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The genetic basis of cardiac function: dissection by zebrafish (*Danio rerio*) screens

Kerri S. Warren[1], Justina C. Wu[1], Florence Pinet[2] and Mark C. Fishman[1*]

[1]*Cardiovascular Research Center, Massachusetts General Hospital, Charlestown, MA 02129, USA*
[2]*Institut National de la Santé et de la Recherche Médicale Unit 36, College de France, 3 Rue d'Ulm, 75005 Paris, France*

The vertebrate heart differs from chordate ancestors both structurally and functionally. Genetic units of form, termed 'modules', are identifiable by mutation, both in zebrafish and mouse, and correspond to features recently acquired in evolution, such as the ventricular chamber or endothelial lining of the vessels and heart. Zebrafish (*Danio rerio*) genetic screens have provided a reasonably inclusive set of such genes. Normal cardiac function may also be disrupted by single-gene mutations in zebrafish. Individual mutations may perturb contractility or rhythm generation. The zebrafish mutations which principally disturb cardiac contractility fall into two broad phenotypic categories, 'dilated' and 'hypertrophic'. Interestingly, these correspond to the two primary types of heart failure in humans. These disorders of early cardiac function provide candidate genes to be examined in complex human heart diseases, including arrhythmias and heart failure.

Keywords: zebrafish; genetic screen; cardiac development; cardiac function; single-gene mutation; contractility disorder

1. INTRODUCTION

From its first beat the vertebrate heart is distinct from those of its evolutionary chordate ancestors. These more primitive hearts are believed to have resembled those of present day tunicate embryos (Fishman & Chien 1997; Romer 1959; Randall & Davie 1980). The tunicate heart has but one chamber, a simple, single-layered myogenic tube which alternates its site of pacemaker activity and direction of peristaltic pumping. Amphioxus lacks a heart, but develops a unidirectional circulation with blood propulsion by myogenic vessels. While amphioxus does have some endothelial cells on the inner surface of the heart tube, neither tunicate nor amphioxus has a continuous endothethial lining (Randall & Davie 1980).

All embryonic vertebrate hearts have two chambers, an atrium and ventricle. The low-pressure atrium is probably analogous to the more primitive hearts. The real vertebrate 'innovations' are an endothelial-lined vasculature and a chambered heart, particularly one which is sufficiently muscular to generate high systemic pressures. With this, vertebrates developed the means to perfuse larger and more complex tissues. However, the function of the vertebrate heart additionally requires a carefully orchestrated rhythmicity to prevent backflow. Valves between the chambers help to prevent reflux. Equally important is the development of a conduction system which directs coordinated contraction of each chamber, with a pause at the atrioventricular junction to allow the downstream ventricle time to fill. As the heart rate needs to adapt to varying metabolic demands without disrupting this intrinsic coordination, a single controlling pacemaker region also becomes essential to the function of the vertebrate heart (Fishman & Chien 1997; Fishman & Olson 1997).

What genes and pathways underlie these evolutionary innovations in the vertebrate heart? We have approached this question using genetic screens in zebrafish (*Danio rerio*). We have found that even the formation of complex organs such as the heart are tractable to large-scale screens (Stanier *et al.* 1996; Chen *et al.* 1996). In particular, single-gene mutations may remove or perturb individual cardiac components in an informative and specific manner. For example, mutations disrupt formation of specific chambers or layers of the heart, and do so in relative isolation from other effects. Therefore we can speak of genes required for 'ventricle' pathways or 'valve' pathways or 'endothelial' pathways. Each such gene, once cloned, provides an entrance point to discover the other elements of the pathway, by biochemical or genetic methods. These results suggest that for experimental purposes the form of the vertebrate heart can be evaluated as the sum of individual units, or 'modules' (Fishman & Olson 1997). We have recently explored whether cardiac function can similarly be dissected by mutagenesis.

