

Regulation in the heart field of zebrafish

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SUMMARY

In many vertebrates, removal of early embryonic heart precursors can be repaired, leaving the heart and embryo without visible deficit. One possibility is that this 'regulation' involves a cell fate switch whereby cells, perhaps in regions surrounding normal progenitors, are redirected to the heart cell fate. However, the lineage and spatial relationships between cells that are normal heart progenitors and those that can assume that role after injury are not known, nor are their molecular distinctions.

We have adapted a laser-activated technique to label single or small patches of cells in the lateral plate mesoderm of the zebrafish and to track their subsequent lineage. We find that the heart precursor cells are clustered in a region adjacent to the prechordal plate, just anterior to the notochord tip. Complete unilateral ablation of all heart precursors with a laser does not disrupt heart development, if performed before the 18-somite stage. By combining extirpation of the heart precursors with cell labeling, we find that cells anterior to the normal cardiogenic compartments constitute the source of regulatory cells that compensate for the loss of the progenitors.

One of the earliest embryonic markers of the premyocardial cells is the divergent homeodomain gene, *Nkx2.5*. Interestingly, normal cardiogenic progenitors derive from only the anterior half of the *Nkx2.5*-expressing region in the lateral plate mesoderm. The posterior half, adjacent to the notochord, does not include cardiac progenitors and the posterior *Nkx2.5*-expressing cells do not contribute to the heart, even after ablation of the normal cardiogenic region.

The cells that can acquire a cardiac cell fate after injury to the normal progenitors also reside near the prechordal plate, but anterior to the *Nkx2.5*-expressing domain. Normally they give rise to head mesenchyme. They share with cardiac progenitors early expression of *GATA 4*. The location of the different elements of the cardiac field, and their response to injury, suggests that the prechordal plate supports and/or the notochord suppresses the cardiac fate.

Key words: Regulation, Heart field, Zebrafish, *Nkx2.5*, *GATA 4*, Cell fate, Notochord, Cardiac cell

INTRODUCTION

Early vertebrate development is highly regulative. In other words, normal organs, with normal morphology, are formed even after focal destruction of their progenitors. This implies that cells have instructive positional information, either from the organ field or adjacent structures, so that field size is adjusted and cells recruited in the event of injury. However, the origin of the reparative cells and the mechanism of their fate determination are unknown.

We focus here on heart development in zebrafish embryos in order to address the issue of field regulation. The heart field of the postgastrula embryo is the region of the lateral plate mesoderm that contains cardiac progenitors. This region was first described as a heart field because, after explantation, from chick (Rawles, 1943) or frog (Sater and Jacobson, 1989, 1990), it could generate beating heart tissue. Lineage tracing confirmed that a subset of cells from this region normally contribute to the heart. This has been accomplished by regional labelling, using [³H]thymidine (DeHaan, 1965; Rosenquist,

1985) or extracellular application of DiI (Garcia-Martinez and Schoenwolf, 1993; Gourdie et al., 1995) at the time of gastrulation and shortly thereafter by β -galactosidase-expressing retrovirus (Mikawa et al., 1992).

Regulation in the heart field has been shown in the axolotl, which forms of a normal heart even after the removal of all mesoderm defined as cardiogenic (by its capacity to beat in culture) (Copenhaver, 1924). This was taken to imply that repair has been instituted by cells that normally are in other fields. Indeed, explantation experiments reveal that a broad swath of mesoderm in the vicinity of heart progenitors can adopt elements of a cardiac fate, given exposure to particular extrinsic influences, such as foregut endoderm (Sater and Jacobson, 1990) and BMP2 (Schultheiss et al., 1997). This suggests that the source of repair after injury to the cardiac field is cells in the nearby mesoderm, presumably those with cardiac potency revealed in explant culture. However, this has not been evaluated by lineage studies in vivo.

The earliest vertebrate heart field marker is the divergent homeodomain protein, *Nkx2.5*, the vertebrate homologue of

Drosophila tinman (Bodmer, 1993; Lints et al., 1993; Tonissen et al., 1994; Evans et al., 1995; Schultheiss et al., 1995). *Nkx2.5* appears to be important for vertebrate heart development. In all vertebrate species examined, *Nkx2.5* is expressed in the lateral plate mesoderm and later in the heart tube (Komuro and Izumo, 1993; Lints et al., 1993; Tonissen et al., 1994; Evans et al., 1995; Schultheiss et al., 1995; Chen and Fishman, 1996). Overexpression of *Nkx2.5* in zebrafish and *Xenopus* creates larger hearts (Chen and Fishman, 1996; Cleaver et al., 1996).

