Table 1). However, a size-reduction of the mutant hearts remains detectable with these markers even at later stages (Fig. 3K,L and not shown). We conclude that *fgf8* expression partially overlaps with *nkx2.5* in heart precursor cells and that Fgf8 is required for expression of *nkx2.5*. Interestingly, the requirement may be stronger in the posterior part of the *nkx2.5* domain, since this expression is completely lost in *acerebellar* mutants (Fig. 3G,H). This early defect in mutant embryos may account for the subsequent loss of heart structures.

Fgf8 can rescue early expression of cardiac genes in *acerebellar*

The requirement of *acerebellar/fgf8* during the earliest stages of cardiac gene expression raised the possibility that Fgf8 is an inducer of heart development. To further examine this issue, we injected synthetic RNAs encoding *fgf8* or a dominant negative Fgf receptor into wild-type and *acerebellar* embryos. In addition to the described dorsalizing effect (Fürthauer et al., 1997; Reifers et al., 1998), *fgf8* injection can restore *gata4*, but not *nkx2.5* expression in mutant embryos at the 6-somite stage (Fig. 4A,B). 14 out of 45 (31%) *acerebellar* mutant embryos show strong *gata4* expression after *fgf8* injection on the injected side. Surprisingly, none of the injected mutant embryos re-expressed *nkx2.5* in response to *fgf8* overexpression by RNA injection (n=26; but see below); *lacZ* control injections gave no effect (not shown).

While *gata4* expression is restored in mutant embryos, neither *nkx2.5* nor *gata4* expression was induced outside their normal expression domain following *fgf8* RNA injection into wild-type or *ace* mutant embryos (Fig. 4A and not shown). This suggests that competence to respond to Fgf8 signaling is restricted to these subregions of lateral plate mesoderm including the prospective heart primordium.

We suspected that the failure to rescue *nkx2.5* expression in the mutant embryos by RNA injections could be due to the dorsalizing influence of *fgf8* overexpression during gastrula stages (Fürthauer et al., 1997; Reifers et al., 1998). To examine this possibility further and to test whether Fgf8 signaling could occur directly within the heart field during postgastrulation stages, we implanted Fgf8 protein-coated beads at the 5- to 6-somite stage into the lateral plate mesoderm of wild-type and *acerebellar* mutant embryos. 8 out of 10 operated mutant embryos strongly reexpressed *nkx2.5* on the bead side; implantation of PBS beads (n=6) gave no effect (Fig. 4E,F). Interestingly, implantation of Fgf8-soaked beads into wild-type embryos also results in a slight posterior expansion of the endogenous *nkx2.5* domain (Fig. 4G). These data indicate that *gata4* expression is dependent on Fgf8 signaling, while *nkx2.5* expression requires in addition to Fgf8 another factor, which may have been delocalized during gastrulation by the dorsalizing effect of *fgf8* RNA injections (see Discussion). Taken together, these experiments show that Fgf8 signaling is required but not sufficient for *gata4* and *nkx2.5* expression during early somitogenesis stages.

To further study the requirement of Fgf signaling for the expression of *nkx2.5* and *gata4*, we analysed the expression in wild-type embryos where Fgf signaling has been blocked either pharmacologically or by injecting RNA encoding the dominant

