Development 127, 4959-4969 (2000)
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DEV8746

Characterization of a novel subset of cardiac cells and their progenitors in the *Drosophila* embryo

Ellen J. Ward and James B. Skeath*

Department of Genetics, Washington University School of Medicine, 4566 Scott Avenue, St Louis, MO 63110, USA
*Author for correspondence (e-mail: jskeath@genetics.wustl.edu)

Accepted 30 August; published on WWW 24 October 2000

SUMMARY

The *Drosophila* heart is a simple organ composed of two major cell types: cardioblasts, which form the simple contractile tube of the heart, and pericardial cells, which flank the cardioblasts. A complete understanding of *Drosophila* heart development requires the identification of all cell types that comprise the heart and the elucidation of the cellular and genetic mechanisms that regulate the development of these cells. Here, we report the identification of a new population of heart cells: the Odd skipped-positive pericardial cells (Odd-pericardial cells). We have used descriptive, lineage tracing and genetic assays to clarify the cellular and genetic mechanisms that control the development of Odd-pericardial cells. Odd skipped marks a population of four pericardial cells per hemisegment that are distinct from previously identified heart cells. We demonstrate that within a hemisegment, Odd-pericardial cells develop from three heart progenitors and that these heart progenitors arise in multiple anteroposterior locations within the dorsal mesoderm. Two of these progenitors divide asymmetrically such that each produces a two-cell mixed-lineage clone of one Odd-pericardial cell and one cardioblast. The third progenitor divides symmetrically to produce two Odd-pericardial cells. All remaining cardioblasts in a hemisegment arise from two cardioblast progenitors each of which produces two cardioblasts. Furthermore, we demonstrate that *numb* and *sanpodo* mediate the asymmetric divisions of the two mixed-lineage heart progenitors noted above.

Key words: *Drosophila*, Heart, Odd-skipped

INTRODUCTION

In this paper, we focus on the cellular and genetic mechanisms that regulate organ formation. The *Drosophila* heart is a simple organ made up of a limited number of cells and cell types (Rugendorff et al., 1994; reviewed by Bodmer and Frasch, 1999) and thus provides an ideal model system in which to dissect the processes that control organ development. The heart, or dorsal vessel, forms from the dorsal-most cells of the mesoderm (Bate, 1993; Dunin Borkowski et al., 1995). A subset of these cells acquire heart cell fates and align to form the heart – a simple tube that runs along the dorsal midline of the embryo or larva (Rugendorff et al., 1994).

The heart is composed of two major cell types: cardioblasts and pericardial cells (Rizki, 1978; Bate, 1993; Rugendorff et al., 1994). Cardioblasts express muscle-specific proteins, coalesce to form the heart tube and are the contractile cells of the heart. Pericardial cells flank and are loosely associated with cardioblasts but do not express muscle proteins. The role of pericardial cells in heart development and function is poorly understood. In this paper, we collectively refer to pericardial cells and cardioblasts as heart cells.

Present models of heart development propose that all heart progenitors develop from a specific location in the dorsal mesoderm demarcated by the region of intersection between *tinman* (*tin*) expression in the dorsal mesoderm and the transverse stripes of *wingless* (*wg*) expressing ectodermal cells (Riechmann et al., 1997; Bodmer and Frasch, 1999). Consistent with this, no heart cells appear to develop in embryos homozygous mutant for *tin* or *wg* (Bodmer, 1993; Azpiazu and Frasch, 1993; Wu et al., 1995; Park et al., 1996). *tin* appears to be required for the development of all dorsal mesodermal derivatives while *wg* has been shown to promote the development of the mesodermal derivatives that develop beneath ectodermal *wg*-expressing cells (Park et al., 1996; Jagla et al., 1997). Thus, all heart cells are thought to require the concerted action of *tin* and *wg* to form. Owing to the restricted domain of mesodermal cells affected by loss of *wg* signaling, only those mesodermal cells located directly beneath the ectodermal *wg* stripe are thought to respond to the *wg* signal (Bodmer and Frasch, 1999).

Gene expression analyses identify multiple types of cardioblasts and pericardial cells (see Fig. 8). For example, analyses of the expression of the homeobox-containing genes *even skipped* (*eve*), *tinman* (*tin*), and *ladybird-early* (*lbe*) and *ladybird-late* (*lbl*) identify three distinct populations of pericardial cells (Frasch et al., 1987; Bodmer, 1993; Azpiazu and Frasch, 1993; Jagla et al., 1997). Two pericardial cells in each hemisegment express *eve* and are located dorsal and lateral to the cardioblasts (see Fig. 8 for orientation of cells

within the heart). These cells are called Eve-pericardial cells and they also express *tin*. Four other pericardial cells in each hemisegment are found just ventral to the cardioblasts: all of these cells express *tin* and two of them express the *ladybird* genes, but none expresses *eve* (Jagla et al., 1997). We refer to this group of cells as the Tin-pericardial cells even though all identified pericardial cells express *tin*. Two other markers, the zinc-finger homeobox-containing gene *zfh1* and the epitope recognized by the anti-pericardial cell monoclonal antibody, each appear to label all of these pericardial cells (Lai, 1991; Yarnitzky and Volk, 1995). Morphological studies identify the classical population of pericardial cells as residing immediately lateral to cardioblasts; however, at present no molecular markers identify this population of pericardial cells (Rugendorff et al., 1994).

Expression of *tin* and the *ladybird* genes also identify different types of cardioblasts. Six cardioblasts develop in each hemisegment, and all cardioblasts express the MADS box gene *Mef2* and require *Mef2* function for their proper differentiation (Bour et al., 1995; Lilly et al., 1995). In this paper, we use Mef2 protein expression to mark cardioblasts and to distinguish them from pericardial cells. *tin* is expressed in four contiguous cardioblasts per hemisegment, the anterior two of which co-express *lbl* and *lbe* (Bodmer, 1993; Azpiazu and Frasch, 1993; Jagla et al., 1997). The third pair of cardioblasts express neither *tin* nor the *ladybird* genes. Within a hemisegment these markers identify 12 heart cells (six cardial and six pericardial cells) and six distinct subtypes of heart cells.

Relatively little is known about the lineages of heart cells. Nonetheless, preliminary lineage analyses of Eve-heart cells has lead to a model whereby individual heart progenitors are thought to produce either cardioblasts or pericardial cells but not both (Park et al., 1998; Bodmer and Frasch, 1999). However, additional lineage-tracing studies are required to determine whether the lineage of all other identified heart cells conform to this model and to determine definitively whether bipotent cardioblast/pericardial heart progenitors exist.

Genetic experiments have identified a set of genes that control the asymmetric divisions of many progenitor cells in various tissues. These genes include *numb*, *sanpodo* and members of the *Notch* pathway (see Dye et al., 1998; Skeath and Doe, 1998 and references therein). The requirement of these genes to promote asymmetric divisions was initially uncovered through work on the *Drosophila* nervous system. However, more recent experiments demonstrate that *sanpodo*, *numb* and the *Notch* pathway control the asymmetric division of many mesodermal/muscle progenitors including those that produce the Eve-pericardial cells (Ruiz-Gomez and Bate, 1997; Carmena et al., 1998; Park et al., 1998). These results suggest that other heart cells may arise via the asymmetric division of bipotent heart/muscle progenitor cells.

sanpodo, *numb* and the *Notch* pathway interact to enable single progenitor cells to produce differently fated sibling cells (A,B) (see Uemura et al., 1989; Rhyu et al., 1994; Spana and Doe, 1996; Guo et al., 1996; Dye et al., 1998; Skeath and Doe, 1998 and references therein). Loss of function mutations in *sanpodo* or genes of the *Notch* pathway equalize sibling fates and produce identical fate transformations, e.g. A,A. Loss of *numb* function also equalizes sibling cell fate with both siblings adopting fate B. Epistatic analyses indicate that *sanpodo* and the *Notch* pathway are epistatic to *numb*. The Numb protein, which contains a phosphotyrosine-binding domain, is membrane associated and segregates exclusively into one daughter cell upon progenitor division. Careful descriptive, phenotypic and genetic analyses demonstrate that Numb acts autonomously in the cell that inherits it to block Notch signaling (Guo et al., 1996; Spana and Doe, 1996). The molecular mechanism through which Numb opposes Notch signaling is unknown; however, Numb has been shown to interact physically with Notch (Guo et al., 1996). Functional Notch signaling proceeds unimpaired in the daughter cell that does not inherit Numb and triggers cell fate B. During some asymmetric divisions the Notch pathway requires *sanpodo* gene function to induce cell fate B; however, the molecular basis of this requirement is unclear. Thus, together the Notch pathway, *sanpodo* and *numb* mediate asymmetric divisions.

