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Fashioning the vertebrate heart: earliest embryonic decisions

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SUMMARY

Our goal here is to set out the types of unitary decisions made by heart progenitor cells, from their appearance in the heart field until they form the simple heart tube. This provides a context to evaluate cell fate, lineage and, finally, morphogenetic decisions that configure global heart form and function. Some paradigms for cellular differentiation and for pattern generation may be borrowed from invertebrates, but neither *Drosophila* nor *Caenorhabditis elegans* suffice to unravel higher order decisions. Genetic analyses in mouse and zebrafish may provide one entrance to these pathways.

Key words: pattern, evolution, polarity, organ, myogenesis, mouse, zebrafish

INTRODUCTION

We have accumulated a wealth of details about vertebrate heart development, but we still lack unifying paradigms. This status is not unique to the heart among vertebrate organ systems and, in fact, the heart is better studied than most. What we are missing with regard to vertebrate organs are the type of systematic approaches and developmental principles gleaned, for example, from studies of patterning of body form and generation of bimodal cell fate decisions, underpinned by the genetic analysis of *Drosophila* and *C. elegans* development. What are the steps of organ development and can they be revealed in an informative manner by single gene mutations? For example, can we define genetic and biochemical pathways that regulate global organ size and form? that generate component parts, such as chambers? that pattern boundaries and relative body position of organs? that generate a seamless circulation? that establish heart rate and blood pressure appropriate for homeostatic needs?

We establish here a base to examine these issues in heart development, grounded in experimental embryology, and indicate where we see unifying themes. The review focuses, first, upon the heart field, the embryonic clustering of cells evident soon after gastrulation from which the heart originates, and, second, upon generation of the heart tube, the earliest formed heart structure. The distinction is artificial, in that decisions as to heart tube form and function begin in the field. In the search for unifying themes, the scope of this review is limited to the earliest stages of development, because they are nearly identical among all vertebrates, unlike the subsequent septation of the chambers and of the outflow tract, which varies between species depending upon the utilization of lungs. Thus, we provide only a brief discussion of these later stages of cardiac morphogenesis (e.g. involving cardiac neural crest and septation), which also are reviewed elsewhere (Kirby and Waldo, 1990; Olson and Srivastava, 1996).

The third focus, or actually theme throughout, is how the vertebrate cardiovascular system differs from that of the presumptive evolutionary chordate ancestor. This is done in the spirit of asking what components are needed to be added to the primitive chordate to make a vertebrate heart? At best we can tell there are two essential new ingredients: (1) vertebrates all have a continuous endothelial lining to the heart and vessels and (2) vertebrates have developed a second chamber in the heart, one designed for generating high systemic blood pressure. This, in turn, brought a requirement for valves and a conduction system constituted to ensure that blood flows in one direction. In addition, unlike its midline or symmetrical predecessors, the vertebrate heart is laterally asymmetric, residing on the left and looped to the right. We find no evidence that this concatenation of components was present in evolutionary ancestors of vertebrates (although important elements are found in some invertebrates, such as cephalopods (Martin, 1980), animals not believed to be on an evolutionary line with vertebrates). The conservation of these elements among all vertebrates may reflect the difficulty of changing one part in isolation from the others during embryonic development. This is understandable given that the cardiovascular system is distributed throughout the body, is linked to all organs and is essential for survival. Developmental interdependence of tissues in general may provide an important limitation on variability in morphology (Hall, 1992). One issue raised in the review is whether some of the "new" components of the vertebrate heart may be dissected free by mutational analysis of development and revealed as modular innovations, summing to add a "new" vertebrate cardiovascular system akin to the "new" vertebrate head (Gans and Northcut, 1983). Some credence for this simplistic approach is provided by the the discovery in

zebrafish of single gene mutations that selectively remove or perturb, individually, some of these “new” elements.

FORMATION OF THE VERTEBRATE HEART FIELD

The heart field is the region of the embryonic mesoderm that contains the cardiac progenitor cells. Under certain experimental conditions, the heart field can be extended to neighboring regions that are not coextensive with the area normally fated to give rise to the heart. For example, mesoderm adjacent to the normal heart-forming area will generate beating tissue after explantation into neutral solution or after removal of the normal heart-forming region (Copenhaver, 1926, 1939). The field, therefore, is a region competent to respond to inductive signals (Jacobson and Sater, 1988; Waddington, 1932), is larger than the area fated to produce the organ (Jacobson and Sater, 1988) and reflects developmental potency rather than fate.

We have outlined below the timing of some important steps of early heart development in chick, frog and fish. Naturally, experimental manipulations have not been done with humans, and few with mouse in the tracking of the early paths of heart precursors, but an attempt is made in Table 1 to correlate the expected timing of decisions in those mammalian species with amphibians, fish and avians, species in which the position and migration of heart precursors have been more intensively studied.

Timing of heart development

Avian

In chicks, the cells destined to form the heart arise in the epiblast lateral to the primitive streak, invaginate through the streak and migrate rostrolaterally to form part of the lateral plate (Garcia-Martinez and Schoenwolf, 1993; Rosenquist, 1970; Rosenquist and DeHaan, 1966). Fig. 1 is a diagram of the relative positions of cells that will form the heart, following their migrations from the primitive streak, to lateral plate and heart tube. As shown, the ingression pattern along the streak at Hamburger Hamilton (HH) stage 3a (early streak) matches

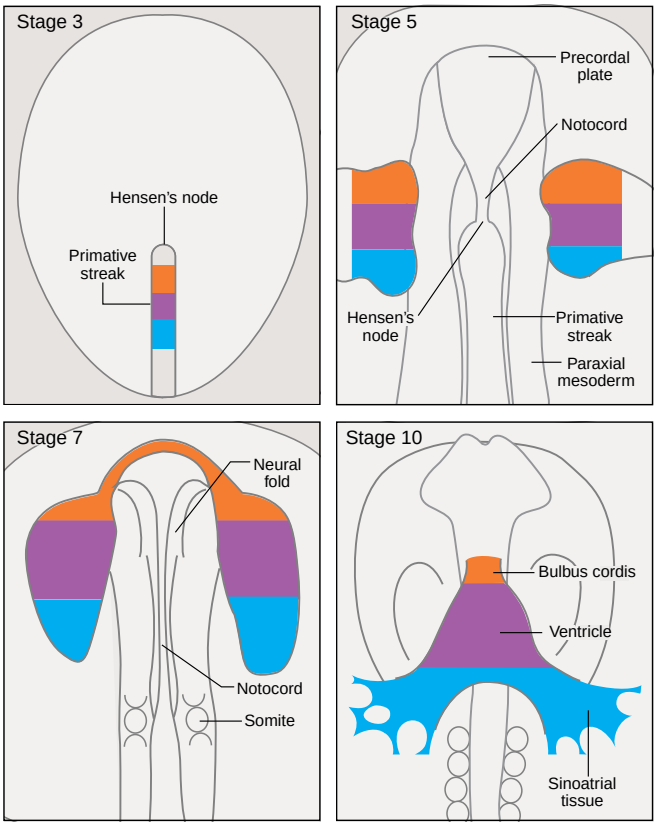

Fig. 1. Schematic of the location of heart precursors in the chick embryo, from a ventral view at the stages indicated. The color patterns are to show the region of the heart to which precardiac cells will eventually contribute. The relative anterior-to-posterior positions of precardiac cells in the primitive streak (HH stage 3) is retained in the heart field in the mesoderm (as shown in HH stage 5 and 7), and in the heart tube at HH stage 10 (DeHaan, 1963, 1965; Garcia-Martinez and Schoenwolf, 1993). There is no evidence for definitive borders of these lineages in the field. At stage 10, the anterior-most region is termed bulbus cordis (or conotruncus) and is the outflow region connected to the aortic sac.

Table 1. Milestones of early heart development in different species

	mouse	chick	human	frog	zebrafish
Migration of precardiac cells from epiblast	7 dpc (primitive streak)	HH4 (definitive streak)	15-16 days	stage 10	50% epiboly (5.5 hpf)
First evident assembly of myocardial plate	7 dpc (late primitive streak; just presomite)	HH5 head process (19-22hrs)	18 days	~stage 13	8-10 somites (~13 hpf)
Generation of single heart tube initiated	8 dpc (5-10 somites)	HH9 (7 somites)	22 days (4-10 somites)	stage 28	20 somites (ring) (~19 hpf)
Tubular heart starts contraction	8.5 dpc 8-10 somites)	HH10 10 somites (33-38 hrs)	23 days	~stage 33	26 somites (22 hpf)
Looping	8.5 dpc	HH11 (11-13 somites)	23 days	stage 33-36	33 hpf
Cushions form	9.5 dpc	HH17	28 days (30-38 somites	~stage 41	48 hpf

The mouse data are primarily from DeRuiter et al. (1992) and Kaufman and Navaratnam (1981), the chick from DeHaan (1965), Garcia-Martinez and Schoenwolf (1993), Manasek (1968), Patten (1957), Romanoff (1960) and Viragh et al. (1989), the human from Hamilton and Mossman (1972) and Sissman (1970), the frog from Sater and Jacobson (1990) and the zebrafish from Stainier and Fishman (1992).

the eventual anteroposterior placement of progeny in the heart (HH stage 10), with the most rostral cells contributing to the bulbus cordis at the extreme anterior end of the heart and the most caudal to the sinoatrial region, at the extreme posterior end (Garcia-Martinez and Schoenwolf, 1993). Progeny of cells of this region contribute to all layers of the heart tube, including myocardium, endocardium and parietal pericardium, as well as to endothelial cells in the vicinity of the heart. In the more rostral region of the streak, cells also populate head mesenchyme and foregut. Regions within the streak can be reversed without affecting heart formation, so the streak is plastic with regard to position at that time (Inagaki et al., 1993). In the lateral plate (Fig. 1, HH stages 5 and 7), cells retain their relative anteroposterior positions.