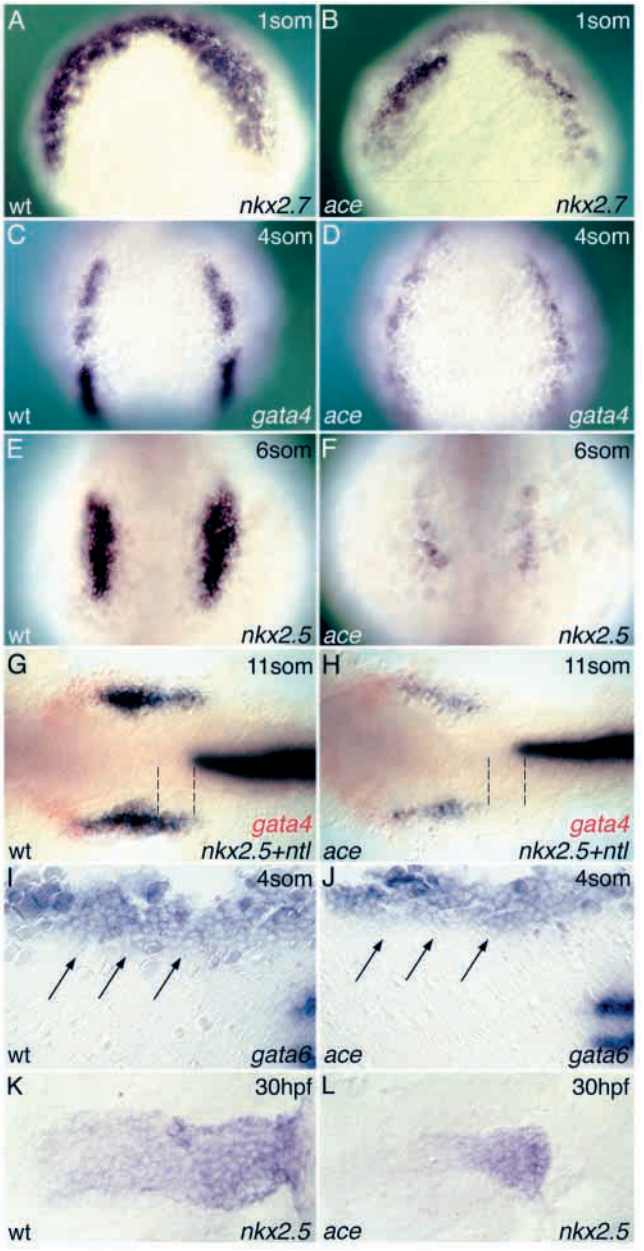

Fig. 3. Fgf8 is required for cardiac marker gene expression. Stages and markers as indicated. (A,B) Expression of *nkx2.7* in wild-type (A) and *ace* (B) embryos. (C,D) Expression of *gata4* in wild-type (C) and *ace* (D) embryos. (E-H) Expression of *nkx2.5* in wild-type (E,G) and *ace* (F,H) embryos. Notice the differential sensitivity of the posterior *nkx2.5* domain (dashed lines). (I,J) Expression of *gata6* (arrows) in wild-type (I) and *ace* (J) embryos. (K,L) Late expression of *nkx2.5* in dissected hearts of wild-type (K) and *ace* (L) embryos. A-F show dorsal views of whole embryos, anterior to the top. Embryos in G-J are flat mounted, anterior to the left. Embryos in G,H are stained for *nkx2.5* and *ntl* (black) and *gata4* (red). Embryos in I and J are double-stained for *myoD* to genotype the embryos (Reifers et al., 1998).

negative Fgf receptor XFD (Amaya et al., 1991). 32 out of 73 (44%) injected wild-type embryos show a strong reduction or even absence of *gata4* expression. In the case of *nkx2.5*, 67% (35 out of 52) of the *XFD*-injected embryos have altered gene expression (Fig. 4C,D). Interestingly, the described effects could only be observed if the misexpressed RNA was located in mesodermal cells (data not shown), suggesting that Fgf signal reception is required only within the mesodermal layer. While these results are consistent with a dependence of *nkx2.5* and *gata4* expression on Fgf signaling in the heart field, they could also be explained by an earlier requirement for Fgf signaling in dorsoventral patterning during gastrulation. To test whether Fgfs are specifically required after gastrulation during early somitogenesis for heart development, we treated wild-type embryos with SU5402, a potent inhibitor of Fgfr1 function (Mohammadi et al., 1997). Since SU5402 blocks Fgfr1 activity by binding to a region identical in all four Fgfrs (Johnson and Williams, 1993), it probably blocks all Fgf signals, including Fgf8. Inhibitor treatment during early somitogenesis results in a phenocopy of the *acerebellar* heart phenotype (not shown), including failure to initiate *nkx2.5* expression (Fig. 4I-K). This confirms that Fgf signaling is required specifically during early somitogenesis for initiation of heart development. As in *acerebellar* homozygous embryos (see above, Fig. 3F and Table 1), *nkx2.5* expression recovers after transient treatment in early somitogenesis (Fig. 4J,M). Continuous inhibitor treatment, however, prevents this recovery (Fig. 4K,N), suggesting that recovery is due to other Fgfs that act during later stages of heart development.

fgf8 itself is later expressed specifically in ventricular tissue (Fig. 2F), and *acerebellar* mutants show strongly impaired ventricle development (Fig. 1O,P). The ventricle defect could either be a consequence of abnormal formation of the primordium, or could represent an independent later function of Fgf8. To distinguish these possibilities, Fgf signaling was inhibited by SU5402 treatment after initial primordium formation, from late somitogenesis onwards. This treatment results in absence of ventricular tissue and apparent enlargement of atrial tissue, indicating that Fgf signaling continues to be required specifically for ventricular development (Fig. 4O,P).

