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Zebrafish *tinman* homolog demarcates the heart field and initiates myocardial differentiation

Jau-Nian Chen and Mark C. Fishman*

Cardiovascular Research Center, Massachusetts General Hospital, and Department of Medicine, Harvard Medical School, 149 13th Street, Charlestown, MA 02129, USA

*Author for correspondence (e-mail: fishman@cvrc.mgh.harvard.edu)

SUMMARY

The fashioning of a vertebrate organ requires integration of decisions of cell fate by individual cells with those that regulate organotypic form. Logical candidates for this role, in an organ such as the heart, are genes that initiate the differentiation process leading to heart muscle and those that define the earliest embryonic heart field, but for neither class are genes defined.

We cloned zebrafish *Nkx2.5*, a homolog of the *tinman* homeodomain gene needed for visceral and cardiac mesoderm formation in *Drosophila*. In the zebrafish, its expression is associated with cardiac precursor cells throughout development, even in the early gastrula, where the level of zebrafish *Nkx2.5* is in a gradient which spatially matches the regional propensity of ventral-marginal cells to become heart. Overexpression of *Nkx2.5* causes formation of disproportionally larger hearts in otherwise apparently normal embryos. Transplanted cell expressing high levels of *Nkx2.5* express cardiac genes even in ectopic locales. Fibroblasts transfected with myc-tagged *Nkx2.5* express cardiac genes. These effects require the homeodomain.

Thus, *Nkx2.5* appears to mark the earliest embryonic heart field and to be capable of initiating the cardiogenic differentiation program. Because ectopic cells or transfected fibroblasts do not beat, *Nkx2.5* is likely to be but one step in the determination of cardiac myocyte cell fate. Its overexpression increases heart size, perhaps by bringing cells on the edge of the field to a threshold level for initiation of cardiac differentiation.

Key words: *Nkx2.5*, heart, zebrafish, myocardial differentiation, *tinman*

INTRODUCTION

The cardiac field, a demarcated region of the embryo which provides progeny to the heart, was first defined as part of the lateral plate mesoderm which, when explanted at gastrulation or neurulation stages, generates beating tissue (Rawles, 1943; DeHaan, 1965). Cells in this region express cardiac markers, including myosin heavy chains (Litvin et al., 1992). By single cell lineage analysis in the zebrafish, we have found that a demarcated zone for generating heart is in evidence even earlier than can be marked by current probes and prior to cardiac specification. This field is in the ventral hemisphere of the late blastoderm, with the ventral cells having peak propensity to form heart which tapers off laterally (Lee et al., 1994).

What transcription factors initiate cardiomyogenic differentiation are not known. The skeletal myogenic factors do not play a role in heart development (Olson, 1993). Recently discovered basic HLH factors, dHAND and eHAND, may be important, but their reduction, even in combination, does not appear to eliminate initiation of heart differentiation, although it perturbs later development (Srivastava et al., 1995). If heart cell differentiation really begins in the field, it is quite likely that the molecules that begin to establish the cardiac cell fate are not the same as those that complete the process. This is because the fate of cells in the field is plastic, such that removal or introduction of a few cells does not perturb the eventual organ (Huxley and De Beer, 1934). Furthermore, not all cells in the field contribute progeny to the heart. These features are true for the heart field of zebrafish (Lee et al., 1994).

Tinman is a homeodomain protein of *Drosophila* which is genetically directly downstream of the mesoderm determining gene, *twist*, and which is responsible for development of the visceral and cardiac subdivisions of the mesoderm (Bodmer, 1993). A similar homeodomain has been noted in vertebrate genes, *mNkx2.5* (or *Csx*) in the mouse (Lints et al., 1993; Komuro and Izumo, 1993), *XNkx2.5* and *XNkx2.3* in the frog (Tonissen et al., 1994; Evans et al., 1995), and *cNkx2.5* in chicken (Schultheiss et al., 1995), all of which are expressed in the precardiac mesoderm. In the mouse, *mNkx2.5* is first detected in myocardiogenic progenitor cells as early head fold stage and its expression remains in myocardial cells to adult (Lints et al., 1993; Komuro and Izumo, 1993). In the frog, *XNkx2.5* and *XNkx2.3* transcripts can be detected by whole-mount in situ hybridization in the cardiac precursors from neurula stage (Tonissen et al., 1994; Evans et al., 1995). In chicken, *cNkx2.5* is first detected in the cardiac progenitors from stage 5 (Schultheiss et al., 1995). Targeted gene mutation of *Nkx2.5* causes arrest of cardiac development at the looping stage (Lyons et al., 1995).

We find that zebrafish *Nkx2.5* is expressed in a spatial and

temporal fashion strongly suggesting that it plays a role early in cardiac myocyte differentiation, and it is capable of causing cells to assume some, but not all, attributes of heart cells, both in vivo and in vitro. In vivo, it can regulate both heart size and at higher doses, ventralize the animal. It is a candidate for initiating cell fate and for tying organotypic size to the essential body plan.

MATERIALS AND METHODS

Cloning of zebrafish *Nkx2.5* cDNA

We generated a zebrafish adult heart cDNA library in λZAPII (Stratagene) using poly(A)$^+$ RNA from adult hearts. We screened the library with probes generated by RT-PCR, using degenerate oligonucleotides based on the tinman domain and homeodomain of *Drosophila tinman* and mouse *Nkx2.5* as primers and RNA from 24-hour zebrafish embryos or adult hearts as templates. Initial denaturation was at 94°C for 1 minute, and was followed by three temperature cycles at 94°C for 1 minute, 56°C for 2 minutes, and 72°C for 2 minutes, with final extension at 72°C for 2 minutes.

Whole-mount in situ hybridization and immunohistochemistry

The digoxigenin-labeled antisense full-length *Nkx2.5* RNA probe was transcribed using T7 RNA polymerase (Promega). Whole-mount in situ hybridization was carried out essentially as described by Oxtoby and Jowett (1993). In brief, embryos were fixed with 4% paraformaldehyde, digested with proteinase K, and hybridized with the zebrafish *Nkx2.5* probe at 67°C. Alkaline-phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) was used to detect the zebrafish *Nkx2.5* signals. After staining with NBT/X-phosphate (Boehringer Mannheim), embryos were refixed with 4% paraformaldehyde and stored in PBS.

