Cardiovascular Development
Prospects for a Genetic Approach

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This review concerns the potential of genetics to unravel cardiac development. From the geneticist’s point of view, the goal is to understand the hierarchy of molecular decisions that go into forming the heart. This can proceed either by perturbing a known gene or by screening for abnormal cardiovascular phenotypes after exposing germ cells to mutagenic agents. The power of the latter approach is that it makes no presuppositions about the role of particular genes, ones that might be based, for example, on the fact that a gene is expressed in the heart. The major potential limitation of a screening approach is that it may not be possible to find cardiovascular mutations in significant numbers, because it is conceivable that many genes important to the development of this organ system also are crucial earlier in development, so that their mutations would be pleiotropic in effect. There are excellent reviews that cover cardiac embryology in detail as well as those that deal with related topics, such as control of myocyte differentiation, vertebrate patterning, mesoderm induction, and cardiac and vascular growth factors, subjects that are not elaborated here.

We know very little about developmental genetics of the cardiovascular. A crucial first step is to define an organism amenable to such analysis. The two best-studied genetic systems are Caenorhabditis elegans (a nematode) and Drosophila melanogaster (the fruit fly), the former of which lacks a heart and the latter of which has a myogenic component of its dorsal vessel that does subserve a contractile function but lacks many important components of a vertebrate heart. In contrast to the vertebrate, the D melanogaster heart is in a dorsal position, and its circulation is open, bathing internal organs in hemolymph, suggesting that its cardiovascular may not bear an evolutionarily ancestral relation to that of the vertebrate.

The Drosophila heart is of interest and relevance in that it is an embryologically early subdivision of the mesoderm, and genes critical for assembly of this germ layer in Drosophila have vertebrate homologues, so that it is reasonable to hypothesize that as for other mesodermal derivatives, functioning of the vertebrate heart may be dependent on them. For example, the maternal Drosophila gene dorsal, a morphogen, regulates the zygotic genes twist and snail, both of which have vertebrate homologues expressed during gastrulation, and the former of which activates tinman, a gene needed for Drosophila heart formation. In mice, tinman-related genes have been identified, and the developmental expression profile of one has been shown to correspond to the region of precardiac mesoderm and adjacent endoderm and to the primitive heart tube. These are reasonable candidate genes for involvement in vertebrate heart formation.

Humans obviously are an inconvenient species for genetic studies. In fact, although most congenital cardiac diseases have a high sibling recurrence rate, only rarely does the inheritance pattern suggest a single-gene defect. There are several potential explanations for the rarity of simple mendelian inheritance of congenital heart defects, but the most likely is that cardiac disorders that affect assembly of the primitive heart tube are lethal, so that embryonic death occurs by 4 weeks of gestation. By this argument, it is only the less severe cardiac anomalies that are seen clinically. Intrauterine development renders any mammal less than ideal for study. The mouse is well suited for testing the role of any specific gene by knockout but not for screening. One important lesson from mouse gene knockout experiments is that the normal patterns of gene expression do not predict the phenotype of the mutant mice, many of which even appear normal. This has been ascribed to redundancy of gene function and may also reflect a lack of assay systems sensitive enough to detect subtle phenotypes and/or lack of environmental stressors that would elicit a phenotype. In any case, whether or not a gene is prominently expressed in the heart may be a poor predictor of its importance in cardiac development or function. Both the mouse and human do provide examples of some important cardiac developmental anomalies affecting single genes. For example, disruption of the elastin gene may be responsible for an autosomal-dominant form of supravalvular aortic stenosis, and the mouse situs inversus gene has been mapped. The axolotl mutant, cardiac lethal, also provides an example of a fortuitous mutation of a gene critical to cardiac development. In this recessive lethal mutant, the heart forms but does not beat, although it can be salvaged by transplantation to a normal host or if cultured with normal pharyngeal endoderm.

Developmental Genetics: Paradigms From Flies and Worms

Recurring themes in D melanogaster and C elegans developmental genetics are “cell-fate decisions” and

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“positional information.” It is worthwhile to examine how far such principles can be pushed in describing vertebrate organogenesis, even if just to anticipate the type of mutations that might occur, and then to consider what additional features may need description.

**Lineage and Fate**

One approach to development is to analyze cell-fate decisions. Every cell has a fate: for a progenitor cell, its fate is described by the behavior of its progeny; for the terminally differentiated cell, fate is the composite of cell behavior and patterns of gene expression.