Table 1. Cardiac marker gene expression in *acerebellar* mutants

Marker gene expression	1 som	3 som	6 som	15 som	22 som	26 som
gata4	nd	–	–	+	+	+
gata6	nd	+	+	+	+	+
nkx2.5	nd	–	–	–	+	+
nkx2.7	–	+	+	+	+	+
bmp4	nd	nd	nd	+	+	+

som: somite-stage. + unaffected, – affected, nd, not detectable.

DISCUSSION

Cardiovascular phenotype of *acerebellar*

Our analysis shows that *fgf8* is expressed in and required for the development of cardiogenic precursors of the zebrafish heart. Homozygous *acerebellar/fgf8* mutants fail to initiate proper gene expression of the cardiac transcription factors *nkx2.5* and *gata4*, resulting in a severely malformed heart. Protein bead implantations and receptor inhibition show that Fgf8 can act directly on the myocardial primordium during

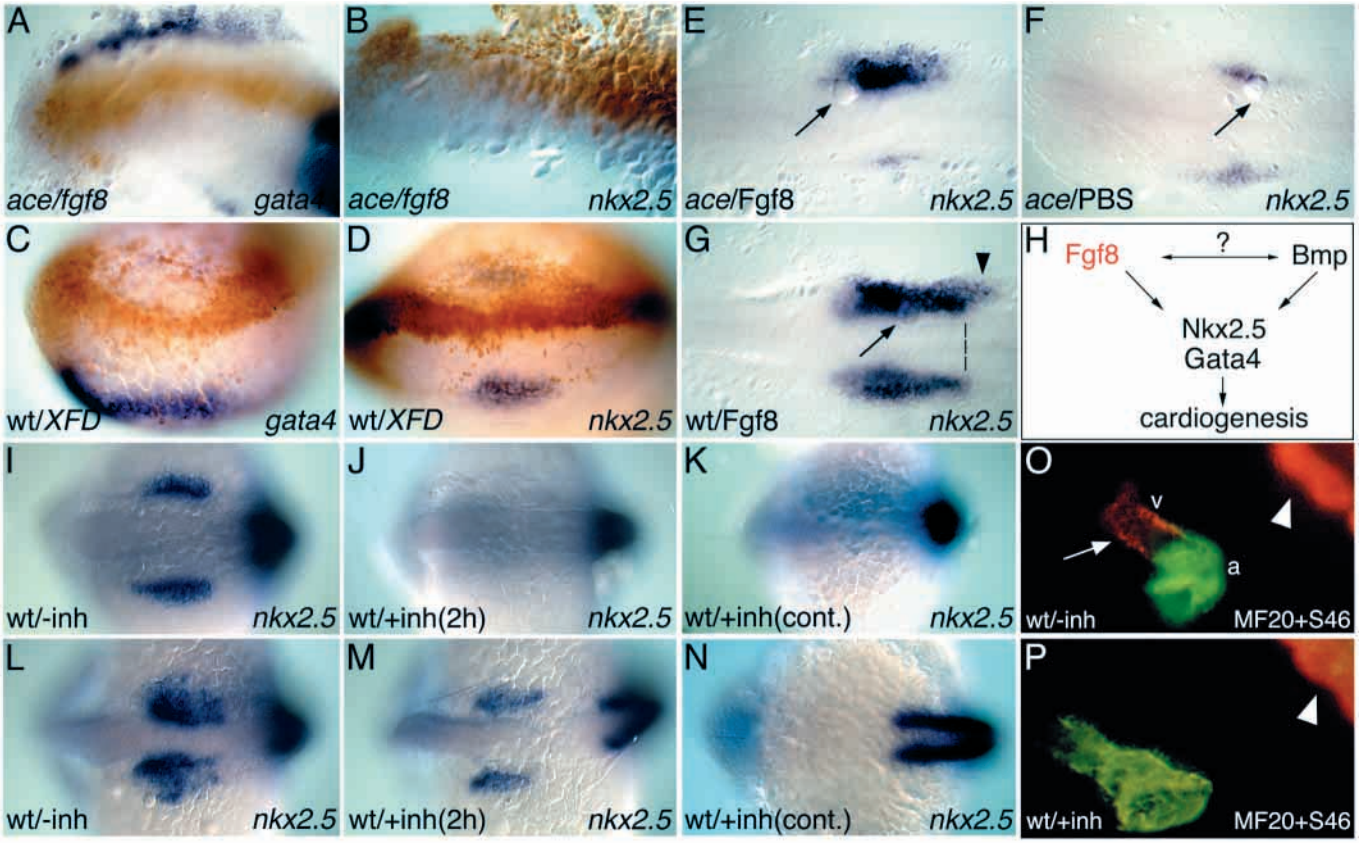

Fig. 4. Fgf8 functions in the heart primordium. (A,B) Misexpression of *fgf8* in *ace* embryos by RNA injection. Localization of *lacZ* co-injected cells with an antibody to β-gal (brown). *fgf8* RNA injection can restore *gata4* (A), but not *nkx2.5* (B) expression in mutant embryos. (C,D) Expression of *XFD* in wild-type embryos by RNA injection. Localization of *lacZ* co-injected cells with an antibody to β-gal. *XFD* RNA injection suppresses *gata4* (C) and *nkx2.5* (D) expression in wild-type embryos. (E,F) Implantation of a Fgf8-soaked bead (arrow) after gastrulation can rescue *nkx2.5* expression in *ace* embryos (E), while PBS bead gives no effect (F). (G) Implantation of a Fgf8-soaked bead (arrow) after gastrulation can caudally extend (dashed line) the endogenous *nkx2.5* expression domain in wild-type embryos. (H) Model of Fgf8 function in cardiogenesis. All embryos are oriented with anterior to the left; A,B,E-G are flat mounted; C,D are dorsal views of whole embryos. *nkx2.5* and *gata4* were detected by in situ hybridization. (I-P) Fgfr inhibitor treatments. (I-K) 10-somite stage, (L,M) 15-somite stage, (N) 18-somite stage, (O,P) 26 hpf. (I-N) *nkx2.5* and *myoD* expression detected by ISH; dorsal view of whole