However, *Nkx2.5* by itself is not sufficient to establish the myocardial cell fate. Its expression is not limited to heart precursors, with the possible exception of zebrafish (Chen and Fishman, 1996; Lee et al., 1996). Its ectopic expression in zebrafish can cause ectopic low level expression of only some myocardial genes (Chen and Fishman, 1996). Furthermore, although *tinman* is required for the heart cell fate in *Drosophila* (Bodmer et al., 1990; Bodmer, 1993), targeted ablation of *Nkx2.5* in the mouse does not block formation of heart precursors or prevent assembly of the primitive heart tube (Lyons et al., 1995). It is not known in vertebrate heart development whether there is a critical timing for expression of the *Nkx2* genes. It is likely that *Nkx2.5* acts in concert with other genes to designate the heart cell fate, including *Nkx2.3* and *Nkx2.7*, genes of the same family and expressed in overlapping patterns (Lee et al., 1996) and with *GATA 4*, shown to have a role in early heart development (Jiang and Evans, 1996; Kuo et al., 1997; Molkenkin et al., 1997) and in maintaining mesodermal cells in a precardiac state (Grepin et al., 1997).

We examine here the spatial and molecular relationships between the heart field cells that normally generate the heart and those that do so after injury. We do so by tracing the late lineages of cardiac progenitors.

MATERIALS AND METHODS

Cell labeling

We injected 1% solution of DMNB-caged fluorescein dextran (Molecular Probes) in 0.2 M KCl into zebrafish embryos at the 1- to 4-cell stage (Melby et al., 1996). Injected embryos were maintained at 25°C until the designated stages, at which time they were placed in an agar ramp and positioned such that the anterior tip of the notochord was directly below the apex of the embryos, allowing access to both lateral plates. The dye was activated by exposing small patches of cells to multiple pulses of 352 nm light generated by a tunable nitrogen pulse laser (Laser Science Instruments), which was focused using a 40× objective on an epifluorescence microscope. Activation was confirmed by visual examination using an epifluorescence microscope equipped to detect fluorescein. Embryos with labeled cells were maintained at 28.5°C until analyzed.

Tissue ablation

Ablations were performed using 442 nm light pulses from the laser. Ablation of cells on a patch perimeter caused purse string contraction and dehiscence of the patch. Cell death throughout the patch was confirmed at the time of the ablation by staining embryos with the supravital dye, To-pro-1 (Serbedzija et al., 1996). Embryos were maintained at 28.5°C until they reached primordial stage 20 (Kimmel et al., 1995). Embryos were then fixed in Dent's Fix (Dent et al., 1989) and stained with the monoclonal antibody MF20 followed by an alkaline-phosphatase-conjugated goat anti-mouse IgG.

In situ hybridization and immunohistochemistry

Whole-mount in situ hybridization was carried out as described previously (Chen and Fishman, 1996) using either digoxigenin-labelled antisense full-length *Nkx2.5* or *GATA 4* RNA probe. Embryos were fixed with 4% paraformaldehyde in PBS, digested with proteinase K and hybridized at 65°C. Alkaline phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) was used to detect the signals. After staining with NBT/X-phosphatase (Boehringer Mannheim), embryos were bleached in 100% methanol, refixed in 4% paraformaldehyde and stored in PBS. The full-length *GATA 4* cDNA, from which the probe was made, was a generous gift of Leonard Zon (Children's Hosp. Boston, MA)

Whole-mount immunohistochemistry was carried out as described previously (Chen et al., 1996). Embryos were fixed in 4% paraformaldehyde in PBS, blocked in 10% normal goat serum and 0.1% Tween in PBS, and incubated with the anti-myosin heavy chain antibody, MF20 (Hybridoma Bank), overnight, at 4°C. Alkaline phosphatase-conjugated anti-mouse IgG antibody (Boehringer Mannheim) was used as a secondary antibody to detect the signals.