Mutational analysis has been used to define the unitary cell-fate decisions made during development of *C. elegans*. By direct visual inspection, the complete ancestral tree, back to the egg, has been established for all of the 959 somatic cells of the adult hermaphrodite.18 The lineage tree of *C. elegans* is essentially invariant. Single-gene mutations can alter development by changing cell-fate decisions so that the lineage is altered. Lineage and fate, therefore, are inextricably linked. Cell fates are controlled by hierarchies of genes, which can be put in order of their function by their epistatic relations.19 Some mutations affect multiple parts of the lineage tree, and some are more discrete in their action. Even if the experimental focus is on a particular region amenable to genetic analysis, such as the egg-laying system of *C. elegans*20,21 or the compound eye of *Drosophila*,22-24 the information may be broadly generalizable in that many of the gene products act widely and with similar function throughout the organism. For example, some genes delay or accelerate decision making with regard to developmental stage (heterochronic mutations25), some cause adoption of an inappropriate new lineage, and some enhance or block programmed cell death.26

**Polarity, Patterns, and Boundaries**

Order extends over many cells in multicellular organisms, both at the level of the whole organism and at the level of its constitutive parts. Some of the means that regulate axial polarity and segmental patterning in *Drosophila* are likely to be applicable to vertebrates, at least in the mechanistic sense whereby a cell’s fate is, in part, determined by its reading of positional information. Certain *Drosophila* genes establish axial polarity, one class determining the anterior-posterior axis and another determining the dorsal-ventral axis. These genes were discovered by dint of their phenotypes when mutated. For example, embryos lacking the anterior determinant, *bicoid*, lack anterior structures, and artificially increased dosages of *bicoid* causes expansion of the head and thorax.27 *bicoid* was the first proved morphogen, meaning a protein distributed in a gradient, the levels of which provide positional information.28 This morphogen gradient is functional within the syncytial *Drosophila* egg and is a product of the maternal genome. It is unclear whether morphogens function in vertebrates and across long distances and intact cells. The best evidence for a vertebrate morphogen is in the limb, where digit identity appears to be regulated by its distance from the region known as the zone of polarizing activity. The action of the zone of polarizing activity can be mimicked by retinoic acid, although retinoic acid seems unlikely to be the morphogen itself.29 Retinoic acid also can delete, in a dose-dependent manner, anterior structures of the frog embryo, suggesting an interaction with axial determinants that function over longer distances.30

One of the clearest types of patterning is segmentation, a property of both flies and vertebrates. Although vertebrate segmentation differs from the fly in several embryologic regards, both appear to depend on homeobox genes. This class of genes was first discovered through study of *Drosophila* mutants, because of the pronounced effects of their mutations on segment identity or body plan. In the mutant *Antennapedia*, for example, flies grow mesothoracic legs in place of antennae. These genes encode transcription factors and are conserved from fly to vertebrate in the region of the 60-amino-acid motif, referred to as the homeodomain, which contains a helix-turn-helix sequence-specific DNA binding activity. In the mouse and human, these genes are organized in clusters on four chromosomes. Each gene in a cluster is expressed along a limited region of the anteroposterior axis of the neuroectoderm and axial mesoderm, and the order of the genes in the chromosomal cluster roughly corresponds to the anterior boundary of anatomic expression.31 In general, targeted mutation of a mouse Hox gene (as the vertebrate homeobox genes are called) affects tissue only in the most anterior region of its normal expression. Several homeobox-containing genes are expressed in the heart and blood vessels, although not restricted to those tissues, and their expression levels and patterns vary during development.32 Certain Hox genes, divergent from the *Antennapedia* class, are prominently expressed in the developing cardiac cushions.33,34 The only member of this family with genetic evidence for a functional role in the cardiovascular is *Hox* 1.5, which when mutated by gene knockout perturbs the heart and branchial arches,35 among other effects, presumably because the cardiac neural crest is included in its domain of expression.