Whole-mount immunohistochemistry was carried out as described by Chen et al. (1996). In brief, embryos were fixed in 4% formaldehyde in PBS for 1 hour, blocked in 0.3% saponin, 10% goat serum, in PBS, for 1 hour, and incubated with anti-myosin heavy chain antibodies at 4°C overnight. Alkaline phosphatase or fluorescein-conjugated anti-mouse IgG antibody (Boehringer Mannheim) were used as secondary antibody. MF20 reacts with both the skeletal and cardiac myosin heavy chain in zebrafish. S46 is specific to the atrial-myosin heavy chain in zebrafish (Stainier and Fishman, 1992). MF20 was purchased from the Hybridoma Bank. S46 is a kind gift from Dr J. Miller.

Nkx2.5 injection

For injection, full length, capped RNA was generated from the *Nkx2.5* plasmid (or, for control, *lacZ*) or *Nkx2.5* lacking the homeodomain (ΔHD)) and injected into zebrafish embryos at the 1- to 2-cell stage. The injected embryos were raised at 28.5°C. Embryos used for analyzing the expression of dorsal and ventral markers were fixed 6 hours after fertilization in 4% paraformaldehyde. Otherwise, embryos were scored after 2 days of development for late effects. Approximately 100 pg of *Nkx2.5* RNA was used for the lower dose injection and 250 pg was used for the higher dose injection.

Cell transplantation

250 pg of *Nkx2.5* RNA was mixed with tetramethylrhodamine dextran and biotin dextran (Molecular Probes) in 0.2 M KCl, and injected into the donor embryos at the 1- to 2-cell stage and blastomeres from the donor were transplanted to the host embryos using methodology as described by Lee et al. (1994), but in an animal pole position. After 36 hours of development at 28°C, embryos were fixed with 4% paraformaldehyde and analyzed with the anti-myosin heavy chain antibodies, MF20 or S46.

Transfection

Full-length zebrafish *Nkx2.5* and an internal deletion construct which lacks the homeodomain (ΔHD) were subcloned to a eucaryotic expression vector, pBMN (Messersmith et al., 1995) (a kind gift from Dr Alex L. Kolodkin), in frame with the myc-epitope. The constructs were then transfected to the zebrafish fibroblast cell line, PAC2 (Lin et al., 1994; a kind gift from Dr Nancy Hopkins), using lipofectin (Gibco). The transfected cells were fixed for immunohistochemistry 3 days after transfection.

RESULTS

Zebrafish *Nkx2.5* is expressed in cardiac precursors

We cloned a zebrafish homolog of *Drosophila tinman*, termed zebrafish *Nkx2.5* because of its homology to *Nkx2.5* genes. Zebrafish *Nkx2.5* shares high homology with *Drosophila tinman* only in the N-terminal tinman domain (TN domain) and the C-terminal homeodomain, which is the DNA binding domain. Its sequence is conserved with other vertebrate homologues in the NK2 domain, which may have transcriptional repression activity (Chen and Shwartz, 1995) (Fig. 1). The predicted amino acid sequence of zebrafish *Nkx2.5* is 98%, 98%, 91%, 92% and 63% identical in the homeodomain to *XNkx2.5*, *cNkx2.5*, *XNkx2.3*, *mNkx2.5* and *Drosophila tinman*, respectively, but is only 73%, 81% and 73% identical to *XeNk2*, *TTF-1* (*Nkx2.1*) and *mNkx2.2*. In the Nk2 domain, zebrafish *Nkx2.5* is 94%, 94%, 83%, and 83% identical to *XNkx2.5*, *cNkx2.5*, *XNkx2.3* and *mNkx2.5*, but is only 78%, 78% and 72% identical to *XeNk2*, *TTF-1* (*Nkx2.1*) and *mNkx2.2*, respectively. In the TN domain, there is an amino acid change, from lysine to arginine, in zebrafish compared to other vertebrate *Nkx2.5* genes and *Drosophila tinman* (Fig. 1B).

Zebrafish *Nkx2.5* expression correlates with the position of cardiac precursors. In zebrafish, cardiac progenitors reach the embryonic axis by the 8-somite stage and form bilateral tubes by the 18-somite stage. These tubes then migrate towards, and fuse, at the midline (Stainier and Fishman, 1993). Bilateral *Nkx2.5*-expressing cells are noticeable by the 10-somite stage (Fig. 2A) and form bilateral tubes ventral to the neural tube by the 18-somite stage, as shown in transverse section in Fig. 2B. These tubes migrate towards the midline, where they begin to fuse at the 20-somite stage (Fig. 2C). The fusion is complete by 24-somite stage (Fig. 2D). The expression of zebrafish *Nkx2.5* in the myocardial cells continues to the adult heart (data not shown). In contrast to other vertebrate *Nkx2.5* genes, where *Nkx2.5* appears to be present in both cardiac precursors and endoderm (Evans et al., 1995; Lints et al., 1993; Schultheiss et al., 1995; Tonissen et al., 1994), we find no evidence of *Nkx2.5* expression other than in the mesoderm in the region of cardiac precursors and in defined heart tissue (Fig. 2E,F).

Zebrafish *Nkx2.5* demarcates the heart field

Zebrafish *Nkx2.5* expression also differs from other described vertebrate *Nkx2.5* genes, and more resembles *Drosophila tinman*, in that it is expressed earlier in embryogenesis. In *Drosophila*, *tinman* is expressed during involution of the mesoderm and then becomes progressively restricted to the cardiac mesoderm (Bodmer et al., 1990). In mouse and frog, *tinman* homologs are not detectable by whole-mount in situ hybridization during gastrulation. However, low levels of *XNkx2.5* and *XNkx2.3* transcripts are detected by RNase pro-