Zebrafish mutations which affect cardiac function potentially have great relevance to human heart disease. In humans, the failure of heart muscle to perform its normal function, termed 'heart failure', is a consequence of many perturbations. Occlusion of the coronary vessels causes myocardial ischaemia and death (infarction)

*Author and address for correspondence: Cardiovascular Research Center, Massachusetts General Hospital-East, 149 13th Street, 4th floor, Charlestown, MA 02129, USA (fishman@cvrc.mgh.harvard.edu).

followed by local scarring. Toxins and 'stressors' can injure or impair myocytes. However, heart failure may be not only a disorder of the cardiomyocyte itself, but may also be a result of perturbed signalling between the myocyte and other cells of the heart, such as endocardial cells or fibroblasts. The cellular pathways to heart failure are poorly understood, as are the mechanisms of their activation through genetic or environmental predispositions. Currently, animal models and studies of human dysfunctional myocardium suggest that disturbances in the cytoskeleton (Arber *et al* 1997; Milner *et al*. 1996; Sakamoto *et al*. 1997; Coral-Vazquez *et al*. 1999; Towbin 1998), as well as in membrane receptor, kinase or signal transduction, and calcium-handling pathways can cause myocyte dysfunction or apoptosis (Chien 1999; Cho *et al*. 1999; Hirota *et al*. 1999; Narula *et al*. 1999), through both direct and indirect mechanisms.

Clinically, it has been useful to classify heart failure as 'dilated' or 'hypertrophic', based on anatomical and physiological criteria (Towbin *et al*. 1999). Dilated cardiomyopathy, the most common form, is defined as impaired systolic contraction usually leading to an enlarged ventricle. It is a syndrome with many different aetiologies, primarily coronary artery disease in the Western world, but there is a substantial proportion of idiopathic cases (Chien 1999). To date, at least 30% are thought to have important genetic components, and the few mutations identified thus far are in genes encoding cytoskeletal proteins (Campbell 1995; Towbin 1998; Olson *et al*. 1998). The familial hypertrophic cardiomyopathies are autosomal dominant disorders of the sarcomere (Seidman & Seidman 1998). The initial defect triggers an as yet undefined intracellular cascade which leads to a pathognomic triad of inappropriate myocardial hypertrophy, myofibrillar disarray and impaired diastolic function. In many cases there is a dynamic systolic obstruction to outflow from the ventricle. Restrictive cardiomyopathy, a third and relatively rare category of heart failure in the Western world, is often associated with infiltrative diseases and in some cases may also have a genetic basis (Kushwaha *et al*. 1997). In all types of cardiomyopathy, a large proportion of cases have familial but non-Mendelian patterns of transmission, suggesting the interaction of several genes and the environment. As for other complex genetic disorders, it is unlikely that linkage studies alone will suffice to identify responsible genes, and molecular dissection will require candidate gene association (Risch & Merikangas 1996). Genome-wide functional screens for cardiac dysfunction would, in principle, reveal a cadre of candidate genes for heart failure.

Here we report a new screen targeted at the discovery of mutations which affect the function of the heart, and analyse these mutations, together with those from a prior screen. It is clear that single-gene defects perturb cardiac function in a myriad of ways. Contractility may be affected principally in one or both chambers. Important elements of pacemaking and conduction may be separately perturbed. Although the mutations affect distinct loci and manifest unique phenotypes, from the vantage point of the whole heart these mutations, like human heart failure disorders, may also be broadly classified as dilated or hypertrophic.

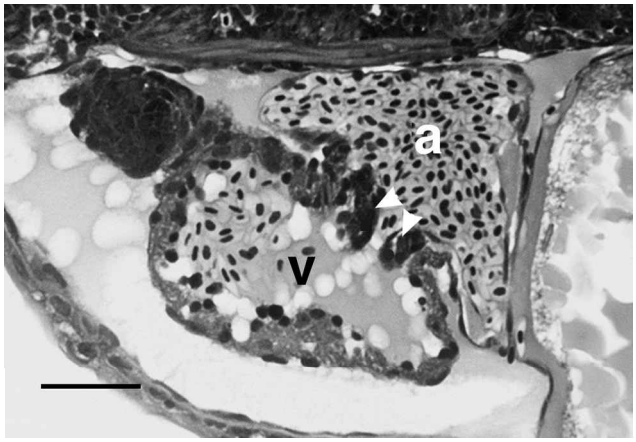

Figure 1. Histology of a normal zebrafish heart at 72 h post-fertilization (hpf), showing differentiation of the heart chambers. Note the larger ventricular myocytes and thicker ventricular wall, in contrast to the thinner atrial walls. a, atrium; v, ventricle; arrowheads, endocardial cushions forming the atrioventricular valve leaflets. Scale bar, 50 μm.