A full understanding of heart development requires the identification of all heart cells and an elucidation of the genetic and cellular mechanisms that control their development. Here we investigate a new population of heart cells defined by the expression of the Odd-skipped protein (Odd) (E. J. Ward, PhD Thesis, St. Louis University, 1997; Ward and Coulter, 2000). We find that Odd identifies a novel population of four pericardial cells per hemisegment and that these cells are distinct from Eve-, Tin- and Mef2 heart cells. The determination that Odd-pericardial cells are distinct from previously identified heart cells brings the total number of heart cells per hemisegment from 12 to 16. Odd-pericardial cells reside immediately lateral and slightly ventral to cardioblasts. We demonstrate through descriptive and cell lineage analyses that Odd-pericardial cells develop from three heart progenitors in each hemisegment and that these progenitors arise in multiple anteroposterior (AP) locations within the dorsal mesoderm. Two of these progenitors divide asymmetrically with each producing one Odd-pericardial cell and one cardioblast. We find that *numb* and *sanpodo* mediate these asymmetric divisions. We also find that within a hemisegment, the remaining two Odd-pericardial cells arise from a single Odd-pericardial cell progenitor and the remaining four cardioblasts arise from two cardioblast progenitors. (Additional studies of the generation of *svp-lacZ* and Tin cardioblasts by asymmetric and symmetric divisions are reported by Gajewski et al. (2000).)

MATERIALS AND METHODS

Genetics

Oregon R was the wild type used. Mutant lines were *sanpodo*^{ZZ27} (Skeath and Doe, 1998) and *numb*^{2} (Uemura et al., 1989). H162 is an enhancer trap insert in *seven-up* (Mlodzik et al., 1990) and referred to as *svp-lacZ*.

Immunohistochemistry and immunofluorescence

The following antibodies were used at indicated dilutions: mouse- and rabbit anti-Odd (1:3000; E. J. Ward, PhD Thesis, St. Louis University, 1997); rabbit anti-Eve (1:5000; Frasch et al., 1986); rabbit anti-Mef2 (1:1000; Lilly et al., 1995); rabbit anti-Tin (1:1000; Azpiazu and Frasch, 1993); mouse monoclonal antibody 3 (mAb3, a pan-pericardial cell marker; 1:5; Yarnitzky and Volk, 1995); mouse anti-Zfh1 (1:1000; Lai et al, 1991) mouse anti-β-galactosidase (1:2000; Promega); rabbit anti-β-galactosidase (1:15000; Cappel).

Immunohistochemistry and immunofluorescence analyses performed as described in Skeath et al. (1995), and Ward and Coulter (2000).

Lineage-tracing experiments

Random *lacZ*-expressing clones were created using the FLP/FRT lineage tracing system as described in Postigo et al. (1999) with the following modifications. Homozygous *hsp70-flp* virgin females were crossed to *Act5C-FRT-Draf-FRT-tau-lacZ* males. Three- to four-hour-old F1 embryos were heatshocked for 7 minutes at 37°C to induce the *flp* recombinase, returned to 25°C and aged until stage 15-16 (Campos-Ortega and Hartenstein, 1997), at which point they were fixed and stained. To avoid problems in data interpretation due to clonal intermingling, we scored Odd- or Mef2-positive clones in embryos that contained fewer than one clone per two adjacent dorsal hemisegments.

RESULTS

Odd expression identifies a new subpopulation of pericardial cells

The pair-rule gene *odd* was first identified in the pioneering genetic screens of Nusslein-Volhard and Wieschaus (1980) and Nusslein-Volhard et al. (1984). In addition to its early pair-rule pattern (Coulter et al., 1990), *odd* is expressed in many other tissues during embryonic and larval development (Skeath et al., 1995; Spana et al., 1995; E. J. Ward, PhD Thesis, St. Louis University, 1997; Ward and Coulter, 2000). Notably, we detected Odd in the nuclei of two rows of mesodermal cells that flank the dorsal midline of late stage *Drosophila* embryos (Fig. 1). The two rows of Odd-expressing cells begin at the boundary of abdominal segments 1 and 2 and extend to the boundary between abdominal segments 6 and 7 (Fig. 1). Analyses of embryos double-labeled for Odd and Mef2 expression demonstrated that the rows of Odd-expressing cells are in close physical contact to, and flank, the cardioblasts (Fig. 2A). Odd-positive cells also resided slightly ventral to the cardioblasts. Immediately anterior to these two rows we detected Odd expression in two bilaterally symmetric clusters of ~20 cells (Fig. 1), which correspond to the lymph glands (Ward and Coulter, 2000).

The spatial dynamics of the Odd expression pattern in the dorsal mesoderm parallels that of proteins known to identify subsets of pericardial cells (see Bodmer and Frasch, 1999). For example, Eve and Tin label subsets of pericardial cells and these cells are found in two rows that flank the cardioblasts in late stage embryos (Frasch et al., 1987; Bodmer, 1993; Azpiazu and Frasch, 1993). Based on the similarity between the spatial pattern of Odd expression and that of known pericardial cell markers, we tentatively identify the Odd-expressing cells as pericardial cells and call them Odd-pericardial cells.

Previous reports have identified two markers, Zfh1 and mAb3, that label all pericardial cells (Lai et al., 1991; Yarnitzky and Volk, 1995). To determine whether Odd-pericardial cells express proteins or epitopes consistent with a pericardial cell fate, we followed the expression of Odd and either that of Zfh1 or the epitope recognized by mAb3 in late-stage wild-type embryos. We found that Odd-pericardial cells co-expressed Zfh1 (Fig. 2D) and the antigen recognized by mAb3 (not shown). These results indicate that Odd-pericardial cells express markers common to pericardial cells and are consistent with our identification of these cells as pericardial cells.

Next we determined whether Odd identifies a new population of pericardial cells or constitutes an additional marker for a previously identified subset of pericardial cells. To investigate this question, we double labeled late-stage wild-type embryos to detect Odd expression and the expression of either Eve or Tin. We did not detect co-expression of Odd with either Tin or Eve (Fig. 2). Since between them, Eve and Tin label all previously identified pericardial cells (Frasch et al., 1987; Bodmer, 1993; Azpiazu and Frasch, 1993), we conclude that Odd identifies a new subpopulation of pericardial cells that also express Zfh1 and mAb3. Odd-pericardial cells reside ventral and medial to Eve-pericardial cells and dorsal and lateral to Tin-pericardial cells (Fig. 2 and data not shown; see Fig. 8 for schematic orientation of heart cells). These results demonstrate that Odd identifies a new population of pericardial cells. Based on their position in the heart, Odd-pericardial cells probably correspond to the 'classical' pericardial cells that are defined by morphological studies to reside immediately lateral to the cardioblasts (Rugendorff et al., 1994).