tection assay at the end of gastrulation (Evans et al., 1995; Lints et al., 1993; Tonissen et al., 1994). In chick, *cNkx2.5* transcripts can be detected during gastrulation, but only after stage 5 (Schultheiss et al., 1995). In the zebrafish, *Nkx2.5* expression is ubiquitous prior to gastrulation (not shown). This expression is likely to be predominantly maternal in origin because zygotic transcription begins in the zebrafish only at about the 1000-celled stage (Kane and Kimmel, 1993). *Nkx2.5* becomes regionalized abruptly to half of the margin of the embryo at 50% epiboly, just at the onset of gastrulation (Fig. 3A). This crescent is at the ventral margin, as shown in Fig. 3B, using *goosecoid* as a dorsal reference. The *Nkx2.5* expressing cells then migrate in the hypoblast (the term used to refer to deeper cell layers during zebrafish gastrulation; Fig. 3C). This position correlates with the position of cardiac progenitors on the fate map. Lineage tracing shows heart precursors to originate from cells in the ventral marginal zone of the blastula, where they involute and converge towards the embryonic axis (Lee et al., 1994). There is a gradient of propensity to generate heart progeny, which peaks at the margin at the ventral axis, and diminishes in a gradient dorsally (Lee et al., 1994). *Nkx2.5* expression is in a matching gradient with a ventral peak. The correlation between the pattern of *Nkx2.5* expression and the propensity to become heart cells suggests that there may be a threshold level of *Nkx2.5* which initiates the cardiac program and that more cells achieve this threshold in the ventral and lateral regions than at the dorsal margin. If so, then increased *Nkx2.5* level should bring more cells to threshold and thereby affect heart formation.

Overexpression of zebrafish *Nkx2.5* causes an enlarged heart

We increased the *Nkx2.5* level by injection of zebrafish *Nkx2.5* RNA (100 pg) at the 1-2 cell stage. The resulting embryos develop normally, but have a visibly enlarged heart (29.5% of surviving injected embryos, *n*=112). The embryos have mild edema, but otherwise appear unperturbed. The heart beat and circulation appear normal. Both chambers are proportionally bigger. Comparison of atrial sizes of control and *Nkx2.5* enlarged hearts are shown in Fig. 4A and B, respectively. Uninjected embryos (*n*=65), as well as the embryos injected with *lacZ* (*n*=86), or ΔHD, a *Nkx2.5* RNA in which the homeodomain is deleted (*n*=72), are not so affected.

To quantitate the effect and to examine if the enlargement is due to an increase in cell number, we counted cell nuclei on histological sections by DAPI staining. The cells of embryonic zebrafish heart appear mononuclear. Therefore, we assume that the number of nuclei represents the number of cells of the embryonic zebrafish heart. The enlarged hearts from *Nkx2.5*-injected embryos have 30% more cells than do those of embryos injected with *lacZ* (*Nkx2.5*-injected mean heart cell number = 2438±171.4, s.e.m., *n*=8 hearts, 55±3 sections per heart; *lacZ*-injected mean heart cell number = 1885±38.5, s.e.m., *n*=5 hearts, 52±5 sections per heart; significant, $P<0.05$.) We assume that the variability reflects different effectiveness of injection or retention of RNA.

A

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   1 TTGTCATAGGTACCCCTGATAAATCCCAACCGGATTATCCAAGTGGATCATCATTCCATG
                                                             M    1
  61 GCAATGTTCTCTAGCCAAATGACTTCCACTCCTTTCTCAGTGCGGGACATACTGAACCTG
     A  M  F  S  S  Q  M  T  S  T  P  F  S  V  R  D  I  L  N  L   21
 121 GAGCAGAATCAGGAGGACATGGTCTCCCTGGACATGTCTCAGCGGCTGGACAGCGCCCTT
     E  Q  N  Q  E  D  M  V  S  L  D  M  S  Q  R  L  D  S  A  L   41
 181 ATTCCGACCTCATCTTGCATGCTGTCCACTTTCAAACAGGAACAGTTCATGGAAATGCCA
     I  P  T  S  S  C  M  L  S  T  F  K  Q  E  Q  F  M  E  M  P   61
 241 TCCGGATCCTCTCTCTTCAGCGAAGACCTTCAGGAGGACAAAGGCAACAAAATCAACTCT
     S  G  S  S  L  F  S  E  D  L  Q  E  D  K  G  N  K  I  N  S   81
 301 CTTAACTTCAGTGCTTCAGGCTTTTACGCGAAGAACTTCCTAGAAATGGACTATGTTAAA
     L  N  F  S  A  S  G  F  Y  A  K  N  F  L  E  M  D  Y  V  K  101
 361 GACGCAAAGACAGATGACACATTTGAAAACAAAGAGAAAAAAGACATCGGCTGTTGTCAG
     D  A  K  T  D  D  T  F  E  N  K  E  K  K  D  I  G  C  C  Q  121
 421 GAAGACCCGGGTGAAGATCTGAAGCTGGATGATGCGGACGTGCCCAAGCAGAGGAAGAGG
     E  D  P  G  E  D  L  K  L  D  D  A  D  V  P  K  Q  R  K  R  141
 481 AGGAAGCCTCGAGTTCTCTTCTCTCAGGCGCAGGTGTACGAGCTCCAGCGACGCTTCAAG
     R  K  P  R  V  L  F  S  Q  A  Q  V  Y  E  L  Q  R  R  F  K  161
 541 CAGCAGAAATACTTGTCTGCACCAGAGAGAGACCACCTAGCCAATGTTCTCAAACTCACC
     Q  Q  K  Y  L  S  A  P  E  R  D  H  L  A  N  V  L  K  L  T  181
 601 TCCACACAGGTGAAGATCTGGTTCCAGAACAGACGATACAAGTGCAAGAGGCAGCGTCAG
     S  T  Q  V  K  I  W  F  Q  N  R  R  Y  K  C  K  R  Q  R  Q  201
 661 GATCAGACCCTGGAGATGGTGGGCATCGCACCTCCGAGACGCATCTCTGTGCCAGTTTTG
     D  Q  T  L  E  M  V  G  I  A  P  P  R  R  I  S  V  P  V  L  221
 721 GTTCGGGATGGTAAACCGTGTCTGGGAGACACGTCCACTTACAACACCTCATACAATGTG
     V  R  D  G  K  P  C  L  G  D  T  S  T  Y  N  T  S  Y  N  V  241
 781 GGAATCAATCATTTCACCTACAACACCTACCCTGCGTTTAGTAATTTTCCGAGTCCAGGC
     G  I  N  H  F  T  Y  N  T  Y  P  A  F  S  N  F  P  S  P  G  261
 841 AACTCGAACTACTCATGCAACTATCCGTCGAGCATGTCCAGCATCCAACCTTCACAGTCC
     N  S  N  Y  S  C  N  Y  P  S  S  M  S  S  I  Q  P  S  Q  S  281
 901 AACAGCAACTACATGAACTTTGGTGTTGGGGATCTAAATAACGTNCAGGCTTCATTTCAA
     N  S  N  Y  M  N  F  G  V  G  D  L  N  N  V  Q  A  S  F  Q  301
 961 TCCAGCAGTGTTCCTTCACTACATGGCATCAGAGCTTGGTGAACAAAACTACATTTACCG
     S  S  S  V  P  S  L  H  G  I  R  A  W  *                   314
1021 ACAGCATTCATCTTTCTGTGGACATTTACATTCTAATGATATTTTTGATGATGAATATTA
1081 ATTGATTCTTGAGATATGGTTTGGCTGGGATGTTTT
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B **HOMEODOMAIN**