RESULTS

Normal position of heart precursors occupies a subdomain of *Nkx2.5* expression

To identify the domain of cells in the 10- to 12-somite-stage embryo that normally gives rise to the heart, we performed a fate-mapping experiment by labeling single and small patches of cells (4-8 cells) in different regions of the lateral mesoderm by light activation of DMNB-caged fluorescein dextran. As diagrammed in Fig. 1, these cells were activated at the 10-somite stage (10s i and ii) in the anterior *Nkx2.5*-expressing region (column A), outside of and anterior to *Nkx2.5*-expressing region (column B), or in the posterior *Nkx2.5*-expressing region (column C). The embryonic positions of the progeny were then determined at 33 hours of development (iii and iv). Cells with progeny in the heart under normal circumstances are found only in the lateral plate region in the part of the *Nkx2.5*-expressing region just anterior to the prechordal plate-notochord junction (Fig. 1 column A i and ii; $n=76$). Cells at the same mediolateral position, but more anterior (Fig. 1 column B i and ii), generate progeny in the head mesenchyme adjacent to the eye (Fig. 1 column B iii and iv; $n=55$). Cells in the more posterior lateral plate (Fig. 1 column C i and ii) have progeny in the mesenchyme around the otic vesicle (Fig. 1 column C iii and iv; $n=67$). The relative anterior-posterior position of cardiac progenitors remains the same at the 12- and 16-somite stage, although they move medially (data not shown). By the 16-somite stage, the heart precursors reside under the neural tube and photoactivation of these cells becomes technically difficult.

It is not known if all *Nkx2.5*-expressing cells in the lateral plate mesoderm become myocardium. For that reason, we compared the position of heart progenitors to that of *Nkx2.5* expression. At the 10-somite stage, *Nkx2.5* is expressed in the lateral plate straddling the prechordal plate-notochord junction, extending posteriorly to the level of the otic vesicle and anteriorly to the level halfway between the notochord and the eye (Fig. 2A). Between 10- and 16-somite stage, the *Nkx2.5* pattern of expression moves medially (Chen et al., 1996; Lee et al., 1996). By the 18- to 19-somite stage, *Nkx2.5* is