The dynamics of Odd-pericardial cell development

Odd is the first molecular marker identified for this population of pericardial cells. Thus, we wanted to use Odd expression as a tool to determine (1) the embryonic origin of Odd-pericardial cells and how they develop, as well as (2) the cellular and molecular mechanisms that control their development. Thus, we first performed a careful developmental analysis of Odd-pericardial cell development in wild-type embryos. To increase the precision of these studies, we simultaneously followed the development of Eve-mesodermal cells during the early time points in these studies. Eve-mesodermal cells produce the Eve-pericardial cells and the Eve-positive DA1 muscle. We failed to detect Odd-pericardial cells in a reproducible manner until stage 12/2 (Fig. 3B) even though we detect Eve-mesodermal cells by stage 10 (staging as per Campos-Ortega and Hartenstein (1997) and Klambt et al. (1991)). By stage 12/2 we detect zero to two Odd-pericardial cells in each hemisegment (Fig. 3B). These cells are located just posterior to the ectodermal Odd stripe, roughly midway between the AP position of Eve-mesodermal cells (Fig. 3B). Eve-mesodermal cells develop underneath the *wg* expression domain and thus mark the AP location of *wg*-expressing cells. By stage 13, we detect on average three Odd-pericardial cells beneath and just posterior to the Odd-ectodermal stripe per hemisegment (Fig. 3C) and by stage 14, we detect an average of four Odd-pericardial cells aligned end to end along the AP length of a hemisegment (Fig. 3D).

Odd-expressing cells that arise from the dorsal mesoderm in the thoracic and first abdominal segments give rise to part of the lymph gland (Ward and Coulter, 2000). The development of these cells differs from the development of Odd-pericardial cells in the abdominal segments (not shown). In this paper, we limit our analysis to the development of Odd-pericardial cells.

numb and *sanpodo* control Odd-pericardial cell and cardioblast cell development

Having established a wild-type profile of Odd-pericardial cell development we next wanted to identify the genetic regulatory

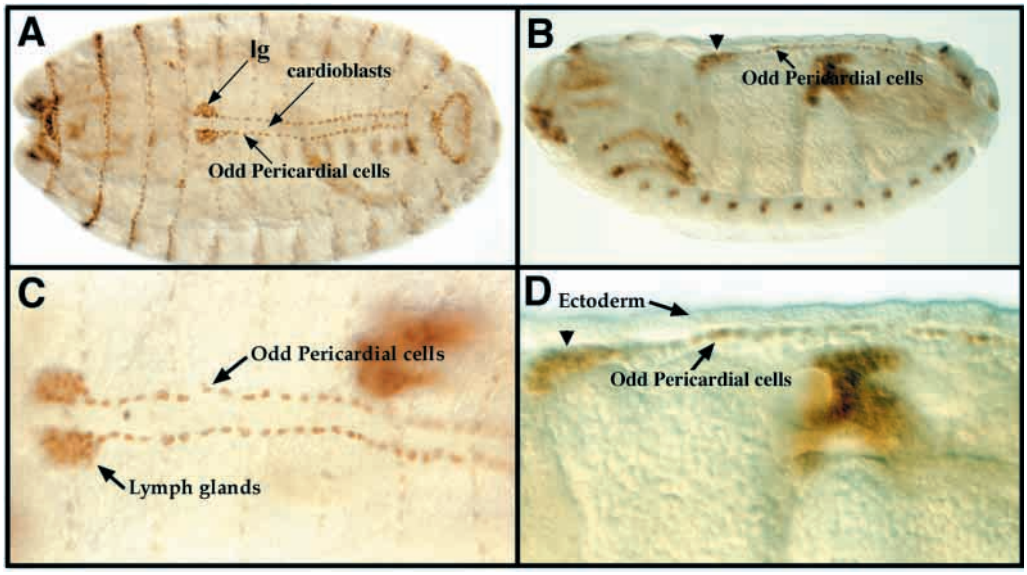

Fig. 1. Odd identifies a new subset of pericardial cells. Low-magnification (A,B) and high-magnification (C,D), dorsal (A,C) and lateral (B,D) views of wild-type embryos labeled for Odd protein. (A,C) Two rows of Odd-pericardial cells reside along the dorsal midline of the embryo; anteriorly these cells congregate in large bilaterally symmetric cell clusters that comprise the lymph glands (lg). (B,D) Odd-expressing cells are located subepidermally and terminate anteriorly in the lymph glands (arrowhead). Anterior is towards the left.

mechanisms that govern Odd-pericardial cell development. Previous research has shown that the genes that control asymmetric divisions regulate Eve-pericardial cell development (Ruiz-Gomez and Bate, 1997; Carmena et al., 1998; Park et al., 1998). Thus, we assessed whether loss of *sanpodo* or *numb* function affect Odd-pericardial cell and cardioblast development. Normally 4.2 Odd-pericardial cells (n>100) and 6.0 cardioblasts (n=100) developed within each abdominal hemisegment of late-stage embryos (Fig. 3). In *numb* mutant embryos, we observed 6.0 Odd-pericardial cells (n>100) and 4.2 cardioblasts (n=47 hemisegments) per hemisegment. Conversely, we detected 7.6 cardioblasts (n=72) and 2.7 Odd-pericardial cells (n>100) per hemisegment in *sanpodo* mutant embryos. Thus, in *numb* mutant embryos we detected roughly two extra Odd-pericardial cells and two fewer cardioblasts per hemisegment. Conversely, in *sanpodo* mutant embryos roughly two fewer Odd-pericardial cells and two additional cardioblasts formed per hemisegment (Fig. 4). These results demonstrate that *sanpodo* promotes Odd-pericardial cell development and opposes cardioblast development. Conversely, *numb* opposes Odd-pericardial cell development and promotes cardioblast development. In addition, they suggest that two cardioblasts and two Odd-pericardial cells arise via the asymmetric divisions of *numb/sanpodo* dependent heart progenitors. These results are consistent with those of Hartenstein et al. (1992) who used a temperature-sensitive allele of Notch to remove Notch function after heart progenitor formation, and found that Notch is required for pericardial cell development. We should also note that loss of *numb* function disrupts the precise alignment of cardioblasts leading to a 'broken rows' of cardioblasts in *numb* mutant embryos (arrows, Fig. 4E).

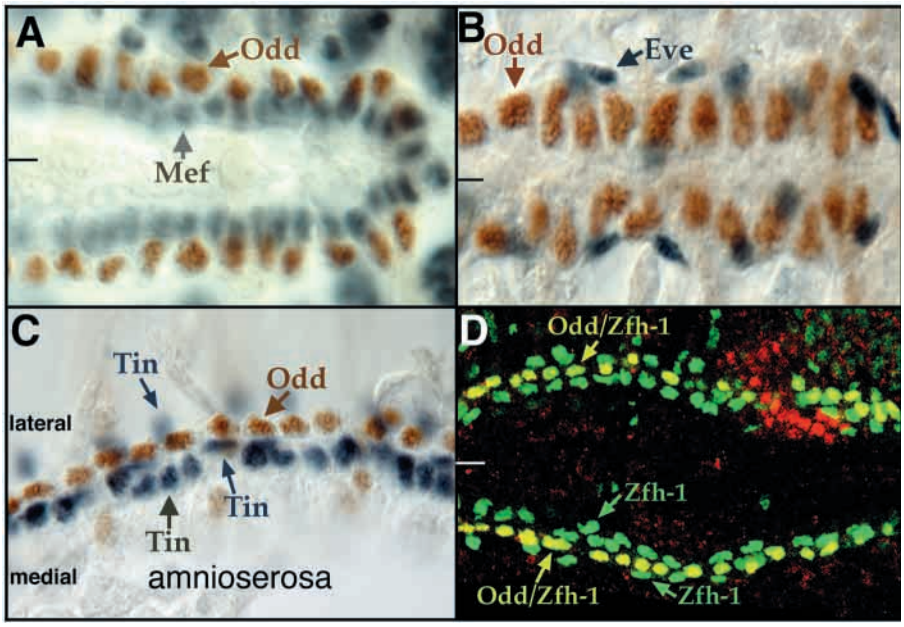

Fig. 2. High-magnification ventral views of stage 15 or 16 filleted wild-type embryos double-labeled for (A) Odd (brown) and Mef2 (blue), (B) Odd (brown) and Eve (blue), (C) Odd (brown) and Tin (blue), and (D) Odd (red) and Zfh1 (green). (A) Odd-expressing cells reside physically adjacent but lateral to Mef2 cardioblasts. (B) Odd-expressing cells are located medial to Eve-pericardial cells. (C) Odd-expressing cells are found lateral to Tin-expressing pericardial cells. Note, this panel shows only one bilateral half of the heart. Lateral is upwards, medial is downwards. (D) All Odd-pericardial cells express Zfh1 (yellow cells). The Zfh1 cells medial to the Odd-pericardial cells (yellow) are Tin-pericardial cells, whilst the lateral Zfh1-positive cells are the Eve-pericardial cells. Anterior is towards the left; horizontal line represents the dorsal midline.