		% of homology
zebrafish *Nkx2.5*	RRKPRVLFSQAQVYELQRRFKQQKYLSAPERDHLANVLKLTSTQVKIWFQNRRYKCKRQR	
XNkx2.5	RRKPRVLFSQAQVYEL**E**RRFKQQKYLSAPERDHLANVLKLTSTQVKIWFQNRRYKCKRQR	98%
cNkx2.5	RRKPRVLFSQAQVYEL**E**RRFKQQKYLSAPERDHLANVLKLTSTQVKIWFQNRRYKCKRQR	98%
XNkx2.3	RRKPRVLFSQAQV**F**EL**E**RRFKQQ**R**YLSAPER**E**HLAN**S**LKLTSTQVKIWFQNRRYKCKRQR	91%
mNkx2.5	RRKPRVLFSQAQVYEL**E**RRFKQQ**R**YLS**PA**ERD**Q**LA**S**VLKLTSTQVKIWFQNRRYKCKRQR	92%
TTF-1	RRK**R**RVLFSQAQVYEL**E**RRFKQQKYLSAPER**E**HLA**SMIH**LT**P**TQVKIWFQN**H**RYK**M**KRQ**A**	81%
XeNK2	**K**RK**R**RVLFS**K**AQ**T**YEL**E**RRF**R**QQ**R**YLSAPER**E**HLA**SLIR**LT**P**TQVKIWFQN**H**RYK**M**KR**A**R	73%
mNkx2.2	**K**RK**R**RVLFS**K**AQ**T**YEL**E**RRF**R**QQ**R**YLSAPER**E**HLA**SLIR**LT**P**TQVKIWFQN**H**RYK**M**KR**A**R	73%
tinman	**K**RKPRVLFSQAQV**L**EL**EC**RF**RLK**KYL**TGA**ER**EII**AQ**K**LN**LSAT**QVKIWFQNRRYK**S**KR**GD**	63%

NK2 domain		% of homology	**TN domain**	
zebrafish *Nkx2.5*	PRRISVPVLVRDGKPCLG		zebrafish *Nkx2.5*	TPFSVRDILNL
XNkx2.5	PRRI**A**VPVLVRDGKPCLG	94%	*XNkx2.5*	TPFSV**K**DILNL
cNkx2.5	PRRI**A**VPVLVRDGKPCLG	94%	*cNkx2.5*	TPFSV**K**DILNL
mNkx2.5	**A**RRI**A**VPVLVRDGKPCLG	83%	*mNkx2.5*	TPFSV**K**DILNL
XNkx2.3	PRR**VA**VPVLVRDGKPC**I**G	83%	*XNkx2.3*	TPFSV**K**DILNL
TTF-1	PRR**VA**VPVLVKDGKPC**QA**	78%	*tinman*	TPFSV**K**DILNL
XeNK2	PRR**VA**VPVLVRDGKPC**HT**	78%		
mNkx2.2	PR**CVA**VPVLVRDGKPC**HA**	72%		

Fig. 1. (A) The sequence of zebrafish *Nkx2.5*. Homeodomain (grey box), N-terminal tinman domain (black box), Nk2 domain (open box). (B) Predicted amino acid sequence comparison of the vertebrate *Nkx* homologs in tinman domain (TN domain), homeodomain, and Nk2 domain.

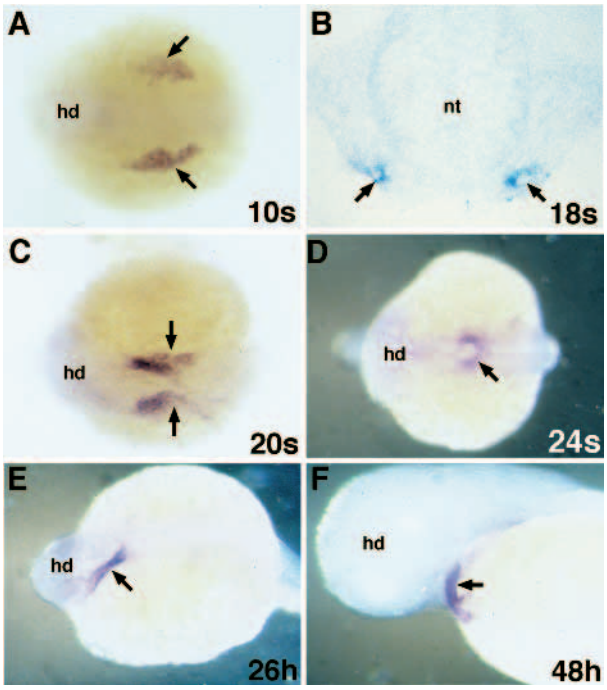

Fig. 2. (A-F) The expression pattern of zebrafish *Nkx2.5*, revealed by whole-mount in situ hybridization, correlates with the position of cardiac precursors. (A) The *Nkx2.5*-expressing cells (arrows) are located on the left and the right of the midline in part of the lateral plate, at the 10-somite stage. (B) Transverse section reveals that the *Nkx2.5*-expressing cells organize in tubes on the right and the left of the midline (arrows) ventral to the neural tube at the 18-somite stage. (C) Starting at 20-somite stage, the two tubes move toward the midline and begin to fuse at the caudal side (arrows). (D) The fusion of the primitive heart tube is complete by the 24-somite stage, shown as a ring-like structure of zebrafish *Nkx2.5*-expressing cells at the midline (arrow). (E) By 26 hours postfertilization (hpf), the heart moves to the left side of the embryo (arrow). (F) *Nkx2.5* expression is restricted to the heart in a 48 hpf embryo (arrow). Anterior to the left in all but B. A, C, D are dorsal views. E is dorsolateral view from the left side; F is left lateral view. hd, head; nt, neural tube.