2. BACKGROUND AND METHODS

(a) *Normal zebrafish heart development and function*

The zebrafish heart tube forms shortly after gastrulation and the first heartbeat occurs before 24 h post-fertilization (hpf). At this stage (roughly analogous to embryonic day 23 in humans), the primitive heart consists of a myocardial tube lined with an inner endocardial layer (Stanier *et al*. 1993). Over the next 24–48 h, the heart rate increases, heart wall motion changes from a peristaltic wave to sequential chamber contractions, and endocardial cushions appear, all serving to propel blood effectively through the chambers with minimal backflow. Blood is received from the sinus venosus and flows to the atrium, ventricle, bulbus arteriosus, and finally to the ventral aorta and peripheral circulation. Cardiac looping is evident at 33–36 hpf. By 72 hpf, the individual heart chambers have morphologically differentiated (figure 1) and express different subsets of sarcomeric proteins. The ventricular cardiomyocytes are larger than atrial cardiomyocytes and the ventricular wall is significantly thicker. Atrioventricular cushions are evident (figure 1) and serve to prevent retrograde flow. Although the heart is essentially formed and functional by the second day of development, the embryo can survive by diffusion even with completely absent cardiovascular function, manifesting minimal secondary deterioration until five to seven days post-fertilization. This provides an analytical advantage when compared with species which develop *in utero* and are completely dependent upon cardiovascular function, because it permits fine distinction among affected elements of the cardiovascular system at the earliest stages.

(b) *Cardiac dysfunction*

The heart of the embryonic zebrafish is directly visible through the transparent embryo. Deviations from normal contractility or morphology can be seen in real time in the intact living embryo under a microscope. Likewise, the presence of an effective circulation may also be directly assessed by observing the flow of erythrocytes pumped from the heart to the vessels of the head and trunk. The ease of scoring cardiac failure in zebrafish makes them ideal for screening for abnormalities of heart function.