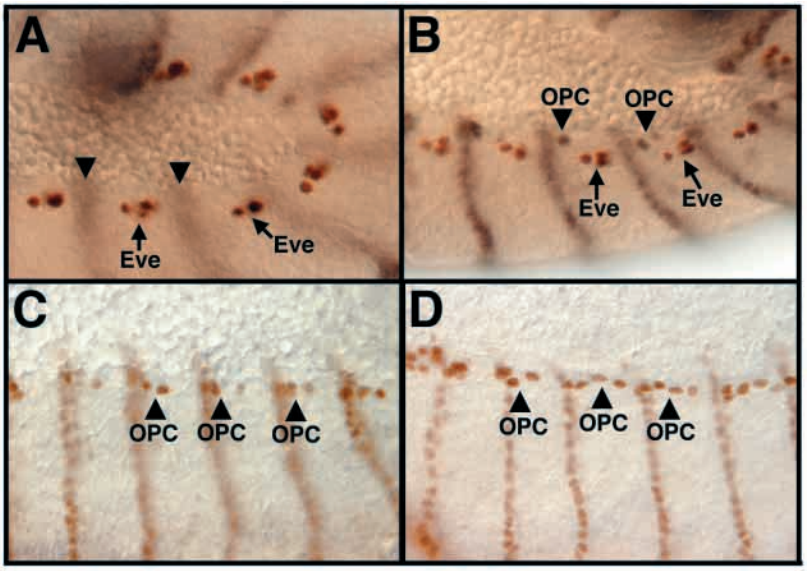

Fig. 3. Odd-pericardial cell development. High-magnification lateral views of wild-type abdominal segments at early stage 12 (A), stage 12/2 (B), mid-stage 13 (C) and stage 14 (D). In A and B, Odd-positive cells are black and Eve-positive cells are brown; in C and D Odd-positive cells are brown. (A) Eve-mesodermal cells (arrows) are found in each hemisegment in early stage 12 embryos; these cells are located midway between Odd-stripes (arrowheads). (B) Odd-pericardial cells (OPCs) first emerge around stage 12/2 immediately posterior to the ectodermal Odd stripe (arrowheads) roughly midway between Eve mesodermal cells (arrows). (C) Three Odd-pericardial cells are detected by mid-stage 13, two are located immediately posterior to the ectodermal Odd-stripe (arrows) and one is located directly below the ectodermal Odd-stripe. (D) By stage 14, the Odd-pericardial cells have aligned in a row across the AP extent of each hemisegment. Anterior is towards the left.

Odd-pericardial cells and cardioblasts arise from mixed- and pure-lineage heart progenitors

Multiple models can explain the reciprocal effects of *sanpodo* and *numb* on cardioblast and Odd-pericardial cell development. For example, one model predicts that two mixed-lineage heart progenitors each divide to yield one cardioblast and one Odd-pericardial cell. A second model predicts the existence of four progenitors: two would divide with each producing one Odd-pericardial cell and one cell of unknown fate; the other two progenitors would divide each producing one cardioblast and one cell of unknown fate. In these and other models, loss of *numb* or *sanpodo* function would equalize all asymmetric divisions and could result in the observed Odd-pericardial cell and cardioblast phenotypes.

We used lineage-tracing assays to distinguish between these models and to determine whether any Odd-pericardial cells and cardioblasts share a common ancestry. We reasoned that if individual heart progenitors divide to produce both cardioblasts and Odd-pericardial cells, then we should identify lineage clones that contain both cell types. Conversely, if cardioblasts and Odd-pericardial cells do not arise from a common progenitor, then clones should contain one of these cell types but not both. To trace the lineage of cardioblasts and Odd-pericardial cells we used the FLP/FRT lineage tracing system of Struhl and Basler (1993) to create random clones of *tau-lacZ* reporter gene expression.

We induced clones during stages 8-9 just as the general pan-mesodermal cell divisions are being completed (Dunin-Borkowski et al., 1995). Thus, we expected to induce clones in mesodermal cells prior to the emergence of cardioblast and pericardial progenitors. To identify the lineage of Odd-pericardial cells we double labeled embryos for β-

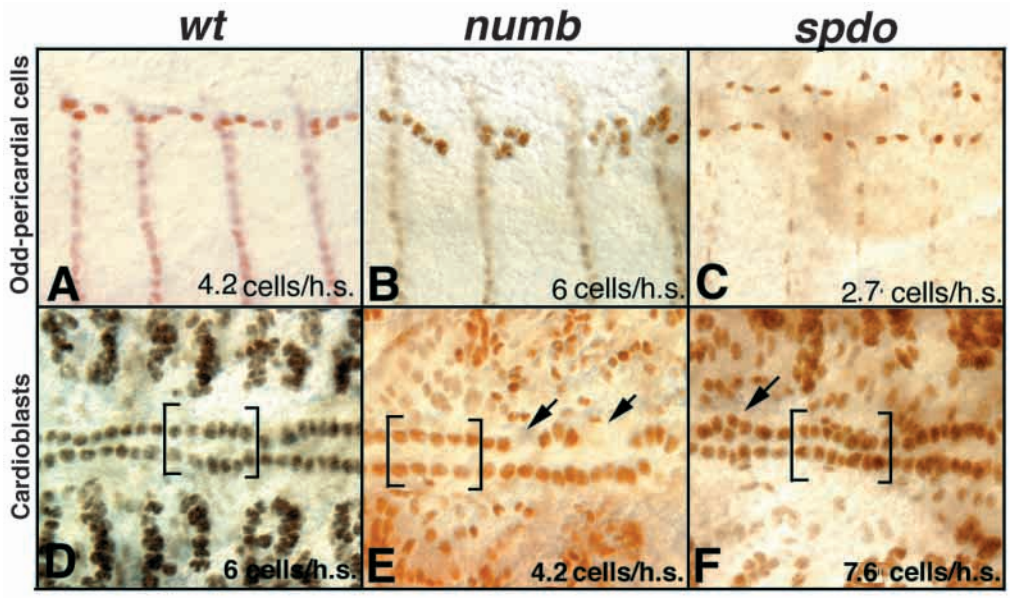

Fig. 4. *sanpodo* and *numb* exhibit reciprocal effects on Odd-pericardial cell and cardioblast development. High-magnification lateral (A,B) or dorsal (C-F) views of stage 14-16 wild-type (A, D), *numb* (B,E) or *sanpodo* (C,F) mutant embryos labeled for Odd (A-C) or Mef2 (D-F). (A,D) In wild-type embryos, 4.2 Odd-positive pericardial cells and 6.0 Mef2 cardioblasts arise per hemisegment. (B,E) In *numb* mutant embryos, six Odd-positive pericardial cells and 4.2 Mef2 cardioblasts arise per hemisegment. (C,F) In *sanpodo* mutant embryos, 2.7 Odd-positive pericardial cells and 7.6 cardioblasts arise in each hemisegment. In D-F, a single segment is bracketed. Average number of Odd-pericardial cells or cardioblasts that arise per hemisegment is given for each genotype in the bottom right of each panel. Anterior is towards the left.