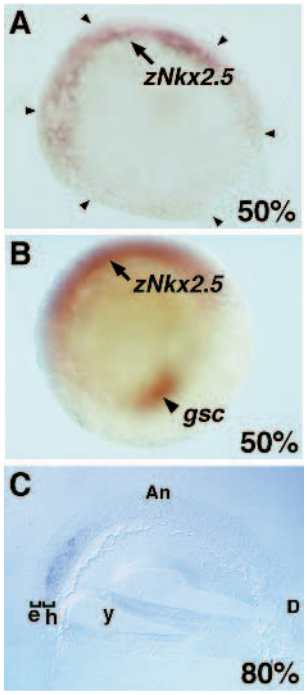

Fig. 3. (A-C) *Nkx2.5* expression can be detected at the onset of gastrulation. (A) It is restricted to the ventral margin of the embryo at 50% epiboly. The yolk is scrapped off intentionally to visualize the gradient of *Nkx2.5* and the arrowheads point to the edge of the embryo. B shows *goosecoid* expression (arrowhead) as a dorsal reference. (C) The *Nkx2.5*-expressing cells migrate in the hypoblast, the term used to refer to deeper cell layers in zebrafish gastrulation. e, epiblast; h, hypoblast; y, yolk; An, animal pole; D, dorsal.

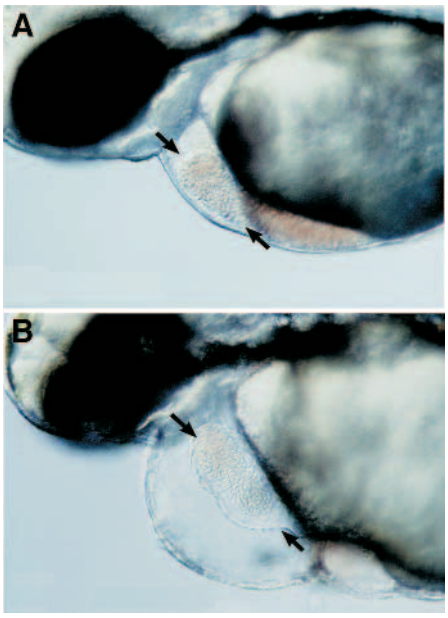

Fig. 4. Injection of *Nkx2.5* mRNA (100 pg) causes an enlarged heart. The atrium of (A) *lacZ*-injected and (B) *Nkx2.5*-injected embryos (arrows point to the border of the atrium). Embryos are oriented with dorsal to the top and anterior to the left.

Disruption of the dorsal-ventral axis by higher levels of *Nkx2.5*

Embryos injected with higher levels of *Nkx2.5* RNA (250 pg) manifest more generalized deficits, which have characteristics referred to as ventralization (Graff et al., 1994), with diminution of dorsal and anterior structures, including the head, eyes, notochord and somites (64%, *n*=104). Fig. 5B shows a severe case, in which the head and eyes are missing, the notochord is diminished, and the somites are fused. More blood cells are observed in the injected embryos. These dorsal-ventral axial effects are evident by the onset of gastrulation, with reduction of the expression of the dorsal marker, *goosecoid* (Fig. 5C,D) and expansion of the domain of a ventral marker, *eve1* (not shown), and of a ventral lateral marker, *gata2* (Fig. 5E,F). Because *Nkx2.5* expression is expressed in the hypoblast and *gata2* and *eve1* in the epiblast (Detrich et al., 1995; Joly et al., 1993), it is likely that these effects are cell non-autonomous.

As described for other manipulations which reduce dorsal-anterior structures in *Xenopus* (Danos and Yost, 1995), there is an accompanying randomization of cardiac looping in the *Nkx2.5* injected fish (55% of the surviving injected embryos loop incorrectly, *n*=104). Such effects are not observed in the control embryos (uninjected, *lacZ* injected or ΔHD injected; *n*=54, 67 and 98, respectively).

Higher doses cause additional cardiac tissue

Many fish injected with these higher levels of zebrafish *Nkx2.5* have cells which express myosin heavy chain in ectopic locations (22%; *n*=104). In nearly all cases such cells are scattered. About half of the time (12.5%; *n*=104), these are accompanied as well by the presence of additional beating structures in the vicinity of the normal heart (Fig. 6A,B). These embryos are quite deformed and have axial defects, so this

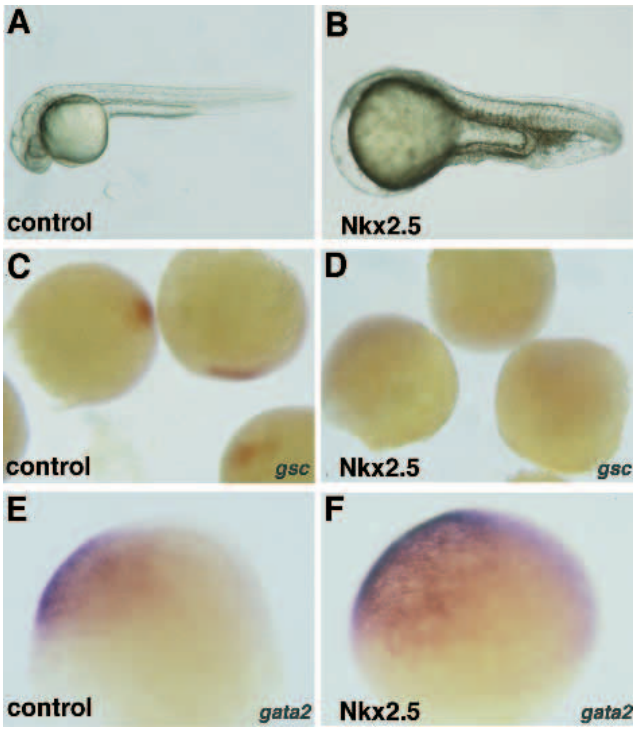

Fig. 5. Dorsoventral axial defects are caused by higher-dose *Nkx2.5* RNA injection. (A) Control embryo after 1 day of development. (B) A severely affected zebrafish embryo after *NKx2.5* injection. The head and eyes are missing, notochord is diminished and the somites are fused. (C-F) Effects on the dorsoventral axis are evident during gastrulation, with (C,D) reduction of the expression of the dorsal marker, *goosecoid*, and (E,F) expansion of the domain of a ventral lateral marker, *gata2* . C and E show embryos injected with 250 pg *lacZ* mRNA, probed with *gsc* and *gata2* respectively. D and F show embryos injected with 250 pg zebrafish *Nkx2.5* RNA, and probed with *gsc* and *gata2*, respectively.

