Introduction: Why study cardiovascular development?

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Socrates, Aristotle, Vesalius, and Galileo are just a few of the long list of notables who have written of their fascination with the rhythmic pulsing of the red spot evident in an opened bird egg. Our curiosity has not abated during the long history of human interest in cardiovascular development. Today we are still fascinated observers of that red spot as we attempt to identify the scientific underpinnings of cardiovascular development and correlate these findings with disease states in humans. Fortunately, our tools have expanded far beyond those of the ancient observers to include phase-contrast microscopy, pulsed Doppler flow, gene sequencing, genetic manipulation, high-performance liquid chromatography, and gel electrophoresis, to name but a few of the techniques the reader will encounter in this book. We are also beginning to appreciate the power of a comparative approach that employs a variety of animal species with different, yet similar, cardiovascular characteristics. Where have these observations of ever increasing sophistication led us?

The primacy of the developing cardiovascular system

We know that the cardiovascular system is the first system to begin functioning in a developing animal. So many of the contributors to this book began their first drafts with these words that, as editors, we felt that we must instead highlight this point once, prominently, at the beginning of the book. The developing heart and circulation do deserve particularly close scrutiny because of their fundamental role in the developmental process. As the system for delivery of raw metabolic substrates and oxygen to the rapidly growing embryo, any constraint on, or limitation of, early cardiovascular performance is likely to constrain all other subsequently developing systems because of their utter dependence on the timely receipt of these materials. Put another way, the embryonic cardiovascular system is an early and potentially severe "choke point" in development.

But study of the developing cardiovascular system is important for more reasons than "just" its role as a vital component of embryonic development. The cardiovascular system is unlike almost all other body systems in that *the cardiovascular system must perform even as it develops and grows.* This situation is emphasized when one looks at the growing mammalian embryo and fetus. Many of the roles of the lungs, liver, urogenital system, and a host of other organ systems are carried out by the corresponding maternal organs. These embryonic organs can undergo prolonged periods of reconfirmation and growth without the need to perform their eventually intended function. Similar examples are found in embryonic and larval fishes, amphibians, reptiles, and birds, where certain organ systems do not come "on-

line" until shortly before, or at, hatching or birth. The cardiovascular system, however, enjoys no such luxury as a period of growth without need for performance. We still have a great deal to understand about how the heart can coil, twist, and differentiate into chambers as it simultaneously generates ever increasing unidirectional flow of blood into a proliferating vascular bed. The relation between developing structure and developing function remain enigmatically entangled as we try to understand, for example, whether heart shape determines flow patterns or flow patterns determine heart shape.

The rationale for this book

Books in the genre of heart development are generally produced either as a set of published symposium papers when the field is emerging (and will benefit from some direction and synthesis, however incomplete) or when the field is maturing (enough is known for a comprehensive forms). As editors, we speak for each contributor to this book when we highlight the former circumstances as our justification – that our knowledge of cardiovascular development is fragmentary in many important areas, and rudimentary at best in most others. By indicating what *is* known in our respective fields, we all hope that we will clearly highlight what *is not.* To this end, each chapter concludes with a section that outlines some of the important unanswered questions and fruitful future directions to be followed in the next several years. In short, this book is intended to educate: not just with the facts as we know them today but also by highlighting the vast areas of our ignorance so as to challenge future investigators.

The scope and content of this book

This book represents a determined effort to explore the development of the cardiovascular system with a comprehensive approach spanning

Organizational hierarchy (molecules to organisms)
Scientific paradigms (basic science to clinical applications)
Systematic perspectives (simple invertebrates to humans)

The scope is purposefully broad in order to bring together groups of investigators, representing differences in approach, that rarely interact, let alone contribute articles to the same books. The editors encouraged communication between authors during the preparation of the chapters. The result has been not only a consistency of style and cross-referencing between chapters but also an appreciation for the power of this broad approach, which comes across clearly in each chapter, regardless of its own focus.

Development of Cardiovascular Systems: Molecules to Organisms is divided into three parts. In Part I, "Molecular, Cellular, and Integrative Mechanisms Determining Cardiovascular Development," authors explore the mechanisms that form the basis of cardiovascular development. With topics ranging from gene-regulated expression of contractile proteins (Chapter 3) to endothelial cell proliferation (Chapter 6) to a sequential approach to determining cardiovascular function (Chapter 7), this first part lays the groundwork for an understanding of cardiovascular development. The editors are particularly pleased that one of our initial secret fears – that the molecularly oriented chapters would prove to be highly "self-focused" – never materialized. Even the chapters on the most molecular of topics and approaches indicate how the data within complement studies carried out at tissue, organ,

and organismal level and contribute in concert to a greater overall understanding of the heart and vascular system.