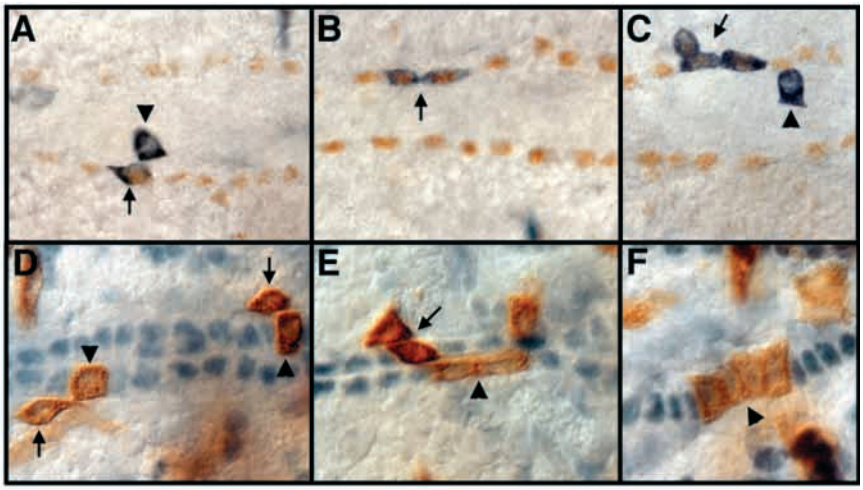

Fig. 5. Cell lineages of Odd-pericardial cells and cardioblasts. High-magnification dorsal views of stage 16 wild-type embryos labeled for β-galactosidase (blue (A-C) and brown (D-F)) to identify clones and Odd (brown; A-C), or Mef2 (blue; D-F). (A) Clone that contains one Odd-pericardial cell (arrow) and one other cell (a putative cardioblast; arrowhead) located medial and posterior to its Odd-positive sibling. (B) Clone that contains two Odd-pericardial cells (arrow). (C) Rare clone that contains three Odd-pericardial cells (arrow) and one putative cardioblast (arrowhead). (D) Two clones that each contain one cardioblast (arrowheads) and one other cell (a putative Odd-pericardial cell; arrows) located lateral and anterior to the cardioblast. (E) A two-cell cardioblast clone (arrowhead) and a cardioblast and putative Odd-pericardial cell (arrow) clone. (F) Rare four cell cardioblast clone (arrowhead). Anterior is towards the left.

galactosidase, to mark clones, and Odd, to identify Odd-pericardial cells. To identify the lineage of cardioblasts we double labeled embryos for β-galactosidase, to mark clones, and Mef2, to identify cardioblasts. Mef2 labels both cardioblasts and somatic muscles (Lilly et al., 1994; Nguyen et al., 1994). However, one can use Mef2 to identify cardioblasts unambiguously, owing to the juxtaposition of Mef2 cardioblasts and the dorsal midline. We identified 52 clones that contained at least one Odd-pericardial cell and 36 that contained at least one Mef2-labeled cardioblast.

Odd-pericardial cell clones fell into two major classes (Fig. 5A-C): those that contained two Odd-pericardial cells (*n*=19; 36%) and those that contained one Odd-pericardial cell and one cell that did not express Odd (*n*=25; 48%). The Odd-negative cell was located medial, slightly dorsal and posterior to the Odd-pericardial cell. We identified Odd-negative cells as cardioblasts because cardioblasts are the only heart cells located medial, slightly dorsal and physically adjacent to Odd-pericardial cells (see Fig. 2 and below). We also observed rare larger clones that consisted of either two pericardial cells and two cardioblasts (*n*=2; 4%), three pericardial cells and one cardioblast (*n*=3; 6%), or two Odd-pericardial cells and one cardioblast (*n*=3; 6%).

As noted, four Odd-pericardial cells develop per hemisegment. The simplest model by which our two major classes of clones could produce four Odd-pericardial cells predicts that one Odd-pericardial cell progenitor and two mixed lineage progenitors develop within each hemisegment. The Odd-pericardial cell progenitor would divide to produce two Odd-pericardial cells and the two mixed-lineage progenitors would each divide to produce one Odd-pericardial cell and one cardioblast. This model predicts a 2:1 ratio of mixed lineage to Odd-pericardial cell progenitors (or clones) and our data most closely fit this model, even though they yield an approximate 1.5:1 ratio of these clone types. Note, our clonal analysis of cardioblast clones and our descriptive analysis of the development of these three heart progenitors support the predicted 2:1 ratio of mixed lineage heart progenitors/clones to Odd-pericardial cell progenitors/clones (see below).

These data also argue against a strict lineal relationship between any of the heart progenitors that produce Odd-pericardial cells. Most notably, the rare four cell clones we

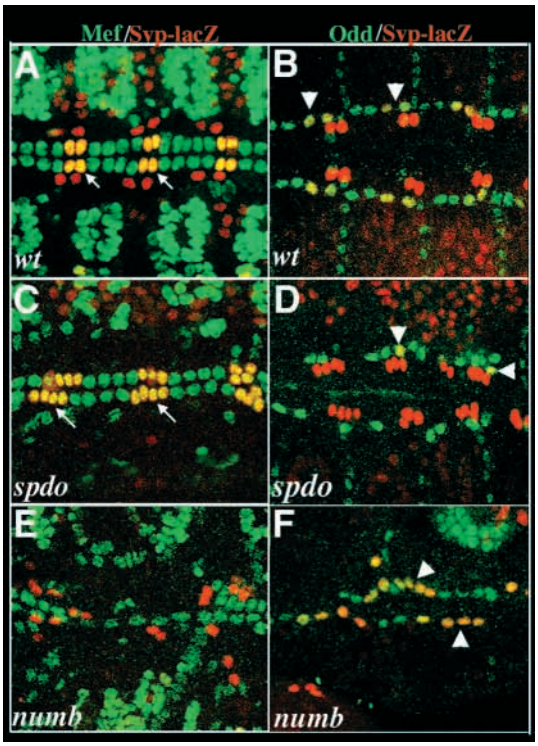

Fig. 6. *svp-lacZ* identifies the *sanpodo*- and *numb*-dependent cardioblasts and Odd-pericardial cells. High-magnification dorsal views of stage 16 wild-type (A,B), *sanpodo* (C,D) and *numb* (E,F) mutant embryos labeled for *svp-lacZ* (red), Mef2 (green, A,C,E) and Odd (green, B,D,F). (A,B) The two medial *svp-lacZ* heart cells per hemisegment express Mef2 and are cardioblasts (arrows, A); the two lateral *svp-lacZ* heart cells express Odd and are Odd-pericardial cells (arrowheads, B). (C,D) In *sanpodo* mutant embryos, nearly all *svp-lacZ*-positive heart cells express Mef2 and acquire the cardioblast cell fate (arrows, C); however, we do observe rare *svp-lacZ*/Odd-pericardial cells (arrowheads, D). (E,F) In *numb* mutant embryos all *svp-lacZ* heart cells express Odd (arrowheads, F) and none expresses Mef2 (E). Anterior is towards the left.