As shown in Fig. 2, of HH stage 8, the lateral plate splits to form two epithelial layers, the somatic mesoderm, which includes migratory precursors for the limb musculature, and the splanchnic mesoderm, which remains an epithelial sheet and includes the cardiac precursors (Manasek, 1968). As the embryo folds ventrally, the splanchnic mesoderm is carried with it, bringing it ventral to the foregut, which is generated as the lateral folds meet in the ventral midline. The precursors of the endocardium are included in the splanchnic mesoderm and

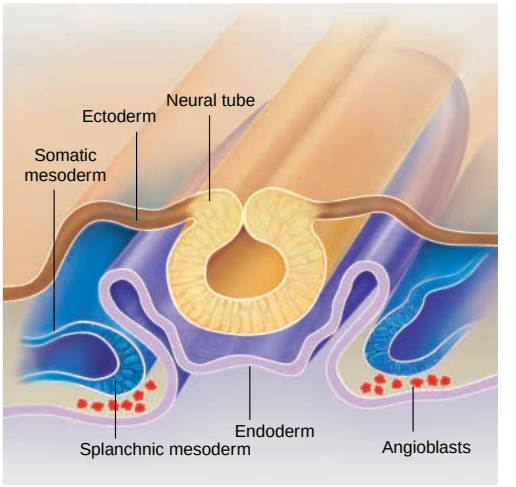

Fig. 2. Chick embryo at HH stage 8, viewed in cross-section in the region of the precardiac mesoderm, looking anteriorly. The embryo is folding ventrally, generating the foregut, and the mesoderm has split into somatic and splanchnic layers, the latter containing the myocardial precursors. The endocardial angioblast precursors are between the splanchnic mesoderm and foregut.

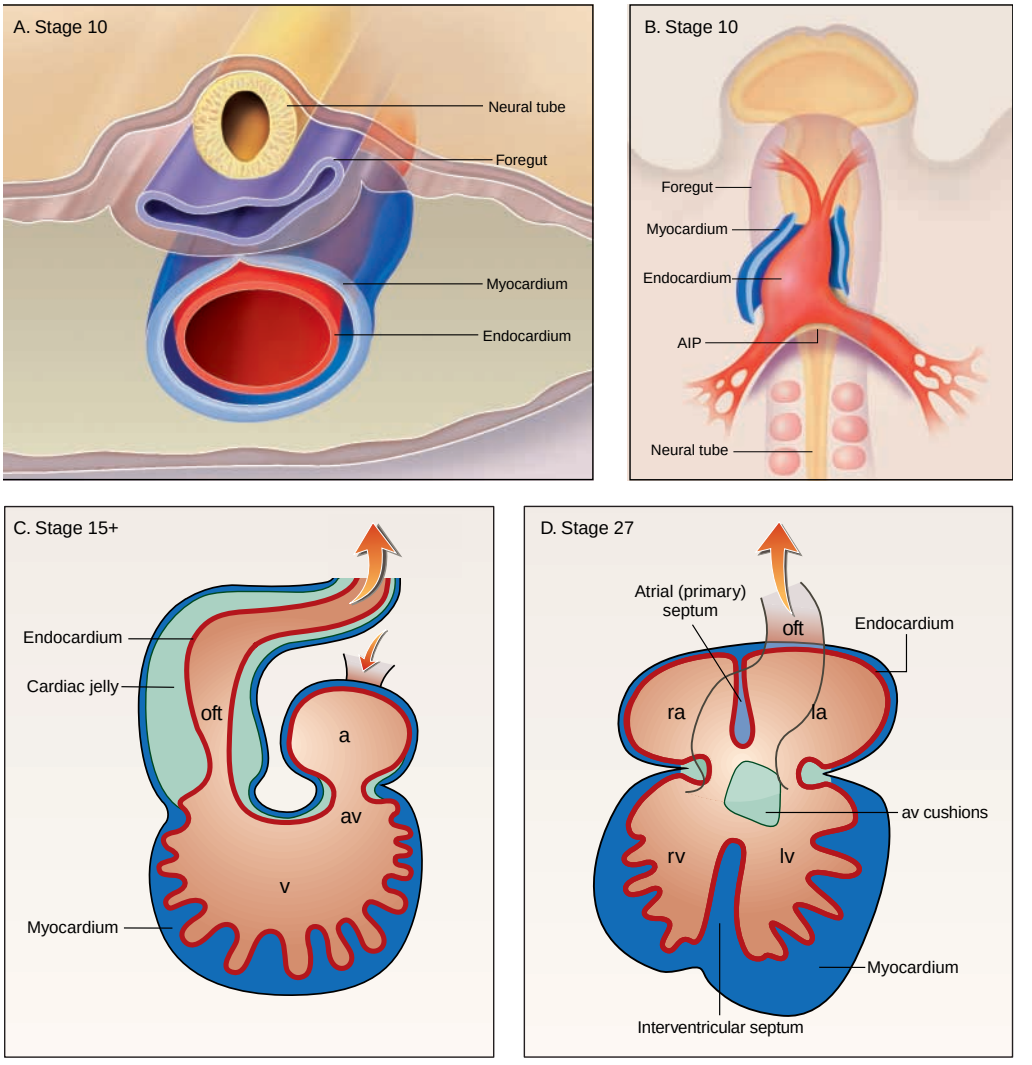

Fig. 3. Chick heart tube at HH stage 10, shown in cross-section (A), and in ventral view (B) and (C) at HH stage 15+ and (D) HH stage 27, when, in the chick, septation is beginning. By stage 10, the ventricular region of the tube is beginning to bend ("loop") to the right. By HH stage 15, the two chambers, atrium and ventricle, are morphologically different, and the ventricle is beginning to thicken by the growth of the wall and addition of trabelculae. The cardiac jelly separates the myocardium from endocardium and becomes thicker especially in the regions where cushions and valves will form, i.e. the outflow tract and the atrio-ventricular junction.
(D) In air breathing animals, there is division of the atrium and ventricle into right- and left-sided chambers by the growth of the interventricular septum, generated first as a coalescence of the trabeculae and by growth of the atrio-ventricular cushions. (C and D modified from Chan-Thomas et al., 1993). a, atrium; av, atrioventricular canal; la, left atrium; lv, left ventricle; oft, outflow tract; ra, right atrium; rv, right ventricle; v, ventricle; AIP, anterior intestinal portal; cushions shown in green).

begin to form separate clusters on the foregut side of the epithelial sheet (Fig. 2) by HH stage 8. They are distinguished from the premyocardial cells by expression of QH1 (Linask, 1992; Linask and Lash, 1993) and *flk*, and lack expression of N-cadherin (Linask, 1992) and muscle genes.

The heart primordia fuse at the midline to form a tubular heart by HH stage 10, as shown in the diagram of Fig. 3A in cross-section and Fig. 3B from a ventral view. The process begins rostrally and proceeds caudally. By HH stage 9-10, the heart begins to bend, or loop, to the right (Fig. 3A). Differentiation, in terms of muscle-specific gene expression, begins in the rostral regions of the cardiogenic plate at HH stage 7, (Bisaha and Bader, 1991; Yutzey and Bader, 1995), even prior to fusion, and proceeds caudally.

Amphibians

The heart-forming regions are part of the leading edge of the mesoderm entering on the lateral sides of the blastopore (Holtfreter, 1938). They are adjacent to endoderm fated to become stomach or esophagus (pharyngeal endoderm). With anterior migration of the lateral mesoderm, the precardiac region comes to lie, by the neurula stage, lateral to the neural folds of the hindbrain and adjacent to the endoderm of the esophagus, stomach and duodenum (Jacobson, 1961). By the tailbud stage, the heart-forming mesoderm has moved ventrally. In *Taricha torosa*, the heart forms between tail-bud stages 28-31 and beats at larval stage 34. The equivalent stages of *Xenopus* are shown in Table 1. Explantation of the lateral edge of the blastopore lip can form beating tissue in culture and lateral mesoderm explants can form beating tubular hearts if transplanted with other adjacent tissues (see below). Regulation is quite remarkable in urodeles and this plasticity is retained until quite late in development, such that normal hearts form after removal of the entire ventral lateral mesoderm from tail bud (stage 27) *Amblystoma punctatum*.

Fish

In the zebrafish, the heart precusors, assessed by intracellular injection of fluorescent dextran, are in the ventral marginal zone at the mid-late blastula stage (Lee et al., 1994; Stainier et al., 1993). Cells ingress and migrate towards the forming axis to generate two tubes, one on either side of the midline, by the 21-somite stage, and thereafter fuse to form an intermediate cone which grows and rotates, forming the heart tube by 24 hours postfertilization (Stainier et al., 1993). Transplantation between embryos show that the field is plastic, at least until gastrulation, in that ectopic blastula cells introduced to the heart field at the late blastula stage are incorporated into the heart (Lee et al., 1994).

Questions about the field

The heart field has several properties, shared with fields of other organs (Huxley and De Beer, 1934): (1) a border, (2) within the border, a gradient of propensity to become heart, and (3) an ability to regulate, when a few cells are introduced or removed, without perturbing the ultimate form of the heart. What are the relationships of these field properties to the generation of heart form? Is the field polar or patterned, and if so, how, and by what interactions within the field and with neighboring structures? Are there borders between cellular compartments, which will contribute to different chambers? How does the progressive assumption of cardiac cell fate relate to the generation of heart form? What gauges the overall size of the heart? In this regard, there is feedback between the heart field and neighboring tissues, in that a normal heart can form after addition or removal of cells from the field. Is there a means by which the embryo can compare field size to the dimensions of neighboring fields or to that of the whole animal? Currently there are no definitive answers to these questions, but they nevertheless serve as a framework to discuss issues related to organ development and to assist in the interpretation of discrete effects of single gene mutations.

Polarity and patterning in the heart field

The vertebrate heart tube is aligned along the anterior-posterior axis. Arterial flow is directed from the ventricle, at the anterior end of the heart, through the ventral aortic vessel and the branchial arches, and subsequently travels posteriorly to the dorsal vessel. Blood flow returns to the heart through the venous system to the atrium, which lies at the posterior end of the cardiac chamber. (In the fish, the early heart tube is reversed because the yolk sac pushes the atrium anteriorly. Once the yolk is absorbed, it, too, has outflow anteriorly). The orientation of the chambers is reflected in all prior stages. In the chick, cardiac progenitor are aligned with a similar polarity even within the epiblast (Fig. 1). In the zebrafish, retinoic acid application at the onset of gastrulation truncates the heart in a directional manner, starting from the ventricular end (Stainier and Fishman, 1992). Parts of chambers are deleted at intermediate doses, suggesting that there is positional information imparted to the heart field independent of chamber allocation, perhaps related to axial polarity of the embryo.