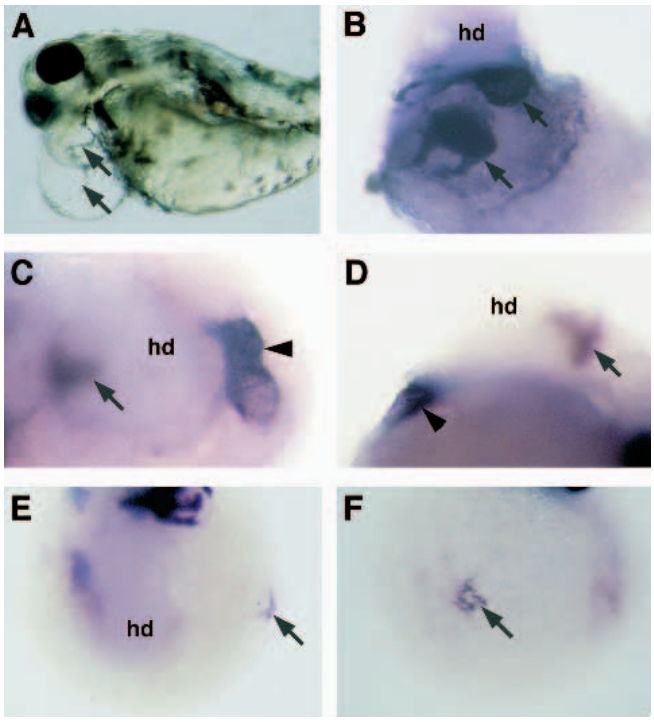

Fig. 6. Injection of higher doses of *Nkx2.5* mRNA (250 pg) causes ectopic myosin heavy chain expression. In the chest region, this is evident as (A) two beating hearts (arrows), shown in their two pericardial sacs or (B) with MF20 labeling (arrows). Elsewhere, patches of ectopic MF20 immunoreactivity may be found (C,D) in the brain (arrows) or (E,F) on the yolk (arrows). The other MF20 immunoreactive structure is the heart (arrowhead). Embryos are oriented with dorsal to the top and anterior to the left in A,D and F. B is a ventral view and C and E are dorsal views of the embryo. hd, head.

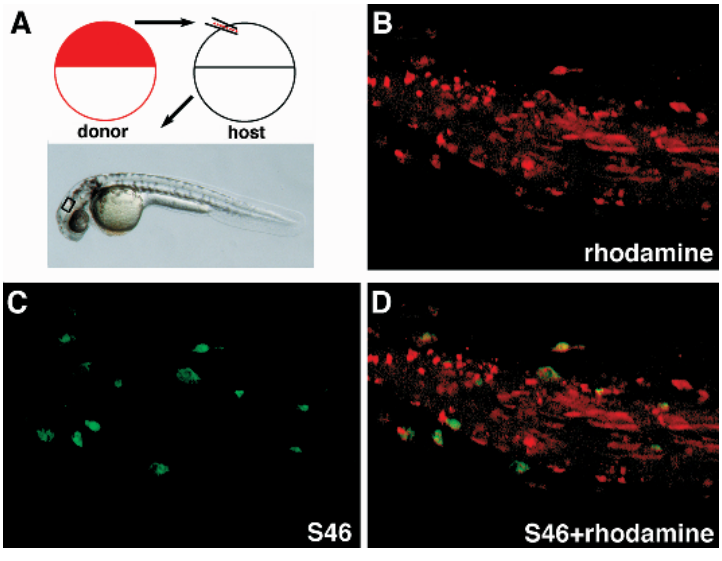

Fig. 7. Transplanted *Nkx2.5*-expressing cells express cardiac markers in ectopic locations. (A) Schematic of method used to coinject zebrafish donor embryos at the 1- to 2-cell stage with *Nkx2.5* RNA and tetramethylrhodamine dextran as described by Lee et al. (1994). A few cells were transplanted to the animal pole of an unlabelled host. They subsequently populated the brain or trunk. B, C and D are confocal microscope images of the brain region shown in A. (B) Donor cells labeled by tetramethylrhodamine dextran. (C) A subset of cells in the same region are reactive to the atrial-specific antibody, S46. (D) A superimposed image of B and C, showing that all S46 immunoreactive cells are also rhodamine-labeled, and hence derived from the donor.

effect may represent a block to fusion of cardiac progenitors (De Haan, 1959) rather than generation of new heart tissue. Rarely, there are patches of cells expressing myosin heavy chain which can be in the brain (2%; *n*=104) (Fig. 6C, D) or on the yolk (4%; *n*=104) (Fig. 6E,F). These patches are immunoreactive for MF20, which also recognizes skeletal muscle, or, in separate experiments, for S46 which, in zebrafish, is specific for the cardiac atrium (Stainier and Fishman, 1992) (not shown).

Transplanted cells express cardiac genes at ectopic locations

The generation of ectopic cardiac tissue in these deformed embryos could be due to cell-autonomous activation of cardiac genes, but, alternatively, could be the result of disruption of the embryonic axis or the interruption of the normal migratory paths of precardiac cells. To examine whether zebrafish *Nkx2.5* can autonomously initiate cardiac differentiation, we transplanted *Nkx2.5* overexpressing cells, marked by tetramethylrhodamine-conjugated dextran, into late blastrula unmarked hosts, at positions where they do not contribute to the heart (Kimmel et al., 1995, 1990; Lee et al., 1994) (Fig. 7A). Embryos subject to this procedure appear normal and, in all embryos raised successfully (*n*=26), all of the transplanted cells migrate to the brain or trunk, as the fate map predicts. This suggests that the migratory path of the transplanted cells is not distorted by overexpression of zebrafish *Nkx2.5*. In most of the embryos (20/26), a subset of transplanted cells become myosin heavy chain-immunoreactive in the ectopic locations, such as the brain and the trunk. Interpretation of MF20 labeling in the trunk is complicated by its concomitant marking of the skeletal muscle along with cardiac muscle. However, because a subset of the transplanted cells also express the atrial-specific marker, S46, it is clear that zebrafish *Nkx2.5* can initiate adoption of the cardiac fate, even in ectopic locations (Fig. 7B-D).