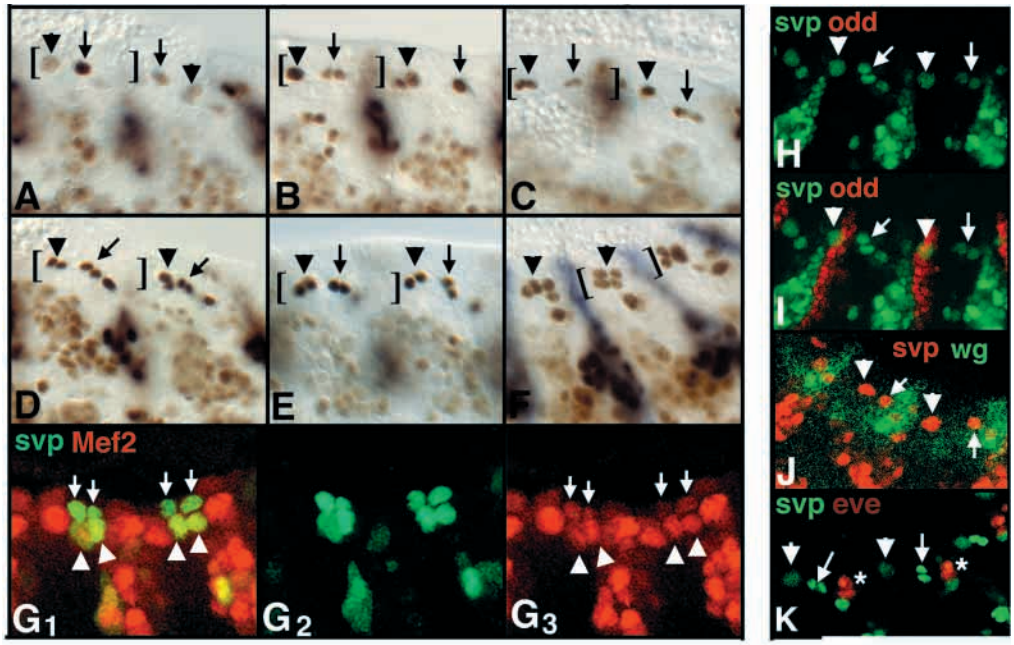

Fig. 7. The mixed lineage heart progenitors develop from multiple AP locations in the dorsal mesoderm. High-magnification lateral views of late stage 11 (A,H-K), early stage 12 (B,C), stage 12/4 (D), stage 12/3 (E), stage 12/1 (F) and stage 13 (G) embryos labeled for *svp-lacZ* (brown in A-F, green in G-I,K, red in J), Engrailed (blue in F), Mef2 (red in G), Odd (red in I), Wg (green in J) and Eve (red in K). Note, in H, I and K, *svp-lacZ* is green but *svp-lacZ* is red in J. (A) In wild-type late stage 11 embryos, two heart progenitors express *svp-lacZ* in each hemisegment (arrows/arrowheads; in panels A-F a single hemisegment is bracketed). (B-D) At the beginning of stage 12 (B,C) these cells divide and produce two pair of *svp-lacZ*-positive siblings by stage 12/4 (D). (E,F) During the latter part of stage 12, the two pair of siblings congregate together. (G_{1-3}) By stage 13 two of the four *svp-lacZ* heart cells can be identified as cardioblasts by high level Mef2 expression (arrowheads); the other two cells are identified as pericardial cells by low level Mef2 expression (arrows). (H,I) The anterior-most *svp-lacZ* heart progenitor (arrowheads in H-K) develops from dorsal mesodermal cells located directly beneath the ectodermal Odd-stripe (red in I). (J) The posterior *svp-lacZ* heart progenitor (arrows H-K) develops from dorsal mesodermal cells located either immediately anterior or just at the anterior edge of Wg-expressing ectodermal cells (green in J); the anterior *svp-lacZ* heart progenitor develops midway between Wg-stripes (arrowheads, J). (K) Relative location of *svp-lacZ* heart progenitors (arrows, arrowheads) to the progenitors to the Eve-mesodermal cells (asterisks). Eve-mesodermal cells develop from dorsal mesodermal cells located directly beneath Wg-expressing ectodermal cells. Anterior is towards the left.

identify fall into two classes: those that contain two Odd-pericardial cells and two cardioblasts (*n*=2) and those that contain three Odd-pericardial cells and one cardioblast (*n*=3). The presence of these two clone types is incompatible with a strict lineal relationship between any two of the three Odd-pericardial cell progenitors.

Specification of cell fates in the dorsal mesoderm appears to occur during early stage 11 (Carmena et al., 1998). Two general pan-mesodermal cell divisions precede these events and occur during stages 7 and 9, while spatially more distinct mesodermal cell divisions occur during stage 11 and stage 12 (Bate, 1993). We induced clones towards the end of the second pan-mesodermal cell division and detected only two and four cell clones. Thus, we interpret four cell clones as arising from clones induced prior to the second pan-mesodermal division and two cell clones as arising from clones induced after the second pan-mesodermal division. Taken together these data appear to favor a model whereby the initial pan-mesodermal divisions produce a pool of uncommitted mesodermal cells upon which patterning and cell fate specification mechanisms act during stage 11 to commit these cells to specific fates (e.g. mixed lineage or Odd-pericardial cell progenitors). In this model, any two of the three progenitors of Odd-heart cells could be siblings at some frequency even though they are not specified in a lineage dependent manner.

Clones that contained Mef2-labeled cardioblasts also fell into two major classes: those that contained two adjacent cardioblasts (*n*=16; 44.4%); and those that contained one cardioblast and another cell located immediately lateral and anterior to the cardioblast (*n*=18; 50%). We identified the non-cardioblast cell in these clones as Odd-pericardial cells because Odd-pericardial cells are the only heart cells that reside immediately lateral to cardioblasts (also see below). As with Odd-pericardial cell clones, we observed rare larger clones: one contained four cardioblasts (*n*=1; 3%), and one contained two cardioblasts and one Odd-pericardial cell (*n*=1; 3%).

As noted, six cardioblasts develop per hemisegment. The simplest model by which the two major classes of cardioblast clones could yield six cardioblasts per hemisegment predicts that two cardioblast and two mixed lineage progenitors arise in a hemisegment. The two cardioblast progenitors would each divide to produce two cardioblasts and the two mixed-lineage heart progenitors would each divide to produce one cardioblast and one Odd-pericardial cell. This model predicts a 1:1 ratio between cardioblast and mixed lineage progenitors (or clones) and our data, which yield an approximate 1:1 ratio between these clone types, fit this model well. Together with the lineage data from Odd-pericardial cells these results indicate that five heart progenitors produce the six cardioblasts and four Odd-pericardial cells that develop in each hemisegment (see Fig. 8).

Our cardioblast lineage data do not exclude the possibility that the two cardioblast progenitors are strictly linearly related. However, we observed only a single four-cell cardioblast clone among the 36 cardioblast clones identified, even though we induced *flp* expression at a stage that should activate *flp* in the parental (or even grand-parental) cells of these progenitors. Thus, we favor the model proposed above that all heart progenitors are selected from uncommitted pools of cells by