Direct marking experiments in the chick indicate that polarity is retained within the lateral plate (Fig. 1) (DeHaan, 1963; Garcia-Martinez and Schoenwolf, 1993; Rosenquist and DeHaan, 1966). The polarity of the cardiogenic plate is evidenced in several ways. One is a preconfiguring of the beat rate gradient intrinsic to different regions of the heart. If the embryonic field is explanted, or physically separated so as to form separate beating vesicles, the right rostral, pre-conus, region beats most slowly while the left caudal, pre-atrial region beats the most rapidly (Satin et al., 1988). If lateral plate pieces are transplanted before stage 5-7, the transplanted tissue adopts the beat rate of the host region, suggesting the presence of information external to the transplanted tissue. Evidence of early polarity is also provided by chamber-specific gene expression, which begins regionally in the lateral plate (Yutzey et al., 1994).

One suggested source of such patterning information is the endoderm. Precardiac cells are in contact with, and appear to migrate along, the endoderm (DeHaan, 1963). As observed in real time in the chick, this movement appears to be random, but, by HH stage 6, the movement becomes directed anteriorly, as cells move towards and over the anterior intestinal portal and towards the midline. Is there orienting information on the endoderm? A cephalad-increasing gradient of fibronectin exists at the endoderm-mesoderm interface in the lateral plate, and its perturbation interferes with heart formation in the chick (Linask and Lash, 1988). Mice rendered fibronectin-deficient by targeted gene replacement are able to generate a heart tube, although it is deformed (George et al., 1993), suggesting that

fibronectin haptotaxis is unlikely to provide all such orienting information.

Currently, it is not clear how cells sort out positionally in the field. In *Drosophila*, cells of a particular imaginal disc compartment prefer to associate together during development, a property due to their uniform expression of a selector gene, such as *engrailed* in the posterior compartment of the wing disc (Lawrence and Morata, 1976). It is not known whether there are sharp boundaries between atrial and ventricular "compartments" in the field, nor whether there is a distinctive pattern or coherence to migratory paths of cells with different fates.

Cell fate decisions within the heart field

Inductions in the field

Three tissues that neighbor the cardiogenic plate have been proposed as regulators of heart development: the endoderm, the neurectoderm and the Organizer. There are also likely to be signals within the field itself. In general, studies of these interactions have used the generation of beating tissue or the expression of cardiac genes as experimental endpoints, thereby providing information directed more to cell fate, specifically myocardial cell fate, than to organ form.

Endoderm

It has been clear from the earliest studies of amphibian heart development that mesoderm alone is insufficient to generate heart tissue in explant culture (Ekman, 1921; Stohr, 1924). As heart formation proceeds, there is a progressive decrement in the need for supportive signals. In urodeles, removal of the endoderm blocks heart formation entirely if removed prior to the tail bud stage (Balinsky, 1939; Jacobson, 1961), but only partially when removed later (Jacobson, 1961). The heart supporting ability of endoderm diminishes with distance from the normal heart primordia (Mangold, 1956).

Exactly what stage of heart development is endoderm-dependent is debated. In fact, there may be different signals for sequential stages and these may be more or less temporally dispersed in different species. This may explain partially discrepant results between mammals and avians (DeHaan, 1965). Some signals may be needed for the initial steps in the establishment of the cardiac cell fate and others in later steps of cardiac cell-specific differentiation. More directed molecular ablation, or assays for gene expression rather than contractile function, may reveal multiple requirements for endoderm at discrete stages of cardiogenesis (Nascone and Mercola, 1996). Gannon and Bader (1995) have shown that explanted HH stage 4-6 anterolateral mesoderm expresses cardiac-specific genes even in the absence of endoderm, although it does not complete myofibrillogenesis. Removal of endoderm in vivo from one side of chicks with experimental cardia bifida does not block formation of the heart tube on that side, although it does interfere with its contractile function (Gannon and Bader, 1995). Similarly, lack of an endodermal signal in the axolotl mutant, *cardiac lethal*, does not prevent formation of the heart, although it cannot beat (Humphrey, 1972). In the frog, endoderm appears to supplement a signal from the Organizer (Nascone and Mercola, 1995). Hence, endoderm is likely to be needed for steps between the assignment of cell fate and organ form. It is likely to play an instructive rather than a permissive role in initiating some of these decisions, in that non-cardiac mesoderm in the chick can express cardiac markers in presence of anterior-lateral endoderm (Schultheiss et al., 1995).

Ectoderm

The failure to form heart caused by stripping of endoderm can be partially alleviated by simultaneous removal of the neural plate (Jacobson, 1960), suggesting that the neurectoderm may exert an inhibitory influence on heart development. The anterior plate and folds are more inhibitory than are posterior regions. In *Drosophila*, dorsal ectodermal expression of *dpp* is needed for maintenance of dorsal mesodermal expression of the *tinman* gene (necessary for heart development in that species), suggesting that very early interactions of mesoderm with ectoderm may initiate or maintain components of the heart field (Frasch, 1995). *BMP-4*, a *dpp* homolog, is expressed in the ventral ectoderm of zebrafish (A. Chin, J.-N. Chen, M. C. Fishman, unpublished data), overlying the ventral mesodermal *Nkx2.5* expression in the heart field (Chen and Fishman, 1996), suggesting that such inter-germ layer relationships may be conserved through evolution.

Organizer

The heart-forming mesoderm in *Xenopus* flanks the Organizer, the dorsal lip of the blastopore, a tissue that is needed for heart formation. No heart forms if the Organizer is removed during early gastrulation. In culture of early (stage 10) gastrulae, the Organizer supports heart formation (Sater and Jacobson, 1990). The dorsal lip can induce a heart as part of the secondary axis if transplanted ventrally (Sater and Jacobson, 1990). Because signals from the Organizer are important in dorsal-ventral axial determination, the Organizer-dependence of heart formation has been interpreted as indicating a role for axial signals in heart formation. This concept is supported by observations that lithium treatment, which increases dorsal structures, causes development of large radial hearts, while UV light exposure, which causes ventralization, leads to a reduction in heart size. The Organizer appears to provide only a weak or partial signal, and its cardiac-inducing potency is markedly enhanced by endodermal signals (Nascone and Mercola, 1995). This combination of signals appears to act early during gastrulation, as heart mesoderm explanted without them as early as stage 10.5 can form beating tissue.

Intra-field interactions

The nature of the interactions within the cardiac field, or with neighboring fields, are not known. Presumably, such interactions are critical for the generation of the proper number of cardiac precursors, in that the field can maintain normal regulation of organ development despite minor perturbations. One might predict that lateral inhibition between fields, or between chamber precursors, could ensure proper boundary establishment by regulating the number and position of cells with particular fates, as occurs in the *C. elegans* vulva or *Drosophila* wing. There also may be self-reinforcing signals, so-called "community effects". For example, cardiac-specific gene expression can be initiated by epiblast cells of the pregastrulated (HH stage 3) chick when cultured at high density, but not at low density. By HH stage 4-8, cardiac genes are expressed even at clonal density (Litvin et al., 1992).

Responses in the field

Transcriptional regulators

Nkx2-5 homeodomain proteins

A marker of the early stages of the heart field is *Nkx2*-5 (Harvey, 1996), the divergent homeodomain homolog of Drosophila *tinman* (Harvey, 1996). In mouse (Komuro and Izumo, 1993; Lints et al., 1993), frog (Tonissen et al., 1994), fish (Chen and Fishman, 1996) and avians (Schultheiss et al., 1995), *Nkx2-5* is expressed during gastrulation in the lateral plate mesoderm. In most species, *Nkx2*-5 expression is not completely restricted to the precardiac region and is also expressed, for example, in pharyngeal endoderm. Some other related members of the *Nkx2* family, such as *Xnkx-2.3* in *Xenopus*, display similar patterns of expression (Evans et al., 1995). *Drosophila* contains a single member of this family, which is required for heart formation (Azpiazu and Frasch, 1993; Bodmer, 1993). In the mouse, targeted ablation of *Nkx2-5* does not prevent formation of the heart tube, although it does block heart development at the stage of looping morphogenesis, indicating that, if it is critical for the initiation of cardiac cell fate decisions, there must be redundant pathways (Lyons et al., 1995). Overexpression of *Nkx2.5* in frog or zebrafish generates larger than normal hearts in otherwise normal animals (Chen and Fishman, 1996; Cleaver et al., 1996). Interestingly, in the zebrafish, *Nkx2.5* is first localized at gastrulation as a gradient in the ventral-marginal region with a distribution that matches the propensity to become heart (Chen and Fishman, 1996). (There is, in fact, evidence for early expression in frog as well, although it has been difficult to localize by in situ hybridization (Evans et al., 1995)). This suggests that the propensity to become heart is controlled, in part, by achieving a threshold level of *Nkx2-5* and that raising the border zone level by injection brings a larger number of cells to threshold. *Nkx2*-5 seems to be insufficient to specify myocardial cell fate determination by itself. High level overexpression does cause low level expression of cardiac-specific genes in zebrafish, even in ectopic locations after transplantation and after transfection into fibroblasts, but in neither case do the cells beat (Chen and Fishman, 1996), suggesting the need for at least one more signal. Mouse Nkx2-5 binds a variant of the consensus target site for Antennapedia homeodomain proteins (Chen and Schwartz, 1995) and three putative downstream target genes (*M2C-2v* (Lyons et al., 1995; O'Brien et al., 1993), *CARP* (Zou et al., 1997) and *E-hand* (Harvey, R. and Olson, E. N., personal communication; Srivastava and Olson, 1996)) are selectively downregulated in *Nkx2-5*$^{-/-}$ mouse embryos, suggesting a position for Nkx2-5 early in the hierarchical pathway for regulation of the cardiac gene program.

MEF-2 family

Members of the MEF2 family also are expressed in the precardiac mesoderm. These transcription factors, as homo- and heterodimers, bind and activate through A/T-rich sequences present in cardiac and skeletal muscle-specific genes (Iannello et al., 1991; Navankasattusas et al., 1992, 1994; Olson, 1992; Zhu et al., 1993). In mice, *MEF2B* and *MEF2C* are expressed in the precardiac mesoderm at e7.75, followed by *MEF2A* and *MEF2D* (Edmondson et al., 1994; Molkentin et al., 1996). In zebrafish, *mef2C* is expressed in the precardiac mesoderm, followed by *mef2A*, both apparently limited to the premyocardial and not pre-endocardial cells (Ticho et al., 1996). In *Drosophila*, the loss of the single *D-mef2* gene prevents formation of cardiac, visceral and skeletal muscle (Lilly et al., 1995). In the mouse, mutations of the *MEF-2C* gene interrupt the expression of a small subset of cardiac genes and interfere with heart development at the looping stage (Q. Lin and E. Olson, personal communication), while *MEF-2B*$^{-/-}$ mice have normal cardiac development. Serum response factor, a member of the MADS family, as are MEF2 factors, is ubiquitously expressed, and CArG-box-binding sites are present in cardiac-specific genes and are required for their expression in transient assays in cultured muscle cells (Minty and Kedes, 1986). However, the roles of SRF in the in vivo regulation of these genes and in cardiogenesis per se are not yet clear.