**Cardiovascular Development: Application and Limitations of the Paradigms**

**Where Does the Heart Originate?**

It is difficult to assess with precision the timing or location of cell-fate decisions relevant to the cardiovascular system, because its embryology is not resolved at the level of single cells. However, at least the relevant regions of the embryo have been well defined, as shown in the Figure. Most of the first evident progenitors of the heart and blood vessels are in the lateral plate mesoderm (panel A).1,36 The mesoderm (the middle of the three germ layers) forms by involution of cells during gastrulation. Although the shape of the early embryo varies between species (the amphibian being a ball and avian and mammalian being flat sheets), the relative positions of the cardiac progenitors after gastrulation are similar. These cells migrate anteriorly and medially, apparently in contact with underlying endoderm (panel B), and then the right and left regions fuse in the midline to generate the primitive heart tube (panel C), a structure with two concentric cellular layers, inner (endocardium) and outer (myocardium), separated by an extracellular matrix, referred to as the cardiac jelly. The primitive heart tube is oriented, at
least transiently, in an anteroposterior direction (panel D), with venous return posteriorly and aortic outflow anteriorly. Contraction accompanies formation of the tube, as myofibrillar differentiation proceeds in an anteroposterior direction.

Few developmental decisions in metazoans are cell autonomous, and there is evidence that cardiac development also is crucially dependent on cell-cell interactions. For example, in urodele amphibians, removal of endoderm adjacent to the cardiogenic mesoderm prevents heart formation, and in culture, the cardiogenic mesoderm forms the beating heart only if endoderm is added. There is a spatial gradient of inducing capability, so that more posterior endoderm is less capable of cardiac induction. There is debate about the need for endoderm in chick cardiac induction. In *Xenopus*, cardiac induction appears to coincide with the onset of gastrulation, so that by the neurulation stage explanted cardiogenic mesoderm no longer requires pharyngeal endoderm to form the heart. A signal seems to be provided by the dorsal lip of the blastopore. The role of these signals could be to provide information to guide an uncommitted cell to a cardiac fate, to enhance its propensity toward this decision, or to render it competent to respond to other signals. The responsible molecules are not identified, although it has been determined that heart formation from isolated urodele mesoderm is enhanced by transforming growth factor-β (TGF-β) and platelet-derived growth factor, a component of the activin growth factor family is secreted by embryonic endoderm, and activin A induces expression of cardiac-specific myosin heavy chain in animal pole explants of *Xenopus* blastulae. Several genes have been identified in the “Spremann organizer,” a region of the dorsal lip of the blastopore that can induce a second embryonic axis when implanted ectopically, and given the importance of the dorsal lip in cardiac induction, these genes are candidates for involvement early in the cardiac induction cascade.

Induction is also involved in cardiac valve formation. In two regions along the microscopically homogenous endocardial tube (the atrioventricular canal and the outflow tract), endocardial cells delaminate, extend long filopodia into the cardiac jelly, and migrate toward the myocardium. This migration occurs in collagen gel organ cultures, and this system has been used to show that the myocardium releases a signal triggering endocardial cell invasion and that the sites of both the signal and the responding cells are limited to the preavalvular region. Several genes are localized during the period of cushion formation to the atrioventricular canal or outflow tract, including transcription factors, such as G-Hox and *Ev1-I*, growth factors, such as TGF-β, and bone morphogenetic protein-4, and com-
ponents of the extracellular matrix, such as cytotactin and fibulin. In vitro, TGF-β, antisense oligonucleotides inhibit the invasion.

**What Are the Cell-Fate and Lineage Decisions?**

Although it is likely that there are many subtle distinctions between heart cells that elude current anatomic classification, a short list would include atrial myocyte, epicardium, ventricular myocyte, endocardium, endothelium, Purkinje cell, and myoendocrine cell. It is likely that at least some of these lineages can be defined quite early in embryogenesis. For example, just after gastrulation the chick stage-4 mesoderm already contains cells capable of expression of atrial and ventricular myosin heavy chains when cultured in isolation. In the zebrafish, a cardiogenic region is predictably located even in the blastula before gastrulation. It is not known at which stage specific cardiovascular gene programs are activated, except for a cardiac-specific myosin heavy chain, which labels stage-7 chick precardiac mesoderm.

Most of the lineage relations of the cardiovascular are still quite murky. For example, the origins and migratory paths of endocardial cells do not seem identical to those of the myocardial progenitors. The endocardium may derive from a population of lateral plate mesoderm cells, located in the same region as the myocardial progenitors but distinct from them.

These are believed to derive from angioblasts of the lateral plate. Although blood vessels first appear in the yolk sac around blood islands, intraembryonic vessels appear to be derived from intraembryonic angioblasts, which are widely dispersed throughout the mesoderm.