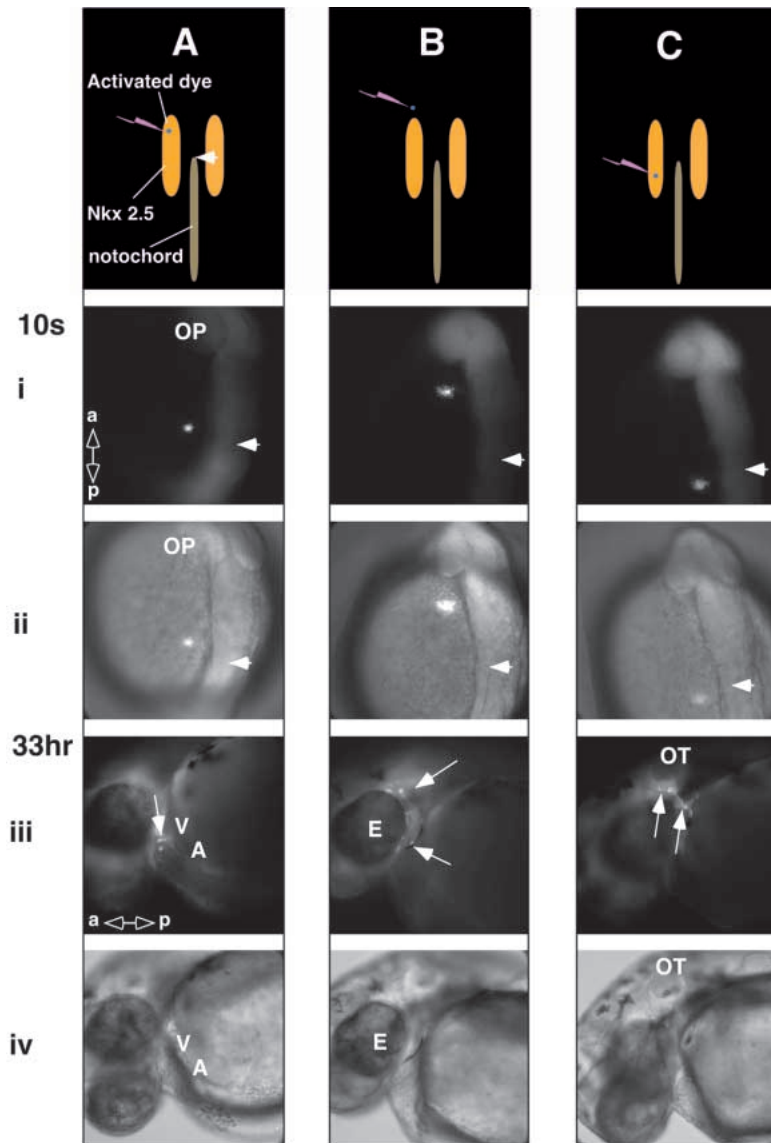


Fig. 1. Fluorescence (A-C, row i and iii) and phase (A-C, row ii and iv) images of cell labeling in the lateral plate mesoderm. The schematic images indicate the location of the dye activation (blue dot) in relation to the notochord (brown line) and *Nkx2.5* expression (yellow). The arrowhead is the notochord tip in all figures. (Ai-iv) Cells labeled in the lateral plate mesoderm just anterior to the prechordal plate-notochord junction at 10-somite stage, give rise to progeny in the heart (arrow) of the 33 hour embryo. (Bi-iv) More anterior cells give rise to cells in the cranial mesenchyme around the eye (arrows). (Ci-iv) More posterior cells contribute labeled cells (arrow) to the mesenchyme adjacent to the otic vesicle. For orientation, the optic cup (OP), eye (E), otic vesicle (OT), ventricle (V) and atrium (A) are labeled.

extinguished in the cells posterior to the notochord tip (Fig. 2C,D), leaving only the anterior cells continuously expressing *Nkx2.5*.

Thus, the anterior extent of *Nkx2.5* corresponds to the region of cells defined by lineage study normally to give rise to heart cells. However, the *Nkx2.5*-expressing cells posterior to the prechordal plate-notochord junction, in which *Nkx2.5* becomes extinguished by the 19-somite stage, do not have cardiac progeny. Therefore, *Nkx2.5* expression does not suffice to

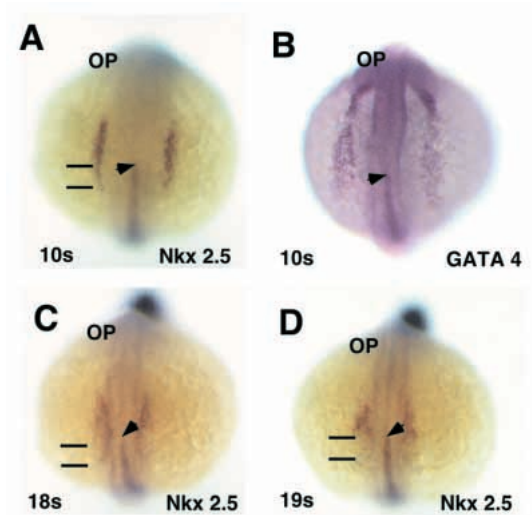


Fig. 2. *Nkx2.5* and *GATA 4* staining. In all of the images, the notochord is stained with the *no tail* and the tip of the notochord is indicated by the arrowhead. (A) At the 10-somite stage, *Nkx2.5* is expressed in the lateral plate straddling the prechordal plate-notochord junction. (B) *GATA 4* is expressed in the same mediolateral level, but extends to the optic cup (OP). (C) At the 18-somite stage, the bilateral *Nkx2.5*-expressing cells converge at the midline at the tip of the notochord, in an X-shape pattern. (D) In the 19-somite-stage embryos, *Nkx2.5* is extinguished in the cells posterior to the notochord tip. In all of the *Nkx2.5* stained images, the bars demarcate the original posterior *Nkx2.5*-expressing region.

confer cardiac fate, at least not if limited to times before the 19-somite stage.