HLH proteins

The Myo D family of basic helix-loop-helix (HLH) myogenic proteins, which regulate skeletal myogenesis, are not present in heart. In skeletal muscle, these proteins heterodimerize via their HLH domain to ubiquitously expressed partners and thereby acquire an increased affinity for the E-box consensus sequence of a variety of muscle-specific target genes. No cardiac "master regulator" has been found thus far. In fact, the cardiac muscle phenotype does not appear to be dominant in cardiac myocyte-fibroblast (Evans et al., 1994). However, recent studies do support an important role for another subclass of HLH proteins in cardiac development (Srivastava et al., 1995). *dHAND* and *eHAND* are novel HLH genes expressed in the lateral plate and heart tube, as well as in the neural crest and branchial arches (Srivastava et al., 1995). If antisense oligonucleotides to both are added simultaneously to chick, heart development arrests at the looping stage (Srivastava et al., 1995). *dHAND*-deficient murine embryos display prominent defects in the morphology of the aortic sac and ventricular chamber and in looping morphogenesis (Srivastava and Olson, 1996). Therefore, the early cell fate decisions of cardiac myocytes can progress in the absence of dHAND and eHAND, but later stages of cardiac morphogenesis may require these HLH proteins.

Other transcriptional regulators

A number of other transciptional regulators have recently been implicated in the early embryonic control of the cardiac muscle gene program. GATA-4, a zinc finger protein, is found in precardiac mesoderm (Heikinheimo et al., 1994; Morrisey et al., 1996) and can bind to consensus GATA sites found in several myofibrillar protein genes. The precise role of the GATA proteins in the hierarchy of cardiac transcriptional factors is not yet known, but the pathology of GATA-4$^{-/-}$ embryonic mice indicates a potential role in formation of the early heart tube (Kuo, Parmacek and Leiden, personal communication; Molkentin and Olson, personal communication). Recent studies have also identified a novel cardiac-restricted ankyrin repeat protein (CARP) that is expressed bilaterally in the cardiac primordia (Zou et al., 1997). CARP interacts with components of the endogenous HF-1a complex that is required for the maintenance of ventricular muscle-specific expression of the *MLC-2v* gene, and appears to serve as a negative regulator of the cardiac gene program.

Myogenesis and lineage diversification

There is a highly dynamic, and species-specific, pattern of contractile protein gene expression in the developing heart field and early heart tube (Lyons, 1994). Although, as a rule, there appears to be a rostocaudal progression of differentiation (Litvin et al., 1992), some genes are expressed in a manner compatible with limitation to a specific chamber or its precursors. For example, an atrial-specific myosin heavy chain is limited to the preatrial region of the field and persists in that chamber (Yutzey et al., 1994). In chick, the earliest expressed myofibrillar proteins noted to date are ventricular MHC and α-actin, which are evident in the lateral plate by stage 7 (Lyons, 1994).

Although atrial and ventricular muscle cells co-express a number of myofibrillar genes, the electrophysiological, contractile, morphological and physiological phenotypes of these two striated muscle cell types are distinct. The initial separation of atrial and ventricular muscle lineages may begin soon after gastrulation, as noted in both chick and zebrafish (Stainier et al., 1993; Yutzey and Bader, 1995). Whether this initial separation is reversible is not clear, and genes expressed in a chamber-specific manner at one time in development may not be so limited at others (Chien et al., 1993).

Because expression of the myosin light chain *MLC2V* gene is the earliest known marker of vertebrate ventricular muscle cell lineages, (O'Brien et al., 1993), it has been an important guide to the molecular bases of lineage diversification. The *MLC2V* gene encodes a contractile protein of both cardiac and slow skeletal muscle. It is expressed in a bilaterally symmetric restricted zone of the cardiogenic crescent (Lyons et al., 1995) and limited to the ventricle throughout embryonic and postnatal development. What restricts its expression to the ventricle is not known. No ventricle-restricted transcriptional factors have been described, but recent work indicates that particular combinations of factors may suffice to confer ventricular specificity to *MLC2V* during murine cardiogenesis, acting through a 28 base-pair *cis* regulatory element, composed of adjacent HF-1a and MEF2 sites (Ross et al., 1996). The factors that occupy these sites are not tissue-restricted, even to heart. Since *MLC2V* is selectively down-regulated in *Nkx2.5*-deficient murine embryos, Nkx2.5 may also play a role in the initial process of ventricular specification (Lyons et al., 1995).

Ventricular lineages continue to diverge as the heart matures. For example, some transgenes are preferentially expressed in the right (Ross et al., 1996) or left (Kelly et al., 1995) ventricular chambers, even if the native genes are not so restricted. A truncated 200 bp *CARP* promoter is expressed predominantly in the conotruncal region (Chen et al., 1996; Chen and Chien, unpublished) and a *lacZ* knock-in inserted into an endogenous C2H2 zinc finger gene (HF-1b; Zhu et al., 1993) is expressed in the muscular septum (V. Nguyen, K. Wollert and K. Chien, unpublished observations). Ventricular muscle cells at the atrioventricular junction express distinct molecular markers (Lyons, 1994) and generate specialized cues for endocardial cushion formation. The Purkinje cells of the distal conduction system are derived from ventricular muscle cells (Gourdie et al., 1995) but have distinctive electrophysiological properties. One of the major challenges in the coming years will be to identify the molecular and positional cues that lead to the appearance of these distinct ventricular muscle lineages, and to determine whether maturational arrest of these lineages, either qualitatively or quantitatively, can acccount for particular congenital heart diseases.

Heart contributions from outside of the heart field

Neural crest and head mesenchyme to outflow tract

The events that serve to separate pulmonary from systemic circulations, especially the separation of the outflow tract of the heart in air breathing animals, are important to note briefly, but beyond the scope and stages of this review (and well reviewed elsewhere (Kirby and Waldo, 1990; Noden et al., 1995; Olson and Srivastava, 1996). A central component of this separation is constituted by cells that are derived from the neural crest. Neural crest cells that are destined for the heart migrate through pharyngeal arches 3, 4 and 6 to the outflow region, where they generate much of the aortopulmonary and conotruncal septa (Kirby and Waldo, 1990, 1995). (Outflow tract endocardium is derived from cephalic paraxial and lateral mesoderm, at the level of the otic placode.) Thus, it is not surprising that removal of the "cardiac" neural crest causes persistent truncus arteriosus, the failure to separate the outflow tract. More difficult to understand is why there is an accompanying defect in myocardial contractility, with consequent ventricular dilatation, apparently due to an intrinsic defect in the myocardium. Perhaps as a consequence of the poor contractility and accompanying ventricular dilatation there is failure to loop properly, which, in turn, may lead to other defects, such as double outlet right ventricle or tetralogy of Fallot (Kirby and Waldo, 1990, 1995). Alternatively, neural crest cells may affect myocardial cell maturation.

Coronary arteries

The myocardium is avascular for many days, in the chick for 6 days after contractions begin at HH stage 10. The coronaries are derived from the epicardial mantle, a grape-like cluster of mesothelium, which appears near the sinoatrial junction in contiguity with, and perhaps from, neighboring hepatic mesenchyme, and which then grows, in a caudal-to-rostral direction, to enclose the heart. The epicardial organ is attached to the myocardium at the dorsal mesocardium, from which cells extend to cover the outer surface of the heart, forming the epicardium. The epicardial organ appears to contain the precursors of both coronary smooth muscle and endothelium, which derive from separate progenitors (Mikawa and Fischman, 1992; Mikawa and Gourdie, 1996). This epicardial origin of the coronary smooth muscle lineage differs from the neural crest lineage of the proximal great vessel tunica media. Coronary vessels assemble from local aggregates of angioblasts, join up to generate continuous tubes and puncture through the aortic wall to generate continuity with the aorta.

THE VERTEBRATE HEART TUBE

How the bilateral myocardial plates merge to form a single heart tube and come to enclose the endocardium is debated. The process may differ between species. In the chick, there are two myocardial plates, with endothelial cells between them and the endoderm (Fig. 2). These are kept separate by the ventral mesocardium until its rupture at the time of fusion shortly after HH stage 9 (Viragh et al., 1989). In the mouse, most of the heart

appears to form from splanchnic mesoderm at the front of the horseshoe-shaped field, anterior to the buccopharyngeal membrane (DeRuiter et al., 1992; Kaufman and Navaratnam, 1981), with a subjacent proendocardial layer, so that there may be no need for fusion of the limbs of the horseshoe. In the zebrafish, two apparently intact myocardial tubes are formed with a core of pre-endocardium medially between them, so that there must be a complete redeployment of the myocardial cells to form a single tube around the endocardium (Stainier et al., 1993).

The heart tube begins contractile function essentially as it is forming, although there may be slight differences between species. Although the first contractions are peristaltic, they soon become sequential, with atrial contraction preceding ventricular. The maturation of function is accompanied by specialization of contractile protein machinery. At the time of tube formation, but prior to any morphological evidence of chamber demarcation, cellular differentiation is well underway. Some proteins are expressed in a chamber-specific manner, including, for example, MLC2V (O'Brien et al., 1993) in the mouse and an atrial myosin heavy chain (MHC) in the zebrafish (Stainier and Fishman, 1992). Other genes are homogeneously expressed throughout the heart tube at the onset of contractions. In the 8 dpc mouse, for example, this is true of of MHCα and MHCβ, MLC1A and MLC1V, cardiac α actin, and tropomodulin, tropomyosin, α-actinin, titin and desmin (Lyons, 1994). Some of these subsequently achieve chamber specificity by down-regulation. For example, in the mouse, there is down-regulation of MHCα in ventricular myocytes, and of MHCβ and MLC1V in atrial myocytes (Lyons, 1994). In species with further subdivision into right and left ventricles, there may be a matching preconfiguration of contractile protein expression. For example, an *MLC3F-lacZ* transgene is expressed only in the region of the future left ventricle (Kelly et al., 1995). In a reciprocal fashion, a *MLC2V-lacZ* transgene confers a predominant conotruncal and right ventricular pattern of expression (Ross et al., 1996).