Nkx2.5 activates cardiac gene expression in fibroblast cells

We were curious as to why not every transplanted cell expresses myosin heavy chain. In the transplantation experiment, we are not able to distinguish whether the variability is due to differences of protein levels within transplanted cells or modification by the environment. To address this issue, we transfected the zebrafish fibroblast cell line, PAC2, with zebrafish *Nkx2.5* tagged with the myc epitope, which can be recognized by the antibody, 9E10. About 20% of the transfected cells are positive for 9E10, and hence are presumably generating *Nkx2.5* protein. MF20 and 9E10 have different IgG subtypes, which allows us to do double staining with these antibodies. 90% of zebrafish *Nkx2.5*/myc-expressing fibroblasts adopt these attributes of cardiac cell fate, defined by MF20 labeling (*n*=234) (Fig. 8A,B). In other experiments, about the same ratio of the *Nkx2.5* transfected cells are positive for S46 (not shown; S46 has the same IgG isotype as 9E10, so cannot be simultaneously compared). myc-tagged constructs in which zebrafish *Nkx2.5* lacks the homeodomain do not cause myosin heavy chain expression (*n*=147) (Fig. 8C,D). These data indicate that zebrafish *Nkx2.5* acts cell-autonomously with regard to myosin heavy chain expression, and that this effect requires the homeodomain.

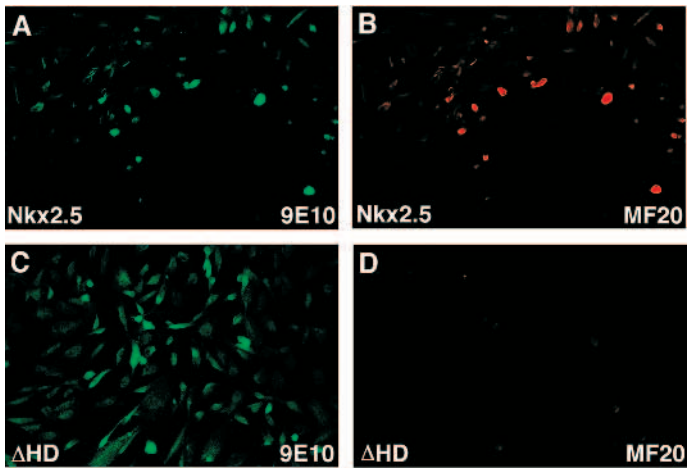

Fig. 8. *Nkx2.5* activates cardiac genes in fibroblasts. A and B are images of the same field after transfection with myc-tagged *Nkx2.5*. C and D are of the same field after transfection with myc-tagged *Nkx2.5* missing the homeodomain. (A,B) All of the cells expressing the product of the transfected gene, as revealed by immunoreactivity for the myc tag (A), are also immunoreactive for MF20 (B). (C) The myc-tagged construct with *Nkx2.5* missing the homeodomain transfects cells equally well, such that they become immunoreactive for 9E10 but (D) no cells express MF20.

DISCUSSION

The zebrafish *Nkx2.5* gene appears tied to generation of the heart. From the site of the early field in the ventral marginal zone, to the lateral plate, and then to the heart tube, its pattern of expression corresponds tightly to the location of heart precursors. Its overexpression enlarges heart size, an effect which can occur in the absence of other effects upon the embryo. Transplanted cells expressing *Nkx2.5* can assume a cardiac fate, even in distant ectopic sites such as the brain, and it causes fibroblasts in culture to express myosin heavy chain. However, these cells do not beat, suggesting that *Nkx2.5* is one step, perhaps the first, in determining cardiac cell fate, but that subsequent signals are needed to complete differentiation.

Nkx2.5 is a conserved metazoan gene related to heart

In *Drosophila*, *tinman* is believed to function just downstream of the maternal mesoderm determining genes, including the transcription factor, *twist*. *tinman* is expressed first throughout the mesoderm, and then restricted to visceral and cardiac mesoderm (Bodmer, 1995; Bodmer et al., 1990). Embryos mutant in *tinman* lack visceral mesoderm derivatives and heart (Bodmer, 1993). The target genes for *tinman* are unknown. Vertebrate homologs are related to *Drosophila tinman* in the homeodomain and in an 11 amino acid stretch near the amino terminus. Unlike *tinman*, they also share a short peptide domain of homology downstream of the homeodomain (Lints et al., 1993). In other vertebrates, they are largely, but not totally, restricted to cardiogenic areas and the heart. In the mouse, *mNkx2.5* is expressed in the pharyngeal endoderm and the tongue, lingual muscle, spleen and stomach, in addition to myocardial cells (Lints et al., 1993). In the frog, both *XNkx2.5* and *XNkx2.3* are expressed in the pharyngeal endoderm (Tonissen et al., 1994; Evans et al., 1995). In chick, *cNkx2.5*

is also expressed in the pharyngeal ectoderm and endoderm (Shultheiss et al., 1995). The functions of *Nkx* genes in these tissues are not yet clear. The heart of mice mutant in *Nkx2.5* fails to develop through looping, a stage apparently sensitive to loss of several genes (Lyons et al., 1995), so that, although clearly critical to heart development, the stage at which *Nkx2.5* acts has not been defined by the targeted mutation. If *Nkx* genes are essential for initiating the cardiac cascade, continued differentiation in mice with the *Nkx2.5* mutation may reflect the presence of redundant pathways. Indeed, *Nkx2.3* is expressed in a similar domain, at least in frog (Evans et al., 1995). Therefore, the loss of function experiments do not preclude a critical role of *Nkx2.5* in early steps of cardiomyogenesis.

Is the zebrafish *Nkx2.5* really essentially distinct from other vertebrate homologs? This seems unlikely. The pattern of zebrafish *Nkx2.5* expression is similar to other vertebrate homologs in that it is expressed in the cardiac precursors. It differs from other vertebrate *Nkx2.5* genes in cardiac specificity and early onset of expression. Although the role of fish *Nkx2.5* might differ from other vertebrates, for example because the dynamics of fish gastrulation are distinctive, it is equally plausible that the embryonic transparency of the zebrafish facilitates visualization of low levels of expression. Along these lines, it is interesting that the expression of *XNkx2.5* is evident during gastrulation by RNase protection at times prior to evident expression by whole mount in situ hybridization (Tonissen et al., 1994; Evans et al., 1995).