Vessel assembly occurs by aggregation of the angioblasts ("vasculogenesis"), by sprouting of preexisting vessels ("angiogenesis"), or by a combination of the two. The central vascular tree is assembled from isolated or small aggregates of angioblasts, i.e., by vasculogenesis, as are the vessels of many organs, including the lung, pancreas, and gut. Some organs, such as the brain, thymus, kidney, and liver are vascularized predominantly by angiogenesis.

One might suspect that if different blood vessels were really formed by significantly different mechanisms, specific mutations might selectively affect specific parts of the vasculature, and this has, in fact, proved true in one zebrafish mutant (B.M. Weinstein, D. Stemple, W. Driever, and M.C. Fishman, manuscript in preparation). Avian and mammalian blood cells are in contiguity with endothelium both in blood islands and in the developing para-aortic region, suggesting to some that there is a common progenitor cell for some embryonic endothelial cell and blood cells, a progenitor cell referred to as a hemangioblast. Later in embryogenesis, hemopoiesis begins in the liver, spleen, and bone marrow. Therefore, at least early in development, there is a shared lineage for myocardial cells, endocardial cells, endothelial cells, and blood cells.

To sort out the cardiovascular lineages, it will be necessary to track decisions at the level of single cells and to account for their progeny. One approach has been to use retroviral infection of precardiac mesoderm with β-galactosidase-expressing retroviruses, which integrate in the genome and hence identify progeny and also which reveal clones of related myocardial cells. In the zebrafish embryo, it is feasible to fill individual blastomeres with fluorescent dextran and continuously track the migratory paths and dispersion of progeny. This study has shown that a cardiogenic region can be defined even before gastrulation. Furthermore, even by the late blastula, different blastomeres are distinguished by having their progeny restricted to a single chamber. This suggests that the cardiogenic lineage is one of the first to be established and that different chambers may arise from different progenitors.

**Does the Heart Have Polarity, and Is It 'Patterned'?**

One feature that distinguishes the vertebrate heart from that of its nearest ancestors, the protochordates, is its functional polarity. The tunicate heart, for example, alternatively pumps blood in either direction, whereas the vertebrate heart has a single site of heart beat initiation, a conduction system for sequential chamber activation, and valves to guarantee unidirectionality of flow. There is also an intrinsic beat rate of cardiac muscle cells, with atrium faster than ventricle, which helps to prevent runaway pacemakers. During development, polarity is evident at stages as early as the precardiac mesoderm, at which stage more posterior tissue is destined to form aortas and more anterior tissue is destined to form ventricle, and if explanted, preatral tissue already beats more rapidly than does preventricular tissue. This beat rate gradient may be imposed from neighboring tissues, in that presumptive cardiogenic tissue transplanted to an ectopic site within the precardiac mesoderm adopts the beat rate of its new site, if the transplantation is performed at an early enough stage. It is possible that cardiac polarity begins to be established even before gastrulation. In the zebrafish, a cardiac progenitor cell region is demarcated by the midlate blastula. Retinoic acid applied transiently at the early gastrula stage of zebrafish causes deletions of the heart tube, progressing from outflow tract to venous inflow region as the concentration increases.

In terms of patterning, the heart is not segmented, but it does have borders between chambers. These can be discerned by the heart tube stage, even before morphological chamber distinction, because different myosin heavy chain isotypes already distinguish preatrium from preventricle. Shortly thereafter, cushions and then valves mark the borders. Work from *Drosophila* suggests that boundaries between cellular compartments may be of particular importance in providing frames of reference for organizing fields between the borders. Therefore, formation of an organ may require not only genes for determining the bulk of the tissue but additional ones for borders between zones. For example, fashioning the wing of *Drosophila* depends critically on the wingless gene, which is used to demarcate the border between the ventral and dorsal compartments. The product of the wingless gene is homologous to the secreted proteins of the Wnt gene family of vertebrates, expressed in developing tissues and, by
gene knockout, important in establishing embryonic form.66

Assumption of Organotypic Structure: The Need for New Paradigms

Although general principles for cell fate and patterning can be extrapolated from the genetics of Drosophila or C elegans, some dynamics of organogenesis have no clear analogy. These include the issues described below.

What Regulates the Shape of the Heart?