What's distinctive about the vertebrate heart tube?

The vertebrate heart differs from the presumptive ancestral chordate both in global size and form and in its component parts. How many genes are involved in this evolutionary change and what attributes of the heart do they determine? Can single genes account for discrete independent additions to a primitive ancestral heart?

It is believed that vertebrates evolved from ancestral cephalochodates (such as amphioxus), and that the cephalochordates evolved, by neotany, from urochordate larvae resembling those of current-day tunicates (Randall and Davie, 1980). Thus, the ancestral chordate would have lacked a specialized endothelial cell lining to the vessels and the heart (Fig. 4), and had instead an interconnected series of sinuses, bathing tissues directly in blood at low pressure. The cepalochordates have an endothelium that lines heart and vessels, although it is not continuous, as is that of vertebrates (Fig. 4). If really like current-day tunicate larvae, the chordate ancestor would have had a tubular and valveless heart, contracting by peristalsis and driving blood around an in-series circulation, but reversing direction of flow every few minutes as dominant pacemakers alternated between the ends of the heart.

Vertebrate hearts differ from the presumptive ancestral chordate with respect to several features that are clearly evident even in the early heart tube stage: (1) an endothelial lining to the heart and vessels, (2) a downstream muscular chamber, the ventricle, which generates flow at higher blood pressures and in a single direction around a circulation, (3) mechanisms to ensure this polarity of flow, including a localized dominant pacemaker and a conduction system designed to direct the propagation of the impulse and timing of chamber contractions, (4) sequential chamber contractions rather than peristaltic motion and (5) valves between the chambers to prevent regurgitation. In addition, vertebrate hearts are bigger and laterally asymmetric. Individually, many of these attributes are seen in invertebrates, but the remarkably uniform constellation appears to be completely conserved among vertebrates and unique to them.

Of course, there is no evidence that these "new" attributes of the vertebrate heart, along with other global changes such as increased size and laterality, actually are modular additions to a primitive heart or that they may be dissected free by single gene mutations. However, it is a convenient, if simplistic, framework and provides one heuristic approach, and recent studies based upon genetic screens in zebrafish do reveal mutations with very discrete effects upon each of the "new" vertebrate additions. We will discuss the global features of size and laterality, and then examine the possibility that many of the "new" vertebrate components are due to the addition of endocardium and its signals.

Vertebrate hearts are bigger

The larger the animal, the greater must be the volume of blood circulated, and, hence, the stroke volume of the heart. Thus,

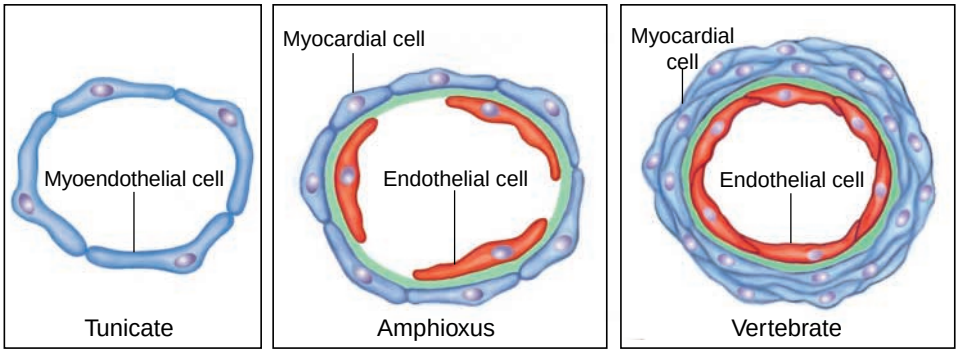

Fig. 4. Evolution of the chordate heart tube, shown in cross section. The presumptive chordate ancestor's heart is believed to have been a tube akin to that of modern tunicates, consisting of a single layer of myoendothelial cells with myofibrils in them, which generates a peristaltic impulse, alternating in origin between ends of the heart, and functioning at low pressures. and without valves (Randall and Davie, 1980). The contractile endostylar artery of amphioxus is believed to be an intermediary form. It generates flow in one direction only and has a discontinuous lining of endothelial cells (Randall and Davie, 1980). The vertebrate heart tube generates higher pressures, so has thicker muscle, and has a continuous endothelial lining.

with the increased size of the vertebrates came a need to increase cardiac output. (Diffusion suffices for gas exchange if the distances between environment and mitochondria are less than 1 mm, the diameter of many fish at hatching (Burggren and Pinder, 1991)). For vertebrates, the mass of the heart, and the volume ejected per beat, are proportional to the mass of the animal. This relationship between heart and animal size holds either when comparing adult vertebrates of different sizes (Holt et al., 1968) or the same species at different stages during growth and development (Burggren and Pinder, 1991). When in development is organ size rendered proportional to the animal? The primitive heart tube forms prior to onset of function, so physiological activity cannot be the primary determinant of the tube's initial dimensions, although it may modify subsequent patterns of growth. There is evidence that, prior to heart tube formation, information is provided to the heart field regulating later growth rate. *Amblystoma tigrinum* grows much more quickly than does *Amblystoma punctatum* (Copenhaver, 1939). After interspecies transplantation of precardiac mesoderm, from *tigrinum* to *punctatum*, hearts grow more rapidly than expected if the rate were that of the host (Copenhaver, 1939). This is not to say that flow and pressure are unrelated to heart growth. Even in the heart tube, flow and pressure changes induced by constricting the outflow tract lead to a host of congenital abnormalities (Gessner, 1966). Along these lines, it is of interest that overexpression of *Nkx2.5*, both in frogs and zebrafish, causes enlarged hearts to form in otherwise normal animals (Chen and Fishman, 1996; Cleaver et al., 1996; Fu and Izumo, 1995). It is conceivable that *Nkx2-5* expression determines the limits of the heart field, i.e. which cells have the potential to become heart. In *Drosophila*, the *Nkx2-5* homolog, *tinman*, is regulated by expression of *dpp*, a BMP-4 homolog, in the adjacent ectoderm (Frasch, 1995). Therefore, one possibility is that determinants of the body axis, such as BMP-4 (Graff et al., 1994), also regulate the size of fields for internal organs. This integration would have the advantage of coordinating organ size with body size, beginning in the field.

Vertebrate hearts are laterally asymmetric

Distributed organ systems, such as the vasculature, must take global embryonic asymmetry into account during assembly in order to connect properly, a task made all the more difficult if, as for blood vessels, parts of the system form locally and subsequently must connect seamlessly. Hence, it is important not that the vertebrate heart is asymmetric per se, but that its inflow and outflow regions are positioned correctly to permit linkage to the other components of the vasculature, and that organ form is rendered appropriate for placement in contiguity with neighboring asymmetric organs, such as the lungs, and for connectivity with the nervous system. Therefore, the key unanswered question is: what genes link cardiac development to embryonic asymmetry? What are the signalling organs and how does the heart read such information?

The vertebrate is asymmetric along the left-right axis from the onset of gastrulation, if not before (Yost, 1995). In fact, recent studies in *Xenopus* indicate a role for maternal Vg-1 in the initial establishment of left-right asymmetry (Hyatt et al., 1996). Much emphasis has been placed on the looping of the heart to the right as the first obvious break in left-right symmetry, which has been the subject of the vast majority of studies of asymmetry. However, there is clear evidence of a right-left distinction between the heart fields and in the positioning of the heart tube, prior to looping. Many studies have been interpreted as indicating that the direction of looping (as the end-point studied) requires a set of signals separate from the physical act of looping per se, and is due to molecules that bias the normal looping system toward the right. In other words, as a result of the abolition of these signals, the heart continues to loop, but 50% of the time looping occurs toward the left, a process termed randomization. While this interpretation may be true in certain circumstances, the number of single gene defects that cause looping problems is too small to be confident that abnormal or randomized "looping" is really looping, in the sense of being mechanistically identical to normal but mirror-image reversed. It may be that, in situations with apparent randomization, the heart manifests no laterality and rather bends due to growth constraints on elongation, or other forces, as development proceeds. These are not necessarily the same forces which would guide normal looping.

Asymmetry in the field

The left and right heart fields have different properties and, if separated, give rise to differently looped hearts. In twins produced by constricting Triton embryos in the midline, the organs of the left-sided twin are positioned normally, whereas the organs of the right twin are reversed in orientation (situs inversus) (Spemann and Falkenberg, 1919). In the normal rat, the bilateral heart tube primordia begin to beat before fusion of rudiments, with the left primordia beating 2 hours earlier and faster than the right primordia (at 9.5 dpf). In the chick, if fusion of the bilateral primordia is prevented by endodermal injury (DeHaan, 1959), two hearts are formed, and the left heart begins beating earlier and with a faster rate than the right. Until HH stage 5-6, the differences between the left and right fields are reversible, such that transplants adopt the properites of the host region, but after that they retain the properties of the donor side (Satin et al., 1988). There are molecular differences evident by this stage, with expression of flectin, a large extracellular matrix molecule, primarily in the left precardiac mesoderm by HH stage7+/8– (Tsuda et al., 1996) and a quantitative increase in QH1, a presumptive endocardial marker, in the right lateral plate at HH stage 7+ (Sugi and Markwald, 1996).

Asymmetry of the heart tube

It is likely that the heart tube is never symmetric, reflecting inherent differences in the two bilateral heart fields from which it forms. In the zebrafish, immediately after the first evidence of fusion of the two tubes, there is both molecular and morphological asymmetry (J. Chen, personal communication). The first beating of the chick heart occurs as soon as the ventricle forms, at HH stage 10, along the right posterior margin of the ventricle (DeHaan, 1965).

Looping

The heart loops to right in all vertebrates, but the mechanism of looping is not known (Taber et al., 1995). It is not normally due simply to constraint of longitudinal growth, in that the heart loops if explanted in culture (Manning and McLachlan, 1990). There is no evidence for marked differences in mitosis or cell death, at least after the initial formation of the heart tube

(Stalsberg, 1969, 1970) (although such differences might occur earlier, in the progenitor population, and have gone unnoticed). Although there are cell shape changes, that accompany looping, the heart still loops when these are abolished, for example by disruption of microtubules (Icardo and Ojeda, 1984). Actin filament disruption does prevent looping (Itasaki et al., 1991). One suggestion is that the heart loops due to a constraining force of the dorsal mesocardium, which is attached to the inner curvature (Taber et al., 1995). The process of looping is extremely important in aligning component of the heart tube so that, in higher phyla with septated ventricles and outflow tract, the inflow and outflow tracts are brought into proximity, in turn placing the aortic root between the mitral and tricuspid valves. The septation of the outflow tract is thereby placed directly over the septum between the ventricles (Kirby and Waldo, 1995). Thus, abnormal looping may lead to congenital defects in the inflow and outflow tracts.