After the groundwork is laid in the first section of the book, Part II, "Species Diversity in Cardiovascular Development," explores the great variety of "pumps and pipes" associated with cardiovascular development found within the animal kingdom, and the approaches by which this diversity can be investigated and categorized. Chapters 9 and 10 set the tone with considerations of evolution, development, and their interaction. They are followed by a six-chapter survey of animal cardiovascular systems and their development. Ironically, even as diversity of patterns is emphasized and celebrated in these chapters, what emerges as well is prominent commonalities in cardiovascular development. It would appear that the earlier in development one conducts experiments, the more broadly applicable are the findings to all species. This means that experimenters focusing on early development can take a cosmopolitan approach, employing any combination of species that facilitates the acquisition of new and useful data. For example, the explosion of zebra fish onto the developmental scene in the last few years is not because they are interesting animals in their own right (although see Chapter 12 on fishes' cardiovascular development) but because a few key features of zebra fish biology, particularly the ease with which genetic mutagenesis can be induced, lend themselves to answering developmental questions, including those concerning cardiovascular development (see Chapter 1), not easily garnered from other sources. These data, so easily acquired, are generally transferable to vertebrates as a whole, including humans. Of course, much of what we know of hemodynamic development comes from the study of the chick embryo (see Chapters 7 and 15). The long-standing assumption that findings on the chick are broadly applicable to all vertebrates has been verified by studies of fish (Chapter 12), amphibians (Chapter 13), and rodents (Chapter 7), which show that in many respects there is a "vertebrate" pattern of early physiological development that will almost certainly hold for larger mammals, including humans. (This topic is discussed more fully in Chapter 13.)

In Part III, "Environment and Disease in Cardiovascular Development," attention is on external factors influencing cardiovascular development and how the resulting malformations can be managed clinically. Following two chapters that deal with environmental influences on cardiorespiratory development (Chapter 17) and its modeling (Chapter 18), perspectives are provided on more clinically oriented topics that range from principles of abnormal cardiac development (Chapter 19), through therapeutic treatments (Chapter 20), to management of human patients (Chapter 21).

Development of Cardiovascular Systems: Molecules to Organisms concludes with a synthesis of, and perspective on, a dynamic field as it currently stands, and provides projections for the future.

The book also offers a compendium of nearly 1500 references. Reflecting the explosive growth in this field, more than half the references have been published just in the 1990s. Having a single reference list was an active pedagogical decision to allow for paper cross-referencing. Thus, each reference concludes with an indication of each chapter in which it is cited. This should prove a useful tool that complements the Index as the reader tracks down information on particular topics. Additionally, our hope is that a biomedically oriented student searching for a reference on development of, for example, starling relationships will be intrigued by seeing this paper cited in the chapters on fish and amphibian development and will then read these additional chapters to gain a comparative perspective. Similarly, our hope is that the curiosity of the comparative zoologist will be sufficiently

aroused by seeing a familiar paper cited in a clinically oriented chapter that he or she will read that chapter as well.

As is evident from even a quick scan of this book, the advanced tools and approaches being brought to bear on all aspects of cardiovascular development are yielding new information at an enormous rate. The field of cardiovascular development, however, is in an early era of growth and development, when new questions are unearthed more quickly than we can answer them. In many ways, that rhythmically pulsing "red spot" remains as enigmatic a beacon to us now as it was to the philosopher-scientists of history. Our intention is that this book will help to remedy that situation through the organization and display of current information and through the stimulation of continued interest.

Part I

Molecular, cellular, and integrative mechanisms determining cardiovascular development

1

Genetic dissection of heart development

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Introduction

Along with the evolution of the closed chordate circulation, which contains blood at relatively high pressures, came the evolution of a highly muscular heart with valves separating low- and high-pressure chambers and of a vasculature lined throughout by endothelium (see Chapter 9). How is this seamless tubular system fashioned?