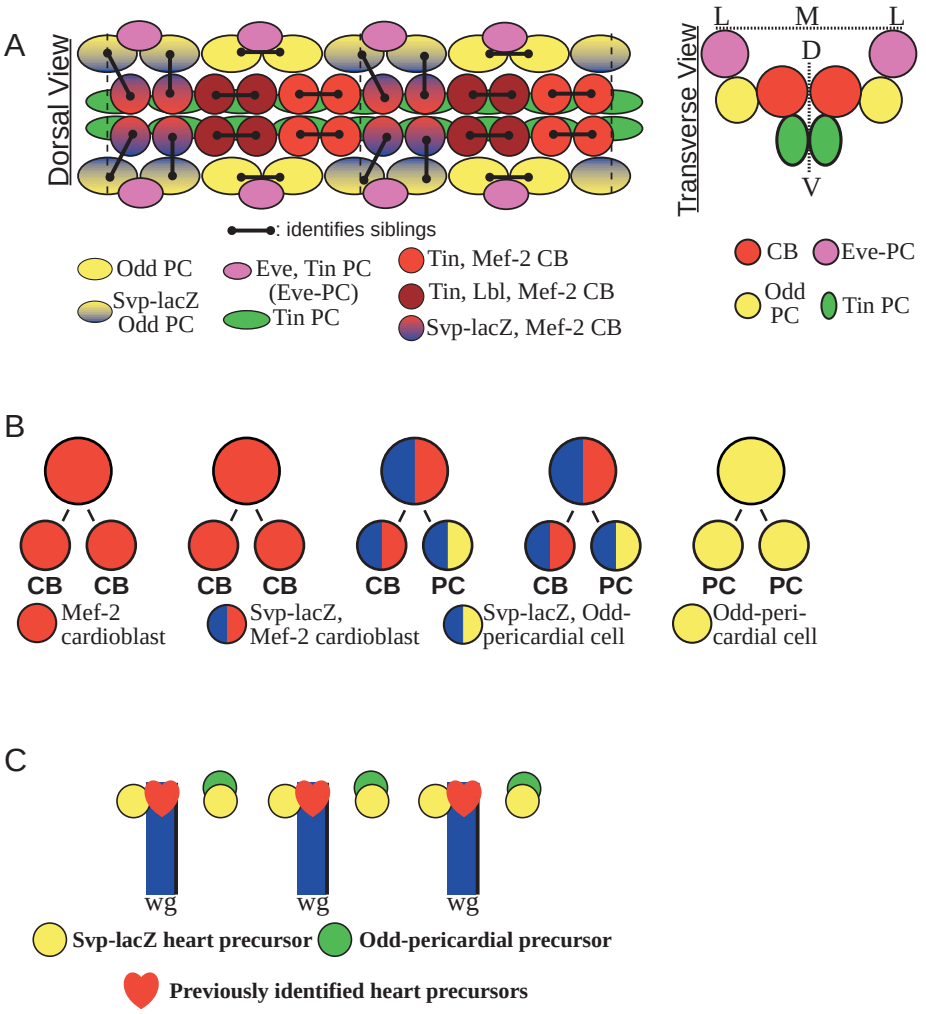

Fig. 8. (A) Schematic representation of a dorsal (left) and transverse (right) view of a mature embryonic heart showing the relative position of all known heart cells in stage 16 wild-type embryos. Lines that connect heart cells indicate sibling relationships. D, V, M, L refer to dorsal, ventral, medial and lateral, and provide orientation cues for the transverse schematic. Broken lines identify segmental repeat of heart cells; they do not mark precise segmental boundaries. (B) Cardioblasts and Odd-pericardial cells arise from five heart progenitors: two of which produce mixed lineage heart clones that contain one cardioblast (CB) and one Odd-pericardial cell (PC). (C) The heart progenitors that produce Odd-pericardial cells arise from two distinct AP positions in the dorsal mesoderm. Previously identified heart progenitors arise from dorsal mesodermal cells located beneath *wingless*-expressing cells. Anterior is towards the left.

patterning and cell-fate specification mechanisms. The future identification of four cell clones that contain three cardioblasts and one Odd-pericardial cell would support the idea that the two cardioblast progenitors do not develop in a lineage-dependent manner.

An enhancer trap in the seven-up gene identifies the two mixed-lineage heart progenitors

Towards the end of our lineage analyses we discovered fortuitously that an enhancer trap in the gene *seven-up* labels four heart cells in each abdominal hemisegment (Fig. 6A,B; we refer to this enhancer trap as *svp-lacZ*; Mlodzik et al, 1990; see also Bodmer and Frasch, 1999). Two of these cells reside at the dorsal midline and are cardioblasts as they express Mef2 (Fig. 6A). The other two cells reside just lateral and slightly ventral and anterior to the *svp-lacZ* cardioblasts. These two cells are Odd-pericardial cells as they express Odd. The relative positioning of the *svp-lacZ* cardioblasts and Odd-pericardial cells closely resembles that of the sibling cardioblasts and Odd-pericardial cells marked by our mixed lineage heart clones (Fig. 5). This suggested that the *svp-lacZ* heart cells may identify the four progeny of the two mixed lineage heart progenitors that arise in each hemisegment. If the four *svp-lacZ* heart cells are the progeny of these two progenitors, then loss of *sanpodo* function should convert all *svp-lacZ* heart cells to cardioblasts and loss of *numb* function should convert all *svp-lacZ* heart cells to Odd-pericardial cells. In *sanpodo* mutant embryos, all four *svp-lacZ* cells acquire the cardioblast fate (Fig. 6C,D) and in *numb* mutant embryos all four *svp-lacZ* cells acquire the Odd-pericardial cell fate (Fig. 6E,F). The results from these experiments demonstrate that *svp-lacZ* identifies the progeny of the two mixed lineage heart progenitors and that *numb* and *sanpodo* mediate the asymmetric divisions of these mixed-lineage heart progenitors.

Heart progenitors arise in multiple anteroposterior locations within the dorsal mesoderm

Present models of heart development suggest that all heart cells arise from dorsal mesodermal cells that reside beneath the transverse stripe of ectodermal cells that express Wg protein (Riechmann et al, 1997; Bodmer and Frasch, 1999). However, we first detect Odd-pericardial cells emerging roughly midway between Eve-pericardial cells which themselves arise beneath the Wg-expressing ectodermal cells (Fig. 3). The initial appearance of Odd expression in pericardial cells at the end of stage 12 precludes its use as a definitive marker of the embryonic origin of these cells. However, since *svp-lacZ* labels all four progeny of the two mixed lineage heart progenitors we assayed whether *svp-lacZ* is expressed in the mixed lineage

heart progenitors. If so, we could use *svp-lacZ* as a marker to identify the AP origin of mixed lineage heart progenitors.

We first detect *svp-lacZ* in the dorsal mesoderm during stage 11 in two individual cells (Fig. 7A,H-K): one is located beneath the ectodermal Odd stripe midway between Wg-stripes (arrowheads, Fig. 7H-K); the other is found immediately anterior to, or just at the anterior edge of, Wg-expressing cells (arrows, Fig. 7H-K). During early stage 12 these two cells divide and produce four cells, all of which express *svp-lacZ* (Fig. 7B-D) and Mef2 (not shown); at this stage none of these cells express Odd (not shown). During stage 12 the four *svp-lacZ* heart cells congregate together to form a tight four-cell cluster (Fig. 7D-F). Using confocal microscopy, we found that by stage 13, the four *svp-lacZ*-positive cells could be broken into two groups based on Mef2 expression: two cells express Mef2 at high levels (arrowheads, Fig. $7G_{1-3}$) and two cells express Mef2 at low levels (arrows, Fig. $7G_{1-3}$). Because cardioblasts retain and Odd-pericardial cells extinguish Mef2 expression, we identify the *svp-lacZ* heart cells with high-level Mef2 expression as cardioblasts and those with low Mef2 expression as Odd-pericardial cells. These results suggest that the two *svp-lacZ* heart progenitors arise from two different AP locations in the dorsal mesoderm, at least one of which does not arise from dorsal mesodermal cells located beneath the ectodermal *wg* stripe, the postulated source of all heart cells.

The *svp-lacZ* molecular marker also allowed us to distinguish between *svp-lacZ*/Odd-pericardial cells and the Odd-pericardial progenitor and its progeny. This facilitated the identification of the location of the Odd-pericardial progenitor just prior to its division. We first detect Odd-expression in the Odd-pericardial progenitor at stage 12/0. The Odd-pericardial progenitor is located beneath the ectodermal Odd-stripe and divides shortly after stage 12/0 to produce the remaining two Odd-pericardial cells per hemisegment (not shown). The Odd-progenitor and its progeny reside adjacent and anterior to the Odd/*svp-lacZ*-pericardial cells. These results suggest that the Odd-pericardial progenitor is specified from dorsal mesodermal cells located beneath the Odd ectodermal stripe. However, extensive mesodermal rearrangements occur prior to stage 12/0 (Dunin-Borkowski et al., 1994; Jagla et al, 1997). Thus, it is possible that the Odd-pericardial progenitor is specified in another AP domain (e.g. the *wingless* domain) and that the cellular rearrangements in the mesoderm place this cell beneath the ectodermal Odd stripe prior to the stage (stage 12/0), during which this cell stimulates Odd expression. Markers that identify the Odd-pericardial progenitor earlier in development are required to determine definitively whether the progenitor is born (1) beneath the Odd stripe, or (2) in a different AP domain, migrating beneath the Odd stripe later in development.

DISCUSSION

The results in this paper detail the identification and cellular and genetic analysis of the development of a new population of heart cells – the Odd-pericardial cells. We have shown that Odd-pericardial cells arise from three heart progenitors that develop in the dorsal-most region of the mesoderm (Fig. 8). Two of these progenitors divide to produce mixed-lineage clones that consist of one cardioblast and one Odd-pericardial cell, while the third progenitor divides to produce two Odd-pericardial cells. The pure lineage Odd-pericardial progenitor and one of the mixed lineage heart progenitors appear to develop from mesodermal cells located beneath the ectodermal Odd-stripes midway between Wg stripes. Since all heart cells are thought to arise from dorsal mesodermal cells located beneath the Wg stripe (reviewed in Bodmer and Frasch, 1999 and references therein), these two heart progenitors may identify another source of heart cells within the mesoderm. The second mixed-lineage heart progenitor develops from mesodermal cells located immediately anterior to, or at the anterior edge of, ectodermal Wg stripes. The mixed-lineage heart progenitors divide asymmetrically under the control of *sanpodo* and *numb*. *sanpodo* triggers one daughter cell to adopt the Odd-pericardial cell fate while *numb* opposes *sanpodo*/*Notch* signaling in the other daughter cell, allowing it to adopt the cardioblast fate. Once specified, Odd-pericardial cells align themselves in a row across the length of each hemisegment and thus form two bilaterally symmetric rows of pericardial cells that flank the cardioblasts (Fig. 8).