The normal arrangement of the thoracoabdominal organs is referred to as situs solitus, including, in humans, the right lung as trilobed and the left lung as bilobed, and the stomach and spleen on the left. In humans, 1 in 6,000-8,000 have a mirror-image reversal of all thoracoabdominal organs, with the heart on the right (dextrocardia), a situation called situs inversus. The physiology may be normal, although there is an increased risk of congenital heart disease (Layton and Manasek, 1980). In some cases, visceral sidedness is less clear, a situation referred to as heterotaxy, which is frequently associated with congenital cardiac malformations. In general, most abnormalities of cardiac position or looping are sporadic in nature, but there are families with X-linked (Casey et al., 1993), autosomal recessive and autosomal dominant inheritance (Payne et al., 1995). In humans, Kartagener's syndrome is an autosomal recessive disorder with a defect in dynein arms and affected individuals have mucociliary dysfunction in the lungs, immotile sperm and situs inversus. The heart otherwise usually is normal. In conjoint twins, the left twin has normal situs and the right twin has situs inversus.

The *inversus viscerum* (*iv*) autosomal recessive mutation in mice was discovered by Hummel and Chapman (Hummel and Chapman, 1959). Homozygous animals exhibit randomization, originally believed to be 50% situs solitus and 50% situs inversus, although more recently it has been shown that many actually have heterotaxy (Seo et al., 1992). There is an associated incidence of intracardiac defects (Layton and Manasek, 1980). The responsible gene is not known and efforts to clone it positionally are underway. In the *inv* mouse, a recessive mutation due to fortuitous insertion of a transgene, all homozygous mice have situs inversus, with mirror-image inversions of stomach, spleen, liver, lung and heart (Yokoyama et al., 1993). The insertion is accompanied by deletion and duplication in chromosome 4 (Yokoyama et al., 1995).

Laterality signals

The embryo is asymmetric along the left-right axis at least from the onset of gastrulation. For example, at a gross morphological level, the right side of the node at HH stage 4-5 is bigger than the left (Wetzel, 1929). The earliest asymmetrically expressed gene that has been noted in the chick is the activin receptor-IIa (*cAct-RIIa*), which appears to mediate local suppression of of *sonic hedgehog* (*shh*), which, by stage 4+, becomes restricted to left side of the node (Levin et al., 1995). In turn, *shh* appears to activate expression of the nodal-related gene, cNR-1 in the lateral plate mesoderm. These genes appear to act sequentially, in that activin-soaked beads upregulate *cAct-RIIa* and down-regulate *shh*, and shh upregulates *cNR-1*. Heart looping is randomized if shh is misexpressed on the right side. Whether the cascade involves activin itself, rather than a related protein is not clear, since a targeted activin mutation in mouse does not affect looping (Matzuk et al., 1995).

In different species, the early embryonic asymmetrical pathways may utilize different members of the TGFβ family. For example, in the mouse, *nodal* is expressed in a subpopulation of mesodermal cells extending rostrally from the primitive streak to the caudal region of the developing heart tube (Collignon et al., 1996) and *lefty* is transiently limited, at 8.0 dpc, to the anterior left side of the floor plate and the left side of the splanchnopleure, caudal to the promyocardium (Meno et al., 1996). Gene products need not be expressed asymmetrically to affect the pathway. For example, HNF-3β, although itself symmetric, interacts with nodal (Collignon et al., 1996). None of these genes appear to be in the heart field, so their role is unclear and the targets in the heart primordia unknown.

One important signalling source appears to be the midline. For example, UV irradiation during the first cell cycle in *Xenopus*, or *Xwnt*-8 injection into dorsal midline cells (which are not cardiac progenitors) reduce dorsoanterior structures and randomize looping (Danos and Yost, 1995). It is not feasible from these experiments to pinpoint the responsible source of the signal, in that these manipulations affect assembly of head, notochord, somites and the anterior nervous system. The timing of effects upon looping, however, indicates their actions occur around the neural tube stage; i.e. well before the formation of the heart tube but after the onset of gastrulation (Danos and Yost, 1996). Extirpation of dorsal tissue, causing a gap in the notochord, causes randomization if performed before stage 18. However, not every manipulation that affects looping also affects the dorsoventral or anteroposterior axes. For example, ectodermal injury in *Xenopus*, or perturbation of its extracellular matrix, randomizes cardiac looping if performed at the neurula stage (Yost, 1992).

Laterality effectors

How is left-right information interpreted by cardiac progenitors? What is needed is a molecular asymmetry in the heart that is predictive of cardiac laterality.

One set of asymmetric molecules in the heart tube are *eHAND* and *dHAND*, although neither manifests left-right asymmetry prior to looping, and may rather serve later to help distinguish right from left ventricle. For example, *dHAND* is first in a cranial-to-caudal gradient in the tube and then becomes associated with the right side of the future ventricle (D. Srivastava and E. Olson, personal communication). Targeted mutageneis in mice indicates that *dHAND* is required for normal looping. *Nkx2.5* is expressed symmetrically, but its mutation also interferes with normal looping (Lyons et al., 1995).

The endocardium is a source of vertebrate heart components

The formation of several of the vertebrate heart components that are not evident in presumptive chordate ancestors are

dependent upon the endocardium. These include the cushions and valves, trabeculae in the ventricular chambers, and a thickened ventricular wall, which will be discussed together. In addition, there is recent evidence that a portion of the conduction system, which runs within the interstices of the muscle, is assembled under endocardial control.

Embryonic origin of the endocardium

The endocardial cells arises from within the cardiac field (Eichmann et al., 1993; Sugi and Markwald, 1996; Yamaguchi et al., 1993; Lee et al., 1994). The endocardial progenitors are likely to come from a group of precursors spatially sequestered from myocardial progenitors (Cohen-Gould and Mikawa, 1996; Lee et al., 1994). A separate endocardial lineage is established by HH stage 4 in the chick, at which time the myocardial progenitors vastly outnumber the endocardial progenitors (Cohen-Gould and Mikawa, 1996).

Endocardial cells are first apparent histologically just subjacent to the cardiogenic plate, in contact with foregut endoderm, where they begin to express *flk* and stop expressing N-cadherin, at around HH stage 6-7 in the chick (Linask, 1992). Whether they dissociate from the plate or migrate in from adjacent mesoderm is debated. These cells assemble into a loose vascular plexus underlying the foregut, and coalesce into progressively larger tubes, until eventually generating a single endocardial tube, by HH stage 8+ in the chick (Sugi and Markwald, 1996). Endoderm appears to play a role in directing endocardial development. Explant cultures of HH stage 5 heart-forming regions generate invasive QH1 immunoreactive cells only if cultured with endoderm, or with endoderm-conditioned medium (Sugi and Markwald, 1996). There is debate as to whether the endocardium forms by the merging of two separate bilateral tubes or from a single primordium (DeRuiter et al., 1992). The differences may be species-dependent. In either scenario, there may well be important midline signals to the generation of the endocardial tube, perhaps from the foregut, which in that region, is hypertrophied and different from other endoderm (Sugi and Markwald, 1996). Vascular endothelial growth factor (VEGF) is expressed in the endoderm (Flamme et al., 1994) and could be one endodermal signal.

Signals to the myocardium: trabeculae and muscle growth

Signals from the endocardium to myocardium are important for the generation of trabeculae in the ventricular chamber. By HH stage 13 in the chick, the myocardium begins to evidence an outer, compact layer and an inner, spongy zone, the latter of which becomes trabeculated. Whether trabeculation is accomplished by localized myocardial growth or endocardial invasion, or both, is not clear. Endocardial-lined spaces appear in what was previously the compact myocardium, presumably to enhance oxygenation and nutrient transfer prior to the development of coronary arteries. Groups of myocardial cells become separated from each other, forming the trabeculae, with a myocardial core surrounded by endocardium. Subsequent coalescence of neighboring trabeculae is believed to generate the muscular portions of the interventricular septum and papillary muscles (Andries, 1995; Icardo and Fernandez-Teran, 1987). In humans, the intertrabecular spaces become smaller as coronary arteries develop, but some may remain as Thebesian veins (Brutsaert and Andries, 1992).

Of several potential endocardial-derived signals, at the current time only the neuregulins have a proven role in heart development. Neuregulins are a group of secreted and cell-membrane-associated factors generated by alternative splicing of a single gene. The endocardium expresses neuregulin. Neuregulin action is mediated by tyrosine kinases of the epidermal growth factor family. It is believed that erbB2 and erbB3 form a heterodimer to transduce the signal. erbB4 appears capable of binding and signalling on its own. The myocardium expresses *erbB2* and *erbB4*. (*erbB3* is limited to the endocardial cushions). Targeted gene mutation of *neuregulin* (Meyer and Birchmeier, 1995), *erbB2* (Lee et al., 1995) or *erbB4* (Gassmann et al., 1995) all block development of trabeculae, without noticeably affecting the compact zone. Targeted ablation of several endothelial genes, which play important roles in generating signals, such as *angiotensin converting enzyme* (Carpenter et al., 1996; Krege et al., 1995) and *endothelial nitric oxide synthase* (Huang et al., 1995), have no apparent effect upon heart development, although they are known to affect myocardial function in the adult.

It is clear that, at least in zebrafish, the myocardium can form a heart tube in the complete absence of endocardium, as noted in the *cloche* mutant (Stainier et al., 1995). If anything, the assembly of endocardium may be partially directed by myocardium, in that bilateral hearts form in cardia bifida mutants, complete with endocardium (Stainier et al., 1996). The poor contractility and thinner wall of the ventricle in *cloche* may reflect absence of a signal or, alternatively, effects upon a shared myocardial/endocardial progenitor. Other zebrafish mutants have clear myocardial contractility dysfunction coupled with endocardial deformation and failure to form cushions, but the cell-autonomy of the mutations has not been assessed.