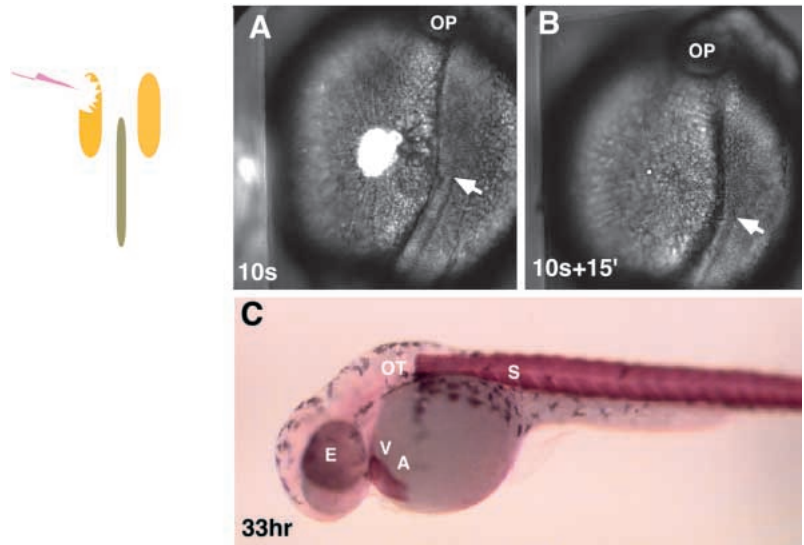
Regulation of the heart field

In order to examine whether and how the embryo could compensate for loss of heart progenitors, we used a pulse laser to ablate the heart-forming region unilaterally in 10- to 12-somite embryos. Immediately after the ablation, the periphery of the ablation contracts, in a purse-string-like fashion, and the dying cells, identified with the supravital dye Topro-1, pinch off the embryo (Fig. 3A,B; also Fig. 5A). By this method, we removed either the entire region of cardiac progenitors ($n=30$) or all of the *Nkx2.5* region ($n=39$) unilaterally. By both morphological and immunohistochemical criteria, the hearts of the previously ablated embryos developed normally (Fig. 3C: $n=65/69$ survivors).

This indicates that the zebrafish heart field is capable of regulation and this regulative ability persists until at least the 16-somite stage in zebrafish.

To examine whether the regulation was an early event, occurring immediately after the injury, or a more gradual event, we ablated either the entire *Nkx2.5* region ($n=50$) or the anterior portion of the *Nkx2.5* the region, containing the cardiac progenitors ($n=55$) unilaterally. Embryos were then fixed at serial time points and stained for *Nkx2.5*. Immediately after

Fig. 3. Phase and fluorescence images of cell ablation in the lateral plate mesoderm. The schematic images indicate the location of the ablation in relation to the notochord (brown) and *Nkx2.5* expression (yellow). (A) Immediately following the ablation of the heart progenitors, the surrounding cells contract in a purse-string-like fashion and the dead cells, fluorescently labeled with Topro-1, are extruded from the embryo. (B) Within 15 minutes, the dead cells pinch off of the embryo. (C) Staining of the embryo at 33 hours with the anti-myosin heavy chain antibody, MF20, shows that both the heart and the embryo develop normally after the ablation. The MF20 antibody labels both the heart and the somites (S). For orientation, the optic cup (OP), eye (E), otic vesicle (OT), ventricle (V) and atrium (A) are labeled.



ablation of the *Nkx2.5* region, there is no expression of *Nkx2.5* (Fig. 4A $n=20$ with ablation of the total *Nkx2.5* region, $n=23$ with ablation of the heart region only). By 1 hour after ablation (at approximately 12- to 14-somite stage), the size of the *Nkx2.5* expression domain is indistinguishable between the ablated and control sides and identical to unperturbed fish,