embryo, anterior to the left. (O,P) Double staining with MF20 and S46 antibodies, lateral view, anterior to the left. (I-N) Embryos have been treated with SU5402 from the 1-somite stage onwards for 2 hours (J,M) or continuously (K,N). (I,L) Non-treated control embryos. *nkx2.5* expression is absent after short exposure to the inhibitor at the 10-somite stage (J), but begins to recover at the 15-somite stage (M). Continuous treatment results in permanent loss of *nkx2.5* expression (K,N). (O,P) Embryos that have been continuously treated with SU5402 from the 18-somite stage onwards show no ventricular tissue (P); compare with the untreated control (O). Arrow marks the ventricle, arrowheads point to the MF20-positive somites. a, atrium; v, ventricle.

postgastrulation stages. We therefore propose that Fgf8 functions in induction of the earliest cardiac gene expression (Fig. 4H).

In addition to its role in cardiac precursor development (see below), *fgf8* is more specifically required in the ventricle of the zebrafish heart at later stages, since this is the most strongly affected structure in *acerebellar* embryos. This finding is consistent with the predominant expression of *fgf8* in the ventricle. In addition to *fgf8*, *fgf1*, *fgf7* and *fgf12* are reported to be expressed in a chamber-specific manner. Additional *fgfs* are expressed during heart development (*fgf2*, *4*, *13* and *16*) (Parlow et al., 1991; Spirito et al., 1991; Engelmann et al., 1993; Mason et al., 1994; Zhu et al., 1996; Hartung et al., 1997; Miyake et al., 1998). Although data concerning their function in heart development are lacking, the presence of these additional Fgfs may account for the variability of the *acerebellar* heart phenotype, since the other Fgfs might partially compensate for the lack of Fgf8. The stronger effect on *nkx2.5* expression in myocardial precursors of inhibitor-treated embryos compared to the expression in *acerebellar* mutants, and the loss of ventricular tissue after late inhibitor treatment are consistent with this possibility.