Cushions and valves

The endocardium is critical to the generation of the heart valves. The cardiac cushions are the primordia of the valves and membranous septae (Eisenberg and Markwald, 1995). They form at two points along the heart tube, the atrioventricular canal and the conotruncal outflow tract (Fig. 3C,D). They are evident first as localized swellings in the cardiac jelly, which are subsequently invaded by endothelial cells.

The properties of both the endocardium and myocardium are distinctive in the cushion regions. The endocardial cells that are localized in the conotruncal and atrioventricular regions express the homeodomain-containing presumptive transcription factor *Msx-2* (Chan-Thomas et al., 1993), the extracellular matrix protein fibulin (Spence et al., 1992), JB3 immunoreactivity (Wunsch et al., 1994) and TGF-β1, which is down-regulated in the rest of the endocardium (Akhurst et al., 1990). In the mouse, *desert hedgehog* is expressed in the precushion endocardium of the AV canal and the truncus arteriosus at 11.5 dpc (Bitgood and McMahon, 1995). At which stage the differentiation from the rest of the tubular heart begins is not clear.

In vitro studies have suggested that the endothelial invasion is, in part, under control of a localized myocardial signal. In explant culture on collagen, endocardial cells from the AV or outflow tract assume a mesenchymal phenotype, but do so only under the influence of AV or outflow tract myocardium. The signal has been proposed to involve proteins secreted from

myocardium into the cardiac jelly, which form 0.1-0.5 μm particulates referred to as adherons, from which several proteins have been extracted, including fibronectin, transferrin and ES/130 (Rezaee et al., 1993; Wunsch et al., 1994). Extracts of these adherons can substitute for myocardium in the explant assay (Mjaatvedt and Markwald, 1989), although the precise role of any of these signals in cushion formation in vivo is unclear. Members of the TGF-β family may play a role, but the evidence is contradictory, in part because there may be species differences as to which TGF-β family members are involved. In the chick, a reasonable candidate is TGF-β3. TGF-β3 is expressed in the AV canal and antisense oligonucleotides to TGF-β3 inhibit the epithelial-mesenchymal transformation in vitro. However, there is also a transcript normally expressed in chick that corresponds to the antisense for TGF-β3 (Potts et al., 1992). There are many other candidates. For example, *BMP-2, -4* and *-6* are expressed in the myocardium of the mouse AV canal and truncus arteriosus at the correct time to play a role (Bitgood and McMahon, 1995; Jones et al., 1991). The invasion may involve action of proteases, in that the endocardial cells express the serine protease, urokinase, which, if inhibited, blocks the migration (McGuire and Alexander, 1993). In addition, these is diminution in expression of N-CAM as the cells invade (Crossin and Hoffman, 1991), coupled to an increase in tenasin, presumably rendering the cells less adherent to each other.

The outflow tract connects the ventricle to the aortic sac. Descriptions of the outflow tract are buried in confusing anatomical terminology (Pexieder, 1995). Sometimes the region is referred to as the outflow tract, sometimes as bulbus, sometimes as conus, and sometimes both, with the part next to the ventricle as conus and the more distal as the truncus (Pexieder, 1995). One useful categorization is that, in species with an interventricular septum, the outflow tract refers to the region within the heart that extends from the secondary interventricular septum to the semilunar valves (Pexieder, 1995). In fish, the short stretch distal to the valve is referred to as either the conus or truncus, depending on the internal structure. The important issue is that both the endothelial cells and smooth muscle cells of this vascular structure include progenitors from outside of the cardiac field, and are rather from the head mesoderm (angioblasts) or neural crest (smooth muscle) (Noden et al., 1995). In addition, the angioblasts for the conotruncal region, unlike most other regions of the endocardium, are not generated in association with endoderm (Noden et al., 1995). The outflow tract is a region often affected in congenital heart disease and in targeted gene mutations in the mouse (Table 2), with consequent secondary valvular and septal defects. These outflow tract defects, which perturb later cardiac development, are prominent among those seen clinically, as opposed to those that affect the early patterning and morphogenesis of the heart tube, the latter of which would be lethal within the first month of gestation.

Vertebrate hearts have unidirectional conduction

Forward flow is generated by properly timed contractions of the two chambers. The cardiac impulse of the vertebrate heart originates in the pacemaker cells of the sinoatrial node, is conducted through the atrium (in some species along specialized bundles) to the atrioventricular junction from whence, after a delay, the electrical signal is propagated to the ventricles along bundles of specialized conduction tissue to the distal Purkinje fibers, which ramify among the contractile myocardium. The lineage of the components of the conduction system is largely unknown. The exception is the distal Purkinje fiber, which appears to develop from a lineage shared with myocardial cells. In the chick, these cells differentiate in close association with coronary blood vessels, perhaps due to a neighboring vascular signal (Gourdie et al., 1995). There is no evidence that central components of the atrioventricular conduction sytem (the bundles) are related by lineage to the distal Purkinje system, although their properties may be similar (Gourdie et al., 1995; Oosthoek et al., 1993). Myocardial cells that are apparent progenitors of conduction system express *Msx-2* (Chan-Thomas et al., 1993).

The rate of the heart beat is set by the fastest pacemaker, which at first is in the ventricle, but soon moves to the inflow region, ultimately to reside in the sinoatrial pacemaker. Other pacemakers can take over the heart beat later in life if the SA node is not functional and each region has its own intrinsic rate, increasing from outflow to inflow regions. This gradient of automaticity rate is established in the heart field. If the field is physically disrupted, each remnant can form an autonomously beating structure, most rapid in the sinoatrial region and slowest in the conal region (Satin et al., 1988). The target genes that mediate the rate regulation are unknown, but candidates would be the channels responsible for the pacemaker potential, the slow diastolic depolarization that brings the membrane potential to threshold. The crucial channel for this activity has been debated, but appears to be I_f, also known as I_h, a hyperpolarization-activated non-selective cation channel that is activated by hyperpolarization and has slow activation kinetics (DiFrancesco, 1995; Baker et al., 1997).

One key transition made by the heart tube during development is the change in pattern of contraction, from a smooth peristaltic wave to sequential brisk contractions of the atrium and ventricle. This acquired physiology depends upon rapid propagation within each chamber and a pause at the atrioventricular junction. The temporal delay between atrium and ventricle appears, at least during early embryonic development, to be due to regions of slowly conducting tissue between the chambers (de Jong et al., 1992). The border zones between chambers are different from the rest of the myocardium, for example continuing to coexpress both atrial and ventricular myosin heavy chains (de Jong et al., 1992), and manifest slow conduction and protracted contraction. They also provide sphincters to prevent retrograde flow before development of valves (Lamers et al., 1991). Tissue from the border region is believed to contribute to the sinoatrial and atrioventricular nodes (Moorman and Lamers, 1994), in the mouse becoming evident around 10.5 dpc (Viragh and Challice, 1983). These nodal tissues are most compact and easily defined as separate in the mammal although they are clearly present in avians (Lamers et al., 1991) and fish (Santer, 1985).

GENETIC DISSECTION OF HEART DEVELOPMENT

Targeted gene ablation in the mouse

Several targeted gene mutations have been noted to affect heart

Table 2. Stages of murine cardiogenesis that are prominently affected by targeted gene mutations

Heart Tube Formation	*GATA-4* (Molkentin et al., 1997; Kuo et al., 1997)
Heart Looping Morphogenesis	*Nkx2.5* (Lyons et al., 1995) *MEF2* (Lin et al., 1997) *dHAND* (Srivastava et al., 1997)
Aortic Arch / Sac Development	*hox-1.5* (Chisaka and Capecchi, 1991) *Splotch/PAX3* (Epstein et al., 1991; Franz, 1993) *Endothelin-1* (Kurihara et al., 1995) *RARα/γ* (Mendelsohn et al., 1994) *RXRα* (Gruber et al., 1996) *Connexin 43* (Reaume et al., 1995) *NT-3* (Donovan et al., 1996)
Conotruncal Development	*RXRα* (Gruber et al., 1996) *SOX4* (Schilling and Kimmell, 1994) *NF-1* (Brannan et al., 1994; Jacks et al., 1994)
Trabeculation	*Neuregulin* (Meyer and Birchmeier, 1995) *RXRα* (Kastner et al., 1994; Sucov et al., 1994) *ERB-2* (Lee et al., 1995) *ERB-4* (Gassmann et al., 1995)
Expansion of Ventricular Compact Zone	*RXRα* (Kastner et al., 1994; Sucov et al., 1994) *N-myc* (Moens et al., 1993) *WT-1* (Kreidberg et al., 1993) *bARK-1* (Jaber et al., 1996) *TEF-1* (Chen et al., 1994) *Dopamine β Hydroxylase* (Zhou and Palmiter, 1995)
Ventricular Septation	*VCAM-1* (Kwee et al., 1995) *Tyrosine Hydroxylase* (Zhou et al., 1995) *PDGF-α* (Morrison-Graham et al., 1992; Schatteman et al., 1995) *RXRα* (Kastner et al., 1994; Sucov et al., 1994) *CXC Chemokine* (Nagasawa et al., 1996) *NT-3* (Donovan et al., 1996)
Atrioventricular Cushion Generation	*RXRα* (Gruber et al., 1996)
Functional Maturation	*αMHC* (Jones et al., 1996) *MLC-2v* (J. Chen and K. Chien, unpublished observations) *IGF-1* (Lembo et al., 1996; Powell-Braxton et al., 1993)

development. Those mutations that perturb laterality and looping are discussed above. To date, the informative cardiac effects of the others are on stages of cardiac development later than the focus of this review. These include, as shown in Table 2, steps of myocardial maturation, including trabeculation and expansion of the compact zone, proper division of the conotruncal region and remodelling of the aortic arch. Many other mutations that are currently noted to be "embryonic lethal" may affect the heart, but the effects of these mutations are frequently pleotropic. Given the dependence of mouse tissues upon blood flow, the dissection of specific effects upon the heart will likely require spatial and temporal restriction of the mutation (Gossen et al., 1995; Kuhn et al., 1995; Metzger et al., 1995; No et al., 1996). Physiological monitoring is now feasible in the embryo (Dyson et al., 1995; Gruber et al., 1996), and will add to the molecular and morphological characterization of the defects and correlations with human congenital heart disease.