In all vertebrates, the heart tube loops and twists to the right side of the embryo. Situs inversus causes randomization of this process such that half of the time the heart tube loops to the left. Since the left-right axis appears to be the last of the three to form and can be defined with respect to the anterior-posterior and dorsal-ventral axes, it has been suggested that right-left bias is imparted by an asymmetric molecule that is aligned by relation to the anterior-posterior and dorsal-ventral axes. Loss of this molecule could result in loss of bias, which would explain the randomization of asymmetry in situs inversus.67 Harder to account for by this theory is a recently described line of mice, all of which have reversed looping of the heart (and reversed sidedness of the viscera) due to insertion of a transgene.68 The action of such biasing genes may occur early in embryogenesis. Focal injury of the ectoderm of a gastrulating frog causes subsequent situs inversus of organs, including the heart, the primordia of which contact the wounded ectoderm during their migration.69

What Regulates Cardiac Size?

Larger organisms have larger hearts, a property that appears to be partially intrinsic to the organ. For example, embryonic heart tissue from the large Amblystoma tigrinum forms a heart larger than that of the host when transplanted ectopically into an animal of the smaller species, Amblystoma punctatum.70 This suggests that although hemodynamic forces certainly regulate the degree of growth of the heart, there also may be inherited programs to control cell number and cellular hypertrophy.

How Do the Heart and Vasculature Form Seamless Connections?

Congenital diseases that occlude flow without providing an alternative route would likely be lethal, but survivable diseases with partial interruption within the heart, which can occur especially in the outflow region, or in the vessels, such as coarctation, suggest that certain regions of the vasculature may be particularly susceptible to dysmorphogenesis. One possibility is that these regions represent the “joints” between tissue segments, which develop from different progenitors or through different mechanisms, such as where neural crest and mesodermal contributions come together. The nonmesodermal component of the heart derives from the neural crest, a population of migratory cells originally from the neural tube.71 Some neural crest cells from the cranial region of the neural folds migrate through the pharyngeal arches and eventually form the aortopulmonary septum and part of the tunica media of the larger arteries that branch from the aorta. Ablation of this region of neural crest causes persistent truncus arteriosus, other outflow tract anomalies, such as tetralogy of Fallot, and interruption of the aortic arch or other great vessels.

The Zebrafish as a Candidate for Cardiovascular Genetics

We have begun to investigate the utility of the zebrafish, Brachydanio rerio, as a vertebrate genetic organism that may be particularly well suited to screening for mutations that perturb the developing cardiovascular.51.64 The heart and vasculature of fish resemble those of humans in essential features through the primitive heart tube stage. (Subsequently, the fish retains a circulation with all cardiac output first traversing the gills and then going directly to the body, rather than generating a respiratory circuit.) The zebrafish is ideal for embryologic studies because fertilization is external, so that all developmental stages are readily accessible, and the embryo is transparent, so that individual cells of the heart and vasculature are visible microscopically.72 Its potential as a genetic organism was first grasped by Streisinger et al.73 It is small and hardy, can be raised in large numbers, is amenable to chemical74,75 and radiation76 mutagenesis and to transgenesis,77,78 and can be grown as clonal lethal-free lines79 or, for short periods, even as haploid embryos generated by parthenogenetic activation of the egg.

Our studies of the zebrafish heart have revealed that the embryonic cardiogenic and vasculogenic regions are predictably located even before gastrulation.51 Analyses of inbred populations or of fish mutagenized by radiation and, in collaboration with Wolfgang Driever (Cardiovascular Research Center, Massachusetts General Hospital), by chemical mutagens reveal that single gene defects clearly affect cardiovascular morphogenesis. For example, we have isolated mutants lacking discrete parts of the heart, such as the valves or a single layer of the primitive heart tube, or of parts of the vasculature; we have found other mutations that result in physiological dysfunction and cause disruption of heart rate or contractility. The responsible genes remain to be identified, but these mutants already have revealed individual steps in the hierarchy that eventsuate in the assembly of the cardiovascular. The mapping and cloning of the mutant genes will be facilitated by the many strains available and their fecundity.

Summary

Genetics is a powerful tool, especially when used in combination with embryology, in the seeking of genes necessary for assembly of the cardiovascular. The first questions must address the types of cellular decisions that are made during development. As for simpler systems in C elegans and D melanogaster, the lineage and cell-fate decisions of the cardiovascular progenitors need to be assessed. In addition it is likely that new paradigms will emerge for multicellular assembly. The study of cardiovascular mutations will define individual genetic steps that define organotypic decisions. A genetic approach is a natural extension of embryology, physiology, and anatomy, fields of great sophistication with regard to the cardiovascular, because, like them, it focuses on integrative biology and on the intact
organism. The zebrafish is particularly well suited to a combination genetic-embryologic study of the fashioning of the cardiovasculature.

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