Cell type diversity in the Drosophila heart

In the mature heart, four Odd-pericardial cells arise in each abdominal hemisegment, immediately lateral and slightly ventral to the cardioblasts (Fig. 8). The identification of a new set of pericardial cells increases the total number of identified heart cells per hemisegment to 16 and also increases the complexity of heart cell types in the embryo. As noted, three different types of pericardial cells were known prior to this study: Eve-pericardial cells, Tin-pericardial cells, and Tin- and Ladybird-pericardial cells (Frasch et al., 1987; Bodmer, 1993; Azpiazu and Frasch, 1993; Jagla et al., 1997). Data presented in this paper identify two additional subtypes of pericardial cells: Odd-pericardial cells and Odd-/*svp-lacZ* pericardial cells. Note, Svp is detected in *svp-lacZ* cardioblasts but not *svp-lacZ*-pericardial cells in late stage embryos. Thus, five different types of pericardial cells are known to exist in the heart. At present, the precise roles pericardial cells play during heart development and function is poorly defined. In this context, it is important to note that embryos mutant for Odd display ectopic Eve-pericardial cells but Odd-pericardial cells appear normal (Ward and Coulter, 2000). In the future, it will be important to carry out careful descriptive/physiological assays of heart development/function in embryos that lack different pericardial cell types to begin to clarify the functional significance of pericardial cells in the heart.

Our studies also identify *svp-lacZ* as an additional marker of a subset of cardioblasts (Fig. 8; also see Bodmer and Frasch, 1999). *svp-lacZ* identifies the two adjacent cardioblasts in a hemisegment that do not express Tin. Of the four remaining cardioblasts all express Tin and the anterior two express Lbl (Jagla et al., 1997) (Fig. 8). Our lineage studies suggest the two Lbl-cardioblasts are siblings and that the two Tin-positive/Lbl-negative cardioblasts are siblings (see below). Gene expression analyses thus identify three types of cardioblasts. At present it is unclear if these different types of cardioblasts have alternate functions during heart development and/or physiology. However, morphological evidence alludes to functional differences between different cardioblast cell types. For example, the alary muscles in the abdomen, which physically link the heart to the dorsal epidermis, attach at regular

segmental intervals to the heart (Rugendorff et al., 1994). In addition, three pairs of lateral valves or ostia perforate the cardioblast tube in the posterior abdomen (Rizki, 1978, Bate, 1993). During the relaxed state, hemolymph enters the heart through these valves and is pumped forward as the heart contracts. Alary muscles probably recognize and attach to one type of cardioblast, while the juxtaposition of two specific types of cardioblasts may permit or trigger ostia formation posteriorly in the heart. Careful descriptive and morphological analysis of the heart in embryos lacking each type of cardioblast may clarify the roles these cells have in heart development and its physiology.

Five heart progenitors produce all cardioblasts and Odd-pericardial cells

Our determination of the pedigree of ten of the 16 known heart cells provides the most comprehensive pedigree analysis of heart cells to date (Fig. 8). Prior to our study, models of heart development proposed that cardioblasts and pericardial cells arise from separate cell lineages (Park et al., 1998; Bodmer and Frasch, 1999). However, our lineage tracing and descriptive analyses indicate that two mixed lineage heart progenitors divide asymmetrically under the control of *numb* and *sanpodo* to produce clones that contain one cardioblast and one Odd-pericardial cell. These studies also indicate that the remaining two Odd-pericardial cells arise from one Odd-positive progenitor and the remaining four cardioblasts, all of which express Tin arise from two cardioblast progenitors. Nearly all two cell cardioblast clones we observed consisted of physically adjacent cardioblasts. Thus, the anterior two Tin cardioblasts (the Lbl-cardioblasts) appear to be siblings as do the posterior two Tin-cardioblasts (Fig. 8). Taken together our lineage data support a model where in each hemisegment two heart progenitors produce mixed cardioblast/pericardial cell clones and the remaining heart progenitors produce clones that contain either cardioblasts or pericardial cells.

Two previous studies assayed the lineage of the Eve-pericardial cells; these studies yielded conflicting conclusions on the lineage of these cells. One study used the FLP/FRT lineage tracing system; their work suggested that each Eve-pericardial cell in a hemisegment arises from a separate progenitor (Park et al, 1998). A second study used detailed descriptive analyses to follow Eve-pericardial cell development; this work suggested that Eve-pericardial cells arise from the same progenitor (Carmena et al., 1998). Clearly, additional lineage-tracing and descriptive assays are required to delineate the exact lineal ancestry of the Eve-pericardial cells as well as the Tin-pericardial cells.

Heart progenitors appear to arise in multiple anteroposterior positions in the dorsal mesoderm

Present models of heart development suggest that all heart cells arise from dorsal mesodermal cells that reside directly beneath the stripe of ectodermal cells that express *wg* (Riechmann et al., 1997; Bodmer and Frasch, 1999). Consistent with this, Eve-pericardial and Ladybird heart cells arise from dorsal mesodermal cells located below the Wg-stripe (Jagla et al., 1997). Our data on the development of Odd-pericardial and *svp-lacZ* heart progenitors suggest that heart progenitors arise from multiple AP positions in the dorsal mesoderm (Fig. 8). Specifically, we find that at least four of the 16 heart cells appear not to arise from dorsal mesodermal cells located beneath Wg stripes. The Odd-pericardial progenitor and one Odd-/*svp-lacZ* mixed-lineage heart progenitor appear to develop from dorsal mesodermal cells located beneath the ectodermal Odd-stripe, midway between Wg stripes. The second Odd-/*svp-lacZ* mixed lineage heart progenitor develops either just anterior to, or at the anterior edge of, Wg-expressing ectodermal cells. These results suggest that different types of heart cells arise in different AP locations in the dorsal mesoderm. Alternatively, some or all of these progenitors may form beneath Wg stripes and then migrate to different AP positions in the heart. The future identification of molecular markers that identify these cells (or their progenitors) earlier in development should clarify this issue. If a population of heart cells is not born beneath Wg-expressing ectodermal cells, then it will be important to determine whether signaling pathways other than the Wg pathway (e.g. Hedgehog signaling) promote their development.

The clarification of the genetic and cellular mechanisms that control *Drosophila* heart development has clear relevance for the analysis of mammalian heart development (Bodmer and Frasch, 1999). Both mammalian and *Drosophila* heart cells appear to be of equivalent embryonic origin. In both cases heart cells arise from the cells that migrate most distally from the point of invagination during gastrulation. In addition, at least some of the molecules that control mammalian and *Drosophila* heart development appear to be evolutionarily conserved. Most notably, Tin and its vertebrate homologs, *Nkx2-5* genes, display similar expression patterns in the developing *Drosophila* and vertebrate hearts, respectively (Harvey et al., 1999). In the future, it will be interesting to see the extent to which the developmental programs that control heart development in flies and mammals are conserved.

We thank Doug Coulter, Nirapuma Deshpande, Teresa Orenic, Bob Holmgren, Deborah Hoshizaki, Manfred Frasch, Bruce Paterson, Zhi-Chun Lai, Talila Volk, Alan Michelson, Ian Duncan and Kathy Matthews and the Bloomington Stock Center for generously providing antibody reagants and/or fly stocks. We obtained antibody 4D4 (Stephen Cohen) from the Developmental Studies Hybridoma Bank developed under the auspices of NICHD and maintained by The University of Iowa, Department of Biology. We also thank Heather Broihier, Alan Michelson and Hemlata Mistry for invaluable critiques of the data and manuscript. J. B. S. supported by the Cancer Research Fund of the Damon Runyon-Walter Winchell Foundation Award, DRS-9 and by an HHMI Research Program for Medical Schools Junior Faculty Award #76296-538202. This work was supported by NINDS grant (RO1-NS36570) and an NSF Grant, IBN-0077727.

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