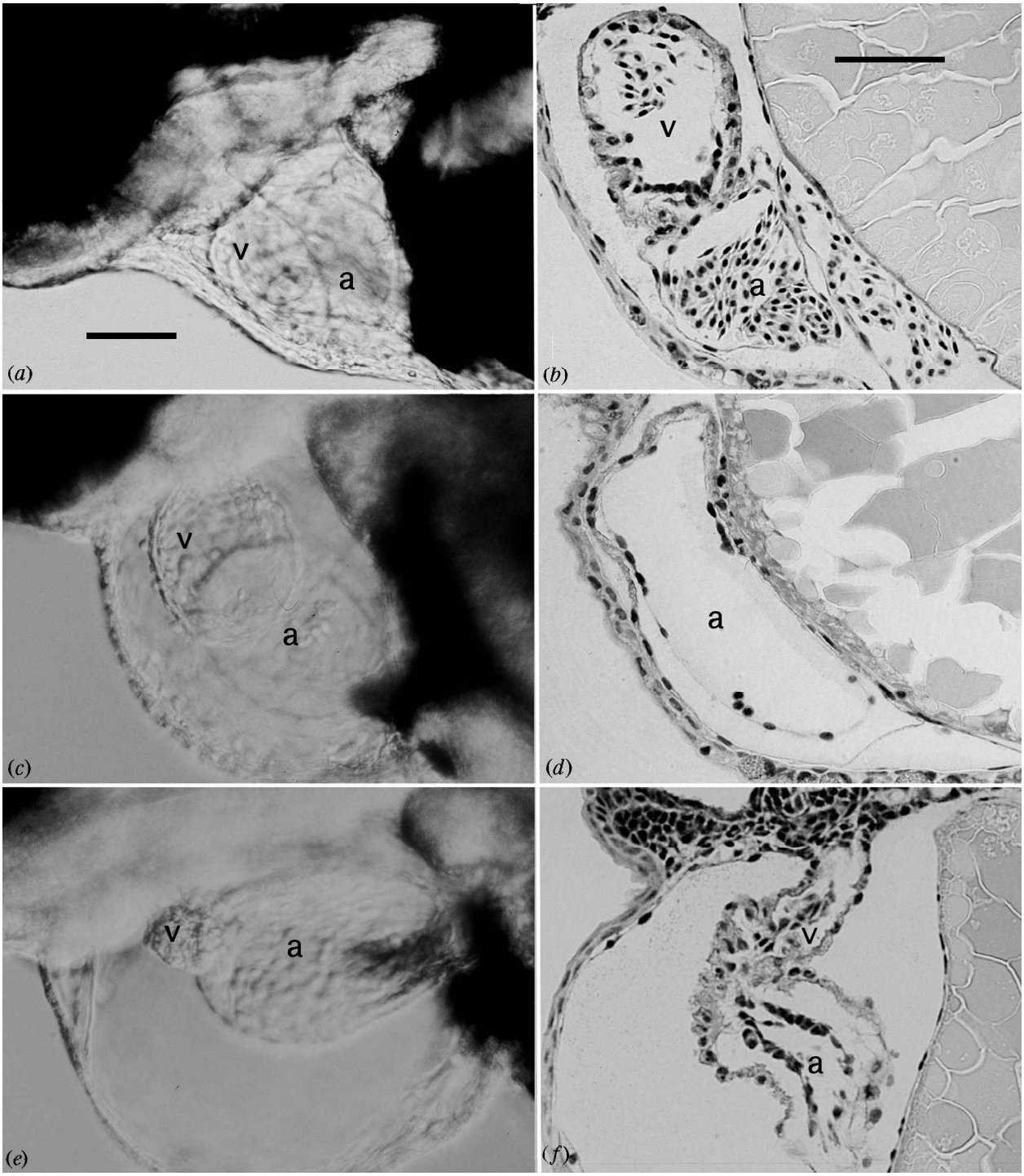

Figure 2. Zebrafish embryos at three days post-fertilization, as shown by Nomarski (*a*,*c*,*e*) and corresponding histological (*b*,*d*,*f*) images of the heart. (*a*) Wild-type embryo, with the ventricle in focus; (*b*) histology of a wild-type heart, showing normal atrial and ventricular tissue architecture; (*c*) dilated mutant, in which the atrium and, to a lesser degree, the ventricle are both hypokinetic and enlarged; (*d*) histology of dilated-type heart, showing the thin-walled, hypocellular and elongated atrium. Atrial enlargement precluded an adequate cross-section of the ventricle on the same section, but in other sections the ventricle appeared only mildly enlarged; (*e*) hypertrophic mutant, in which the atrium is relatively normal but the ventricle is small and compact, with luminal narrowing and near obstruction to blood flow; and (*f*) histology of hypertrophic-type heart, in which the ventricle displays thickened walls with mild cellular disorganization, cells with abundant cytoplasm, and increased cardiac jelly. a, atrium; v, ventricle. Scale bar, 50mm in *a*, *c*, *e* and 100 μm in *b*, *d*, *f*.

(c) *The screen*

Our first large-scale zebrafish screen for cardiovascular mutations was completed in 1994, and identified mutations in cardiovascular structure and function (Stanier *et al.* 1996). To increase the pool of genes defined as important in heart development and function, we undertook a second screen in 1997 using a similar scheme (J.-N. Chen & M. C. Fishman, unpublished data). G_0 generation males were mutagenized by N-ethyl N-nitrosourea, outbred to females of the TL strain for generation F_0. The progeny of $F_2 \times F_2$ crosses were subsequently screened

visually. Recessive mutations were revealed in clutches with 25% of the embryos exhibiting a visible phenotype. All heterozygous pairs were subsequently reconfirmed and mutations maintained by outcrossing.

Cardiac function in F_3 egg clutches was assessed on days 1, 2 and 3 post-fertilization. On the morning of day 1, we screened for the initiation of heart beat, propagation of contraction originating from the sinus venosus, and differences in heart rate. In the afternoon, embryos were scored for cardiac chamber contractility (location, vigour and extent of inward motion of the myocardium during systole), the velocity and direction of circulation, and the ability to clear blood returning to the heart. The day 2 screen continued assessment of contractility and circulation, and also specifically evaluated rhythmicity with respect to the rate, regularity, and conduction of impulses between the now-delineated chambers. Day 3 screening combined the previous days' checkpoints for maintenance of rhythm, contraction, and circulation. Mutants with general degeneration were excluded.

3. RESULTS

(a) *Mutants recovered*

A total of 39 mutants with defects in cardiac function were isolated and confirmed from the second screen, expanding on the 36 mutants (22 genes) from our first screen. Combined, these mutants were categorized as either contractility (59) or rhythm (16) mutants based on the most striking phenotypic abnormality. Embryos with mutations in the contractility category first manifested a defect in the force of myocardial contraction. In three cases the hearts displayed complete akinesis of one or both chambers, which could be due to a primary defect in either contractility or rhythm. Embryos designated as rhythm mutants displayed improper generation or conduction of a coordinated heartbeat, with consequent bradycardia, pausing or asynchronous contraction within or between chambers. Contractility disorders were often associated with arrhythmias, as occurs in human disease. Here we characterize the group of contractility mutants, which is particularly interesting in terms of its relevance to human cardiomyopathies.