The myocyte has been the focus of much of the molecular work to date concerned with heart development. Yet the heart is constituted of cells with many fates, varying in specialized function from conduction to secretion to contraction. Furthermore, it has not been feasible to address at a molecular level essential issues of cardiovascular morphogenesis, especially with reference to larger-scale organotypic decisions (Fishman & Stainier, 1994). For example, are there genes that determine heart size? Are there genes that demarcate chamber borders? Are there single genes crucial to the fashioning of organotypic structures, such as endocardium or valves? Are different vascular beds assembled differently? Although many approaches might be envisioned, genetics has already proven to be powerful in revealing binary decisions during development in *Drosophila* and *Caenorhabditis elegans.* We explore here the possibilities offered by three genetic systems–*Drosophila,* mouse, and zebra fish–to discover the earliest molecular decisions that fashion the cardiovascular system.

Drosophila: The power of genetic screens in invertebrates

Drosophila *heart development*

The fly heart is a simple tubelike organ that is located at the dorsal midline beneath the epidermis and that extends nearly the length of the body. The circulation is open. Although there are regions with "vessels" containing hemolymph in the wings and in other body regions, it is not clear whether the lining cells can be likened to endothelium or rather represent spaces between tissue planes. Hemolymph is sucked into the heart through posterior ostia and ejected anteriorly. Although a valvelike function has been ascribed to the region between the posterior and the narrower, anterior part of the tube, no distinctive structure has been identified. There are three cell types associated with the heart of *Drosophila:* cardial cells, pericardial cells, and alary muscles (Figure 1.1) (Bate, 1993; Rugendorff, Younossi-Hartenstein, & Hartenstein, 1994). Alary muscles connect the heart to the epidermis. Cardial cells express myofibrillar proteins such as actin, myosin, and tropomyosin, and are the

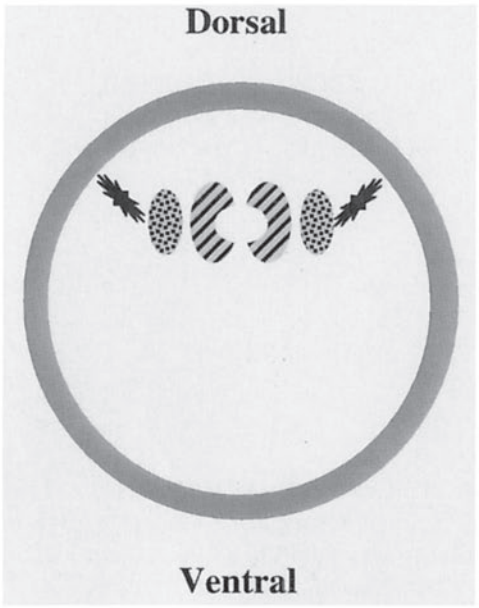

Figure 1.1 Diagram of a transverse section of *Drosophila* embryonic heart. The embryonic fly heart consists of three cell types: cardial cells, pericardial cells, and alary muscle. The cardial cells (*gray areas with stripes*), surrounded by pericardial cells (*gray areas with dots*), form a tube on the dorsal side of the embryo. The tube is connected to the epidermis (*gray*) by the alary muscles (*stars*).

contractile cells. Pericardial cells are believed to be secretory. Unlike the vertebrate heart, there are no endocardial cells or specific chamber boundaries in the heart of *Drosophila.* The circulation is not responsible for oxygen delivery, which is the purview of the elaborate tracheal system.

As in higher vertebrates, heart cells in *Drosophila* are mesodermal in origin. During gastrulation, the mesoderm is generated from ventral cells of the blastoderm, which invaginate into the interior of the embryo along the ventral midline, flatten, and form a single cell layer that spreads dorsally along the inner surface of the ectoderm (Figure 1.2). The dorsal-most cells of the advancing mesoderm are the cardiac progenitor cells. The mesoderm splits into two just ventral to these cells. The inner, or visceral, mesoderm will provide gut musculature, and the outer, or somatic, mesoderm will provide muscle to the body wall. After this separation, molecular markers that characterize the different muscle types begin to be expressed. The two dorsal rows of cardiac mesodermal cells then move toward the dorsal midline and fuse into a tube underneath the dorsal epidermis (Bodmer, 1995). Hence, at a functional and embryological level, *Drosophila* is similar to vertebrates in that its heart contains autonomously contractile cells arranged in a tube arising from bilateral precursors in the mesoderm. It differs in that the heart of *Drosophila* is a dorsal structure, whereas vertebrate hearts are ventral; the heart of *Drosophila* lacks endocardium and closed, endothelium-lined vasculature; the heart of *Drosophila* is not separated into chambers and lacks distinctive valves and coronary circulation. Some, but not all, insect hearts are neurogenic, in that neural stimuli initiate each contraction. To our knowledge this has not been determined for *Drosophila.*