Nkx2.5 and heart cell fate

One proposed role of *Nkx2.5* is in the cascade which drives assumption of the cardiomyogenic phenotype (Bodmer, 1995; Olson and Srivastava, 1996). We provide three lines of evidence to support this. (1) Overexpression of *Nkx2.5* causes ectopic expression of myosin heavy chain immunoreactivity, including that identified by the cardiac-specific antibody S46. Such cells are generally scattered, but occasionally cluster on yolk or brain. (2) Cells transplanted from embryos injected with *Nkx2.5* can express cardiac markers even in ectopic locales, suggesting that the effect is cell-autonomous. (3) Fibroblasts transfected with *Nkx2.5* express cardiac markers.

Nkx2.5 does not appear sufficient to cause expression of the entire repertoire of cardiac genes. Although cardiac-specific reagents for the zebrafish are limited, it is clear that differentiation is incomplete because ectopic cells expressing *Nkx2.5* do not beat. The two hearts noted in some of the deformed embryos are as likely to represent secondary effects of perterbation as they are induction of an ectopic heart. Interuption of midline signals can prevent fusion of left and right primordia, an abnormality termed cardia bifida (DeHaan, 1959). As suggested (Nascone and Mercola, 1995), *Nkx2.5* could be the first step in cardiac differentiation, with subsequent signals needed to finish the process, some presumably from endoderm. This model also is compatible with the observation that *Nkx2.5* is expressed by cells in the early gastrula at a time when cells are still plastic and can assume fates dictated by host locale after transplantation. It is likely that persistence of *Nkx2.5* expression also is needed for cardiac differentiation. In *Drosophila* and vertebrates, not all the cells which express *tinman* or *Nkx2.5* adopt the cardiac fate. In *Drosophila*, *tinman* is first expressed in all mesoderm and gradually becomes restricted to the cardiac precursors (Bodmer et al., 1990). In frog, chick and mouse, it is expressed transiently in both endoderm and cardiac mesoderm (Lints et al., 1993; Schultheiss et al., 1995; Tonissen et al., 1994). In zebrafish, its expression is restricted to the ventral margin at the onset of gastrulation, a position which includes the cardiac precursors but is not limited to them (Lee et al., 1994). If a threshold level of *Nkx2.5* is needed to initiate cardiac differentiation, it may be that not every cell within the field expresses levels high enough or persistent enough to initiate cardiac cell fate decisions.

Nkx2.5 ventralizes the embryo

The expression of zebrafish *Nkx2.5* is restricted to the ventral-lateral margin at the onset of gastrulation. The expansion of ventral determinants and reduction of dorsal and anterior structures caused by zebrafish *Nkx2.5* injection suggests that it interacts with the network of localized factors which set the axial form of the embryo. In all likelihood, this ventralization is responsible for abnormal cardiac looping in these embryos. Reduction of dorsoanterior structures in *Xenopus* by UV or *Xwnt-8* causes a randomization of looping (Danos and Yost, 1995), a condition referred to as situs inversus. Hence, although the effects of *Nkx2.5* on looping may reflect direct effects upon the heart, we believe them more likely to be secondary to reduction in the dorsoanterior structures. How and when the left-right signal is transmitted is unknown, although asymmetrical expression of *activin receptor IIa*, *sonic hedgehog* and *cNR* are evident as early as stage 4 of the chick, and their perturbation can cause situs inversus (Levin et al., 1995).

Nkx2.5 and heart size

Nkx2.5 overexpression causes a large hyperplastic heart. The chamber morphology and function are well-retained, and, aside from some mild edema, the circulation appears to be normal in these embryos. Cardiac dilation is a response to failure or increased load, an adaptation termed Starling's law. However, the enlarged hearts noted here after *Nkx2.5* injection include more cells, indicating that even if there is subtle dysfunction, the enlargement cannot be attributed solely to dilation but also includes an element of hyperplasia. Cleaver, Patterson and Krieg have found recently that *Xenopus Nkx2.5* and *Nkx2.3* cause hyperplasia in injected *Xenopus* embryos (Cleaver, Patterson and Krieg, 1996). It is as though the heart of the *Nkx2.5* overexpressing embryo is designed for a larger animal. In transplantation experiments between newts of different sizes, using tissue from the heart field of the gastrula as donor, Copenhaver noted that heart size did not adjust completely to the size of the host (Copenhaver, 1939). It is possible that overexpression of *Nkx2.5* increases the proliferation rate of cardiac cells. However, a more plausible explanation is that information has been specified by the gastrula stage as to the overall size of the heart. The effect of *Nkx2.5* overexpression could be to raise *Nkx2.5* levels diffusely and thereby bring cells on the edges of the heart field to threshold, or bring more cells in the heart field to persistently express *Nkx2.5* above the threshold. If their position is near or in the field, such cells presumably could receive the additional signals needed to commit them to the heart cell fate.

Organs develop in a highly regulative fashion in vertebrates, as though the precursor regions can adapt to longer-range embryonic determinants of form and size. The region denoted as capable of forming an organ, the field, is larger than that

which eventually does so, and normal organs form even after introduction or removal of cells from the field (Huxley and De Beer, 1934). We speculate that one role for *Nkx2.5* could be as a molecular device to make heart growth proportional to that of the embryo. The expansion of ventral determinants and reduction of dorsal and anterior structures caused by *Nkx2.5* injection could mean that it interacts with the network of localized factors which set the axial form of the embryo so that the *Nkx2.5*-expressing zone would be controlled by the signals which ensure a balance of the relative axial determinants. In some cases, this regulation may be from another germ layer. For example, since *BMP4* (Graff et al., 1994), like *dpp*, is a TGFβ family member, and appears to be a ventral determinant in vertebrates, it might play a role in *Nkx2.5* regulation analogous to that of *dpp* in *Drosophila* (Frasch, 1995; Staehling-Hampton et al., 1994). Therefore, inter-germ layer and axial information could be coordinated and integrated with regard to organ size in a regulative manner beginning early in embryogenesis. The heart is the first organ to form. It will be of interest to see whether other genes could play sizing roles for other organs.

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