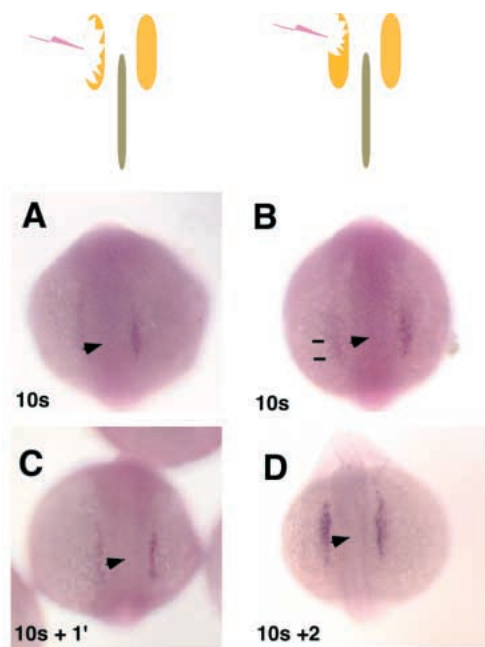


Fig. 4. Recovery of *Nkx2.5* staining after cell ablation. The schematic images indicate the location of the ablation in relation to the notochord and *Nkx2.5* expression. Immediately after unilateral cell ablation in the 10-somite embryo of either (A) all of the *Nkx2.5*-expressing cells or (B) all of the heart progenitors; remaining posterior *Nkx2.5* (marked by bars); there is no *Nkx2.5* expression in the ablated region. (C) 1 hour after the ablation, the *Nkx2.5* pattern of expression reappears, although the level of expression is lower than on the unablated side. (D) By 2 hours after the ablation, *Nkx2.5* expression is normal.

although the level of expression is lower on the ablated than on the contralateral, unablated side (Fig. 4C; $n=15$, $n=15$). By 2 hours after the ablation, there is no detectable differences in either the level or the pattern of *Nkx2.5* expression (Fig. 4D; $n=15$, $n=12$). This indicates that there is a return to normal *Nkx2.5* pattern of expression in the lateral plate prior to generation of a normal heart.

Localized region of compensating cells during regulation

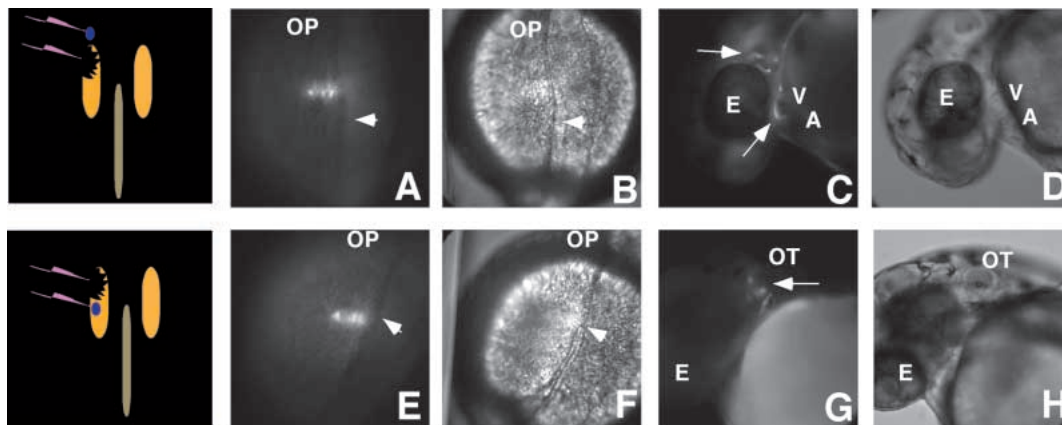
To examine the source of the reparative cells, we traced the fate of cells in the mesoderm surrounding the normal heart precursors after ablation of the heart progenitors. These surrounding cells are not normally fated to contribute to the heart. We find progeny of cells lateral and anterior to the normal cardiac progenitors normally populate the cranial mesoderm adjacent to the eye (Fig. 1, column B). Progeny of the cells posterior to the heart progenitors normally populate the mesenchyme around the otic vesicle (Fig. 1, column C). After ablation, progeny of cells, anterior to the normal heart progenitor region, do contribute to the heart, as well as to head mesenchyme (Fig. 5A,B; $n=18$). On the contrary, progeny of cells posterior to the normal cardiac progenitors do not, even though they express *Nkx2.5* (Fig. 5C,D; $n=16$). This suggests that cells anterior, but not posterior, to the normal heart precursor compartment participate in compensation for the loss of heart precursors.

Therefore, the 'regulatory compartment' of the heart field, the region capable of contributing to the heart after injury, is limited to the region of the lateral plate adjacent to the prechordal plate. Prior to injury, these cells express *GATA 4* (Fig. 2B) and *Nkx2.7* (Lee et al., 1996), but not *Nkx2.5*.