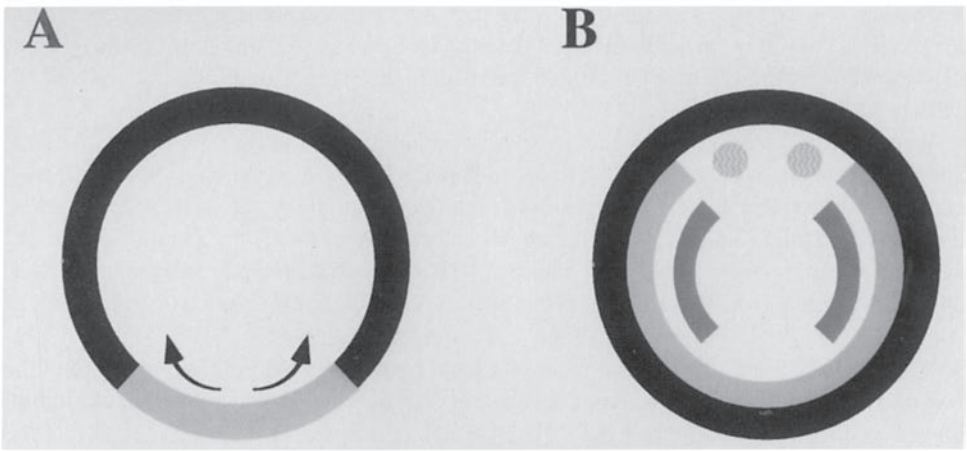

Figure 1.2 Gastrulation and heart formation in *Drosophila.* Diagrams are oriented with the dorsal side to the top. (*A*) Cross section of blastula-stage embryo. The mesodermal cells invaginate into the embryo along the ventral midline and spread dorsally during gastrulation. The black area represents the ectoderm, and the light gray area represents the mesoderm. (*B*) Cross section of the 7-hour *Drosophila* embryo. By this time, the mesodermal primordia of somatic muscle (*light gray*), visceral muscle (*dark gray*), and heart (*black*) have separated.

Drosophila genetics

Most of our knowledge about the genetic cascade responsible for the development of the *Drosophila* heart relates to isolation of a homeobox gene – *tinman* – that lies genetically downstream of the genes that initiate mesoderm determination and upstream of the cascade that distinguishes heart from other mesodermal derivatives. Absent *tinman,* the *Drosophila* embryo does not develop a heart.

The mesoderm arises from ventral cells of the blastoderm, which are distinguished from the dorsal cells by localized activity of the zygotic protein, Dorsal, in their nuclei. Dorsal is actually expressed uniformly around the blastoderm, but ventrally it is transported into the nuclei, where it acts as a transcription factor. Dorsal activates the ventral expression of two genes, *twist* and *snail,* which are expressed in all mesodermal precursors. The product of *twist* is a helix-loop-helix protein, and the product of *snail* is a zinc-finger protein. Mutation in either of these genes prevents mesodermal differentiation (St. Johnston & Nüsslein-Volhard, 1992). The gene *tinman* appears to be necessary for subsequent distinction of mesodermal sublineages and is first expressed soon after *twist* in all mesodermal primordia at the blastoderm stage. The gene *tinman* is dependent upon *twist* for its expression, and *twist* binding sites are found in the promoter region of *tinman.* It is therefore reasonable to assume that *twist* controls *tinman* (Bodmer, 1993, 1995).

The expression of *tinman* persists during gastrulation as the mesoderm invaginates ventrally. Just before the subdivision of somatic and visceral mesoderm lineages, *tinman* expression becomes restricted to the visceral and cardiac mesodermal precursors. By the germ-band-extended stage, *tinman* expression is restricted to the cardiac mesoderm (Bodmer, Jan, & Jan, 1990). Mutations in *tinman* prevent differentiation of visceral and cardiac mesoderm. Genes normally expressed in the early visceral mesoderm, such as *bagpipe,* and those expressed in the cardiac mesoderm, such as *zfh,* are missing from *tinman* mutants,

suggesting that these are genetically downstream of *tinman* (Azpiazu & Frasch, 1993; Bodmer, 1993). Thus, it is currently believed that *tinman* is the crucial link between the general determination of mesoderm by *twist* and the specific differentiation of the visceral and the cardiac mesoderm.