Our analysis of the vascular system in *acerebellar* mutants shows that the defect in circulation is largely, if not exclusively, due to the severely dysmorphic heart. This is consistent with the fact that *fgf8* is expressed during several stages of heart development. *fgf8* expression is closely associated with some or all heart precursor cells from blastula stage onwards and is later on expressed in the ventricle of the heart proper. Since Fgf8 is likely to be a secreted molecule (Baird, 1994; Fernig and Gallagher, 1994; Fürthauer et al., 1997; Reifers et al., 1998), it may very well function in signaling processes in the

heart mesoderm. Consistent with this possibility, the Fgf receptors *fgfr1, fgfr2* and *fgfr4* are expressed during vertebrate heart development (Orr-Urtreger et al., 1991; Peters et al., 1992; Sugi et al., 1995; Thisse et al., 1995). Functional inactivation of the four mouse Fgf receptors causes either no heart phenotype or results in early embryonic lethality preventing analysis of heart development (Yamaguchi et al., 1991; Deng et al., 1994; Arman et al., 1995; Colvin et al., 1996; Weinstein et al., 1998). Our study predicts that Fgfrs probably also function in heart development, possibly in a redundant fashion. In vitro binding studies of Fgf8 protein to Fgfrs suggest Fgfr3 and Fgfr4 as the most likely candidates (MacArthur et al., 1995; Ornitz et al., 1996; Blunt et al., 1997).

Fgf8 requirement for initiation of cardiac gene expression

Our results demonstrate that Fgf8 is necessary for initiation of cardiac gene expression, since the onset and early expression of *gata4*, *nkx2.5* and, less severely, *nkx2.7* in the heart precursor field are affected in *acerebellar* mutant embryos. *acerebellar* embryos also display minor defects in mesoderm during gastrulation (Reifers et al., 1998). These defects are however unlikely to account for the heart phenotype described here, since *fgf8* is expressed in and can function independently in the developing heart precursors. The results of our Fgf8 bead implantations and of the inhibitor treatment both argue that Fgf8 functions in the heart primordium independently of its expression in general endomesodermal precursors during gastrulation (Reifers et al., 1998). Fgf8 function is required for the expression of cardiac genes for different periods of time: from its onset at the tailbud/1 somite stage, *nkx2.7* is very transiently affected, whereas *gata4* and *nkx2.5* are strongly affected until mid- and late somitogenesis, respectively. After this early requirement, the expression recovers at least in the remaining heart cells of *acerebellar* mutants. This recovery most likely reflects functional redundancy with other Fgf family members at later stages in cardiac mesoderm. Recent studies in frog and mouse demonstrated that the *tinman*-related *Nkx* genes in vertebrates are essential for heart development (Grow and Krieg, 1998; Tanaka et al., 1999). In addition, it has been shown in vitro that GATA4 enhances cardiogenesis (Grepin et al., 1997). Since GATA4 may cooperate with Nkx2.5 in activating downstream cardiac genes, and since GATA4 can act as a transcriptional activator of cardiac *Nkx2.5* expression (Durocher et al., 1997; Durocher and Nemer, 1998; Searcy et al., 1998; Lien et al., 1999), it appears that these two transcription factors work within a transcriptional network to drive cardiac development. Since both factors are affected very early in *acerebellar* mutant embryos, we suggest that the failure of early myocardial *nkx2.5* and *gata4* expression may largely account for the severe malformation of the mutant hearts at later stages.

gata6, another cardiac transcription factor of the GATA family (Kelley et al., 1993; Heikinheimo et al., 1994; Laverriere et al., 1994; Jiang and Evans, 1996), is not affected in *acerebellar* mutants. *gata6* expression may therefore occur independently of Fgf signaling. The presence of *gata6*-positive cells also demonstrates that the failure to activate proper expression of *nkx2.7*, *nkx2.5* and *gata4* is not due to the absence of the cardiac precursors, a possibility that was raised by Fgf involvement in cell migration processes (Bodmer and Venkatesh, 1998; Sun et al., 1999).