DISCUSSION

Our focus here is upon the heart field, especially the lineage and spatial relationships between the two cellular components of the field, one normally giving rise to heart progenitors and the other capable of providing progenitors in the event of loss of the

Fig. 5. Cells that replace cardiac progenitors arise anterior to the position of normal cardiac progenitors. Fluorescent (A,C,E,D) and phase (B,D,F,G) images of cell labeling (blue dot) after ablation in 10-somite-stage embryos. The schematic images indicate the location of the ablation and cell labeling in relation to the notochord and *Nkx2.5* expression. (A,B) Labeled fluorescent cells anterior to the ablation of the heart precursors give rise to cells in both the cranial mesenchyme and the heart (arrows; C,D). The purse-string-like contraction of the tissue surrounding the ablation have caused the labeled cells to elongate. (E,F) In contrast, labeled cells posterior to the ablation contribute to the mesenchyme around the otic vesicle but not the heart (arrow; G,H). For orientation, the optic cup (OP), eye (E), otic vesicle (OT), ventricle (V) and atrium (A) are labeled. Images are labeled as described in Fig. 1.



cardiac progenitors. We find that unilateral elimination of the entire compartment of heart progenitors, normally providing all of the cells for both chambers, is tolerated without any evident effect on heart development. The cells that compensate reside in a small compartment just anterior to the cells normally constituting the cardiogenic pool. These cells change their fate under stimulus of adjacent injury, such that progeny populate the heart in addition to their normal head mesenchyme.

As diagrammed in Fig. 6, the normal cardiogenic cells all reside within a subdomain of the region expressing *Nkx2.5* and *GATA 4*. The cells capable of regulation normally do not express *Nkx2.5*, but do express *GATA 4* and *Nkx2.7*. Cells from the posterior *Nkx2.5*-expressing region, adjacent to the notochord, never populate the heart, normally or after injury, suggesting that the lateral mesoderm adjacent to prechordal plate has different properties than that near the notochord despite shared expression of *Nkx2.5*.

The heart field

In the 1000-celled zebrafish, just prior to gastrulation, all

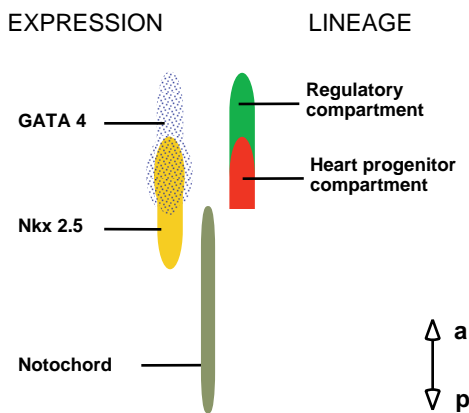


Fig. 6. Diagram of the locations of the pattern of *Nkx2.5* and *GATA 4* expression and the heart progenitor and regulatory compartments. For convenience, the expression patterns are shown only on the right.

cardiac progenitors reside in a ventral marginal swath (Lee et al., 1994). Progeny of these cells populate the heart and other mesoderm, and cells within this ‘field’ have a propensity to become heart cells, but by no means do so predictably. After ingress, the cells migrate medially through the lateral plate mesoderm (Stainier and Fishman, 1992). Tracing of the progenitors of such early injected cells was difficult because of their many and variable fates. Direct microinjection of cardiac progenitors later in their progress through the mesoderm proved not feasible, due to their small size and deeper position relative to the embryonic surface. Hence, we turned to the use of caged dyes (Melby et al., 1996), which we could introduce in the early embryo, and developed a novel means of dye activation in the late embryo using a laser. By this method, we identified a region of lateral plate that predictably provided cardiac precursors. This region is adjacent to the prechordal plate, just anterior to the junction of the prechordal plate and the notochord.

The earliest molecular marker of the heart progenitors is *Nkx2.5*. In mice (Lints et al., 1993; Komuro and Izumo, 1993), frogs (Tonissen et al., 1994; Evans et al., 1995) and chicks (Schultheiss et al., 1995), it is evident first in lateral plate mesoderm and anterior pharyngeal mesoderm, and in lingual muscle later in development. In zebrafish, it is expressed in the ventral marginal zone in a pattern that mimics the cardiac propensity of the cells, and later is restricted to a region of lateral plate mesoderm straddling the prechordal plate-notochord junction (Chen and Fishman, 1996; Lee et al., 1996). In zebrafish, it is not expressed in the endoderm or in non-cardiac muscle (Chen and Fishman, 1996; Lee et al., 1996).