Vertebrate homologues of *tinman* have been isolated from the frog *Xenopus* (*XNkx2.5*) and from the mouse (*Csx/mNkx2.5*) (Komuro & Izumo, 1993; Lints et al., 1993; Tonissen et al., 1994). As expected, there are regions of significant homology between *Drosophila tinman, XNkx2.5,* and *Csx/mNkx2.5.* The amino acid sequences of *XNkx2.5* and *Csx/mNkx2.5* homeodomain show 67% and 65% identity with *Drosophila tinman,* respectively. A 10 amino acid region at the N-terminus (TN domain) is identical in *Drosophila tinman, XNkx2.5* and *Csx/mNkx2.5.* However, an 18 amino acid sequence conserved in the NK2 family is present in *XNkx2.5* and *Csx/mNkx2.5* but absent from *Drosophila tinman.* The structure of the coding region and the homologies between *Drosophila tinman* and its vertebrate homologues are illustrated in Figure 1.3.

The expression patterns of *XNkx2.5* and *Csx/mNkx2.5* include regions of cardiac precursors, although the expression patterns described in mouse and frog begin relatively later in development than does that of *tinman* in *Drosophila.* Transcripts of *XNkx2.5* are first detected in frog embryos at the neurula stage in the bilateral mesodermal regions corresponding to the cardiogenic mesoderm. Expression persists in the heart throughout the embryonic stages to the adult (Tonissen et al., 1994). In the mouse, *Csx/mNkx2.5* transcripts are first detected in the early headfold stage in a crescent of anterior and lateral plate mesoderm, a region corresponding to that of the heart progenitors. Expression persists in the heart tube, embryonic heart, and fetal heart. Pharyngeal endoderm, which is adjacent to the cardiogenic crescent, also expresses *tinman* (Komuro & Izumo, 1993; Lints et al., 1993). Except for the tongue, other muscles do not express *tinman.* Mice mutated in the *Csx/mNkx2.5* gene have recently been obtained by homologous recombination (Lyons et al., 1995). Unlike the *tinman*-deficient fly, in which the heart is absent, the *Csx/mNkx2.5*-deficient mouse does develop a heart, but it fails to loop. The relative normalcy of early heart development without *Nkx2.5* may reflect compensation, perhaps from other members of the *Nkx* family, or may suggest that the singular role of *Drosophila tinman* in heart development has no analogy in higher vertebrates. In terms of functional relatedness, it will be useful to know whether a vertebrate *Nkx2.5* can rescue the *tinman*-mutant *Drosophila.*

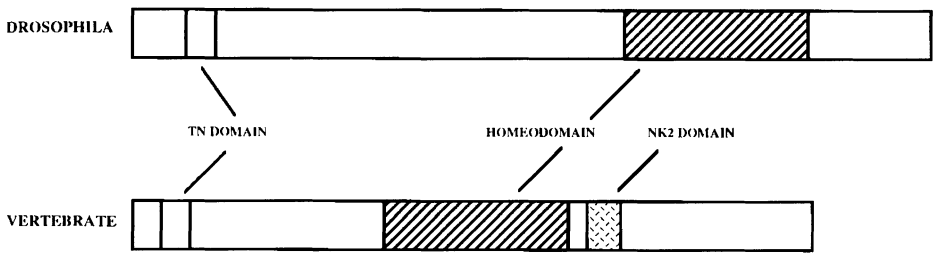

Figure 1.3 Diagrammatic comparison of the cDNA structure of *Drosophila tinman* and its vertebrate homologues. A 10 amino acid sequence at the N-terminus, the *TN* domain, is identical in *Drosophila tinman, XNkx2.5* and *Csx/mNkx2.5.* The homeodomain is highly conserved in *Drosophila, Xenopus,* and mouse. An 18 amino acid sequence downstream of the homeodomain, the *NK2* domain, is found in *XNkx2.5* and *Csx/mNkx2.5* but not in *Drosophila tinman.*