Genetic screen in the zebrafish for the decisions of heart development

The hope of large-scale screens is to discover single gene mutations that reveal key developmental steps and pathways, the latter suggested when multiple mutations have the same phenotype (St. Johnston and Nusslein-Volhard, 1992; Horvitz, 1988). Not all mutations are informative. The effects of some will be too pleotropic to be deciphered and others will be silent because of compensatory or redundant pathways. For example, of the 6000 genes estimated as "essential" by genetic screens of *Drosophila*, 200 are informative about specific roles in embryonic development (Nusslein-Volhard, 1994). If extrapolated to the vertebrate, with 4-5 times the number of transcription units (Gabor Miklos and Rubin, 1996), it is likely that there are less than a thousand genes with critical and specific roles in development which can be revealed by loss-of-function screens.

Neither of the the two invertebrate species most widely used for genetics are ideal for studies of vertebrate organs. *C. elegans* has no heart. *Drosophila* has a contractile dorsal vessel which pumps hemolymph (Ashburner and Wright, 1978; Demerec, 1950; Rizki, 1978) and therefore cannot be considered homologous to the vertebrate heart in the strict (and controversial) sense of sharing a common evolutionary origin and an essential commonality of structure (Bolker and Raff, 1996). Mutations in *Drosophila* cannot speak to vertebrate-specific attributes of form, scale or function. The *Drosophila* circulation is open, without vessels, such that hemolymph is pumped at low pressure to perfuse the tissues directly. The heart has neither endocardium nor chambers and lacks defined structural valves (although certain regions appear to have valve-like function). The contraction of the vessels is peristaltic, rather than brisk and sequential, as is the vertebrate heart. However, vertebrate homologues of genes involved in the generation of *Drosophila* heart cells certainly may play roles in cell fate decisions relevant to single cells, as exemplified by the extrap-

olation from the *Drosophila tinman* gene to the vertebrate *Nkx2-5* genes (Bodmer, 1993; Harvey, 1996).

The mouse is not readily used for screens of early heart development, since the mother is opaque, and it can be difficult to distinguish primary from secondary effects when the heart is involved, because of embryonic dependence upon its blood flow. In addition, large-scale screens using the mouse would be inordinately expensive. This is not to diminish the great importance of targeted mutations of known genes in the mouse for the study of heart development, and their proximate relevance to humans, especially when combined with the emerging power of microphysiological studies in the mouse embryo. However, for large-scale screens of the stages of early heart development discussed here, it seems sensible to consider alternatives. One which has been explored is the zebrafish, *Danio rerio*.

The zebrafish was chosen for a heart development screen for several reasons (Fishman and Stainier, 1994; Stainier and Fishman, 1994; Driever and Fishman, 1996). It is a vertebrate, and its heart is essentially indistinguishable from the embryonic human at the heart tube stage. The embryo is transparent and externally fertilized, so that all steps of development are visible and accessible, and adults can be raised in large numbers at relatively low expense. The zebrafish embryo does not require a functioning heart to live for a few days and to swim and eat, because it is small enough to survive by diffusion (Pelster and Burggren, 1996). The disadvantage is that a genomic infrastructure is only now being constructed, since it is an organism new to the genetic community.

In two large-scale screens using ethylnitrosourea as a presumptive point mutagen, a combined total of 6647 recessive mutations were identified by direct visualization of F3 progeny of mutagenized G0 males (Driever et al., 1996; Haffter et al., 1996). About one-third of these were kept because they appeared interesting. Complementation has not been completed, but current estimates are that about 500 genes from this screen are informative about development (Driever and Fishman, 1996; Granato and Nusslein-Volhard, 1996). These mutations affect many developmental decisions, and include a subset with cardiac effects, many apparently selective for the heart. In fact, what is remarkable is that the mutations can interfere even with individual elements of heart assembly.

Fashioning of heart form, by this analysis, occurs in several genetic steps (Chen et al., 1996; Stainier et al., 1996). As shown in Fig. 5, these include: (1) Fusion of the bilateral tubes, which requires *bonnie and clyde* and *miles apart*. In fish mutant in these genes, two hearts form, one on either side of the midline; (2) chamber generation, which is affected in *pandora*, in which there appears to be a markedly diminished ventricle, and a well-formed atrium is connected directly to the outflow tract; (3) axial positioning of the chambers, which is perturbed in the *heart and soul* mutation, which causes venrticular muscle (labelled with chamber-specific myosin heavy chains) to form within the atrium; (4) concentric growth of the myocardium, which does not occur in *valentine*, *heart of glass* or *santa*. The heart is globally enlarged in these mutants. Given the lack of valves and thin matrix in this class, it is reasonable to speculate that they interfere with endocardial-myocardial signalling; (5) laterality of the heart, which is randomized in a set of mutations that affect the midline (Danos and Yost, 1996; J. Chen, unpublished); (6) valve generation, which is blocked in the mutation *jekyll*, and in which both atrium and ventricle otherwise appear to differentiate normally. Clearly, many other genes are important in heart development, and some may be revealed by additional similar screens (calculations suggest that the first screens did not achieve saturation) or made evident by screens for genetic interactions.

Functional maturation of the heart is affected by other genes. Some interfere with development of the contractile machinery, either of individual chambers or of the whole heart (Stainier et al., 1996). Development of the pacemaker and conduction

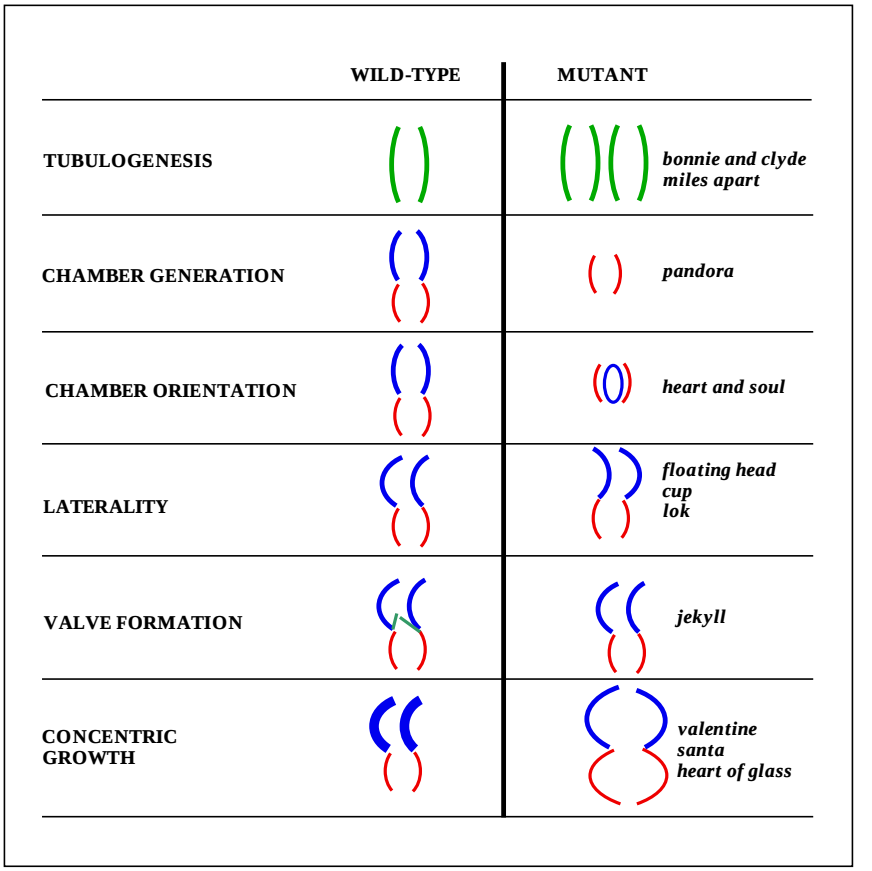

Fig. 5. Genetic steps in fashioning of heart form, determined by large-scale screens in zebrafish. Each step is defined by mutations which perturb it. The wild-type is shown on the left and the change in exemplary mutants diagrammed on the right, along with mutant names. For convenience, the endocardial lining is not shown, although there is a mutation, *cloche*, which lacks endocardium. For each mutation, the remainder of the heart forms reasonably normally. The order is according to the time in development when the mutation is evident, but, of course, the time of action of the genes may be different.

systems also can be dissected into several genetic steps. Pacemaker function is perturbed in *slow mo*, which blocks one kinetic component of the presumptive pacemaker channel, I_h, and is revealed in the intact animal as a slow heart beat (Baker et al., 1997). The exit of the impulse from the sinus node to the heart is blocked in *reggae*, in which impulses activate the heart locally in the region of the node several times before triggering a conducted impulse, the propagation of which appears relatively normal. *tremblor* causes fibrillation of the heart.

This type of analysis points out that the fashioning of vertebrate organs may be decipherable by genetics and partitioned into several informative unitary steps, each key mutation serving as an entrance point to a pathway, for example, to make a ventricle, or design its pattern, or add a valve. In principle, some other components of each of the pathways also may be accessed by genetic means, by identification of enhancers or suppressors. Routine positional cloning of these zebrafish mutants awaits completion of a dense genetic map (Knapik et al, 1996). Large insert libraries are now available (Zhong and Fishman, unpublished data; Zon, personal communication). In the meantime, the embryological analysis of the mutants will begin to determine whether mutations act in a cell-autonomous manner, which can be answered by reciprocal transplantation between wild type and mutant.

Summary: a good place to start

Even if we could find the elusive (and very possibly non-existent) "master gene" families for the myocardial or endocardial cell fates, these would not speak to the higher order organotypic decisions of size, borders, substructures and global form. Nor would they address the design of the organ field, in particular, the ability to adjust to perturbation of cell number. This regulative flexibility presumably serves as an adaptation to the vagaries and uncertainties of embryonic growth that characterize vertebrate development, and which extends over relatively long periods and to large size and complexity. Hence, although it is clear that we should begin to approach organ development by understanding cell fate, it is likely that we will need new paradigms for some of the decisions of organ morphogenesis. It will be particularly interesting also to ascertain how the heart is integrated into body form, adjusting its size and shape to match local anatomy, developing physiological function proportional to the mass and homeostatic needs of the animal, and hooked up seamlessly with vessels and nerves.

The genetic approach to early heart development is complementary to those of molecular and cellular embryology. Steps most amenable to this approach will be in pathways neither redundant nor pleotropic, and several have been revealed in the zebrafish screens, and are interesting in their modularity of effect. The endocardium appears to be a vertebrate addition to the primitive chordate heart, and several of the mutations in both mouse and zebrafish that perturb early heart development do so by interfering with endocardium-dependent processes. The development of vertebrate organ systems may be one arena in which vertebrate-based genetics is needed if organizing paradigms are to be enunciated.

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