Evidence from *Drosophila* suggests that expression of the *Nkx2.5* homologue, *tinman*, while necessary for heart cell fate, is not sufficient unless persistent (Bodmer, 1995; Frasch, 1995). It is expressed early in visceral as well as cardiac mesoderm, and then extinguished, except in precursors of the heart, where it is maintained by instruction of Dpp in adjacent ectoderm (Frasch, 1995). This suggests that it is the proper timing or maintenance of *Nkx2.5* expression that is important for cardiac cell fate. This appears true in zebrafish, as well. Ectopic *Nkx2.5* can induce low-level expression of certain but

not all cardiac genes (Chen and Fishman, 1996). We find that the posterior *Nkx2.5* domain in the lateral plate mesoderm does not correspond to the position of the cardiac progenitors. Expression in this region is extinguished between the 10- and 19-somite stages. The cells that regulate to populate the heart progeny after injury appear to initiate *Nkx2.5* expression only after the insult. Hence, it seems reasonable to assume that *Nkx2.5* is also needed late and in conjunction with other factors. It is interesting that the anterior, persistent, *Nkx2.5* domain is adjacent to the prechordal plate and the posterior, late extinguished, *Nkx2.5* domain is adjacent to the notochord, suggesting that prechordal plate supports, or notochord suppresses, *Nkx2.5* expression.

'Regulation' in the heart field

Assay of the heart 'field' by explantation reveals the presence of cells capable of becoming cardiomyocytes and includes cells not normally so fated. Direct cell tracing in chicken indicates that the extent of the field defined by explantation is broader than the region normally fated to give heart cells (DeHaan, 1965; Rosenquist, 1985; Garcia-Martinez and Schoenwolf, 1993; Gourdie et al., 1995). Thus, it has been assumed that cells nearby the normal cardiac progenitors are relieved of some suppression in culture (Sater and Jacobson 1989). This observation has been extrapolated to suggest that cells nearby the heart precursors can serve to replace such cells in vivo after injury, explaining the amazing 'regulative' ability of vertebrate embryos. The test of this theory requires lineage analysis in vivo to define normal cardiogenic and regulative zones. Therefore, this analysis is an important first step towards understanding the tissue interactions that normally establish and regulate the cardiac progenitor pool.

We find that a small region of cells anterior to the cardiac precursors serves as the source of heart cells after normal progenitors are eliminated (Fig. 6). Progeny of these anterior cells normally occupy head mesenchyme. After injury to the cardiac precursors, they add the cardiac cell fate. Our optical resolution is not sufficient to confidently activate single cells. Hence, we do not know if one cell can give rise to both mesenchymal and heart fates after injury, or if there is a subpopulation of regulative compartment that selectively initiates the cardiac fate. In any case, to our knowledge, this is the first demonstration of the source of replacement cells for an organ field.

The replacement cells share with the cardiac progenitors expression of *Nkx2.7* and *GATA 4*. *GATA 4* has been shown to maintain cells in a precardiic precursor state (Grepin et al., 1997). The function of *Nkx2.7* is not known, but its pattern of expression overlaps with that of *Nkx2.5* for much of early heart development (Lee et al., 1996). This overlap may provide a partial explanation for why *Nkx2.5* mutation in mice does not prevent assembly of a heart tube (Lyons et al., 1995).

One might have anticipated that the reparative cells would be part of the region normally expressing *Nkx2.5*. This is not the case. Although there is a large zone of *Nkx2.5*-expressing cells just posterior to those normally fated to the heart, these posterior cells do not contribute to the heart, either under normal circumstance or after injury. However, after injury, the normal pattern of *Nkx2.5* returns. This suggests that the normal field and/or adjacent structures regulate expression of *Nkx2.5*.

Derepression could be a consequence either of removal of inhibitory activity or stimulation. For example, if the normal cardiac progenitors exerted lateral inhibition, their removal could derepress *Nkx2.5* expression. Alternatively, the movement of replacement, as they fill in the space left by the ablation, might place them in proximity to different regulatory influences. Presumably, the posterior boundary of the heart field, before and after injury, is established by inhibitory midline structures, probably including the notochord.

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