Signaling events in the cardiac primordium

As the loss-of-function situation in *acerebellar* mutants clearly demonstrates, *gata4* expression is initially dependent on Fgf8 function. In addition, *fgf8* is sufficient to restore *gata4* expression in the endogenous domain. This suggests that Gata4 may be a target for Fgf8 signaling. Interestingly, however, *fgf8* RNA injections cannot restore *nkx2.5* expression, while a bead applied after gastrulation as a localized source for Fgf8 protein is able to do so. One possible explanation is that an additional factor is needed for proper *nkx2.5* expression, which might have become delocalized or suppressed by the dorsalizing effect that *fgf8* RNA overexpression has on the zebrafish gastrula (Fürthauer et al., 1997; Reifers et al., 1998). A candidate for this additional signal is BMP2, since BMP2-coated beads or BMP2-producing cells can induce *Nkx2.5* expression in chicken embryos (Schultheiss et al., 1997; Andrée et al., 1998). Previous exposure to BMP2 or another BMP signal in gastrulation could therefore be necessary for responsiveness of *nkx2.5* to Fgf8 induction. Our observation that *nkx2.5* expression is not restored by *fgf8* RNA injections into *acerebellar* mutants is consistent with this possibility, since *fgf8* overexpression during gastrulation suppresses *bmp2* expression (Fürthauer et al., 1997), thereby eliminating the required second signal at early stages. In Fgf8 bead implanted embryos, *bmp2* expression in gastrulation is not affected due to the later stage of implantation, hence allowing rescue of *nkx2.5* expression (Fig. 4E).

Several lines of evidence suggest a role for BMP2 in cardiogenesis. (i) *BMP2* is expressed close to precardiac mesoderm and later on in the heart (Lyons et al., 1989, 1990; Schultheiss et al., 1997; Nikaido et al., 1997; Andrée et al., 1998). *BMP2* mutant embryos show cardiac defects, and a subset of the *BMP2* mutant embryos does not express *Nkx2.5*. BMP2 may therefore be directly required for cardiogenesis, although the alternative possibility that the heart abnormalities are secondary to altered patterning of the gastrula cannot yet be ruled out (Hogan, 1996; Zhang and Bradley, 1996; Kishimoto et al., 1997). (ii) The *Nkx2.5* heart enhancer contains partial consensus sequences for the Smad transducers of BMP signaling. Although this element also contains GATA binding sites that are required for expression (Searcy et al., 1998; Lien et al., 1999), they cannot be sufficient since Fgf8-driven *gata4* expression does not restore *nkx2.5* expression in *acerebellar* embryos. (iii) The general idea that *Nkx2.5* expression is regulated by the BMP pathway is also suggested by *Drosophila* studies (Frasch, 1995). (iv) In vertebrates, Lough et al. (1996) demonstrated in vitro that the combined action of BMP2 and FGF4, but neither factor alone, promotes cardiogenesis in non-precardiac mesodermal explants. Taken together, these data and our observations suggest that Bmp2 may be the second signal needed and that Fgf8 cooperates with Bmp signals to initiate cardiogenesis (Fig. 4H). A similar, but antagonistic cooperation between FGF and BMP signals has been suggested to occur in tooth development (Neubüser et al., 1997).

Interestingly, Fgf8 may be differentially required for the expression of *nkx2.5*. The caudal part of the *nkx2.5* expression appears to be more dependent on functional Fgf8 than the

rostral part, since this caudal subdomain is always affected more strongly in *acerebellar* mutants. These cells contribute to the otic vesicle (Serbedzija et al., 1998), which also shows malformations in *acerebellar* mutants (Brand et al., 1996; S. Léger and M. B., unpublished). The anterior notochord has been shown to produce an inhibitory effect on the caudal-most *nkx2.5* expression (Goldstein and Fishman, 1998), and the possible differential requirement for Fgf8 in this domain suggests that Fgf8 may counteract this inhibitory signal from the notochord in wild-type embryos. This idea is further supported by our Fgf8 bead implantations into wild-type embryos, which result in a caudal extension of *nkx2.5* expression. The same expansion can also be observed in *ntl* mutants lacking a normal notochord, or after notochord ablation in wild-type embryos (Goldstein and Fishman, 1998). Thus, in this circumstance an Fgf signal, probably Fgf8, is even sufficient to activate *nkx2.5* expression in a longitudinal domain of the lateral plate mesoderm.

The involvement of Bmp and Fgf signals in cardiac induction may provide an explanation for the ability of the heart precursor field to regulate itself after laser ablation (Serbedzija et al., 1998), since non-ablated cells in the lateral plate mesoderm, which usually would not give rise to heart tissue, may now be driven into cardiac fate by the inductive signals they receive. Since *fgf8* continues to be expressed in the heart, it may well perform, apart from its role in induction of early gene expression in heart precursors, additional functions in development of the heart, e.g. in polarization or control of proliferation, as has been proposed for other tissues (Picker et al., 1999). Human *fgf8* mutations are not yet known, but given our findings it is conceivable that heart disease would be among the symptoms.

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