(b) *Dilated versus hypertrophic heart mutants*

With the second screen, we more than doubled the total number of heart function mutants recovered (table 1). Combined, the 53 cardiac contractility mutants represent 59 lines, as two of the lines have multiple alleles (complementation analysis of the second screen has not been completed). They display a wide array of functional anomalies. Although they form a spectrum, they may be broadly segregated as either dilated or hypertrophic.

In the 23 dilated mutants, one or both chambers became enlarged in length, width or overall volume when compared with the hearts of normal wild-type siblings (figure 2*a*,*b*). Among different mutants of this category, myocardial function varies from hypokinetic to akinetic, affecting the chamber(s) either globally or focally, and the walls of the heart are thin with a paucity of cells. The layer between the endocardium and myocardium (extracellular matrix, which may represent the cardiac jelly seen in other vertebrates) appears variably increased or decreased, although such changes may simply be a reflection of overall oedema. Figure 2*c*,*d* show a Nomarski view and histology of a mutant in the dilated class, one in which predominantly the atrium is affected.

Table 1. *Classification of zebrafish heart function mutants obtained from the first and second genetic screens combined*

(The numbers in the first three columns represent the number of mutant lines in which atrium alone, ventricle alone or both chambers were affected by functional (i.e. contractility) and/or morphological abnormalities. The last two columns represent the number of mutations which affect initial heart function ($\leqslant 30$ hpf) or have a delayed visual phenotype (> 30 hpf).)

	chamber predominantly affected			onset of visual phenotype	
	atrium	ventricle	both	$\leqslant 30$ hpf	> 30 hpf
dilated	2	2	19	4	19
hypertrophic	0	19	11	7	23

In contrast, the affected heart chambers in the 30 mutants of the hypertrophic group are generally small and thick walled. The lumen of the affected chamber(s) are narrow and appear to leave little space for blood to flow through the heart. The layer between myocardium and endocardium is usually thicker in the affected chamber(s) of these mutants. An example of a hypertrophic heart mutant is shown in figure 2*e*,*f*.

(c) *Phenotypic variation within the two classes*

Within each general category there is marked variation in the location, extent and onset of cardiac abnormalities. In the dilated class, the degree of dysfunction varies from mild to severe, in the most striking cases eventuating in complete akinesis and a 'silent' heart phenotype. Within this class, two mutations affect the atrium, two affect the ventricle, but the vast majority (19) affect both (table 1). Wall motion abnormalities in several mutants selectively affect only focal regions of the chambers.

Within the hypertrophic class, there is variation in the degree of wall thickening (see example in figure 2*e*). This ranges from moderate local thickening at either the inflow or outflow regions of the ventricle, to global concentric thickening, to complete obstruction of the ventricular cavity. In particular, in two mutants the distal ventricle and/or bulbus arteriosus appears to be particularly susceptible to local obstruction. By histological examination, the areas of wall thickening probably correspond to increased density and/or mild disarray of cuboidal myocardial cells, juxtaposed with an increased extracellular matrix and/or oedematous layer (figure 2*f*). In certain mutants, focal outpouchings of myocardial cells develop, forming ventricular aneurysms. One mutant line displays a subdivided ventricle due to a focal constriction in its midportion. In general, cardiac contractility is also diminished in these mutants, although in some lines at early time-points, the atrium appears hyperdynamic, perhaps in an attempt to compensate for poor diastolic function.

Several abnormalities in cardiac function are common to both major categories of mutations. Marked regurgitant blood flow into the sinus venosus, atrium or ventricle

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occurs in most members of both the dilated and hypertrophic groups. This may be due to different causes. Dilated hearts are often accompanied by underdeveloped or incompetent valves. Dilation of the atrioventricular canal and ineffective contraction of the proximal heart chamber also contributes to regurgitation. In the hypertrophic group, reflux may be secondary to inability of the downstream chamber (the ventricle or outflow tract) to accept the volume of blood pumped by the proximal chamber. Rhythm disturbances, such as sinus bradycardia, sinus pauses, slow atrioventricular conduction or disorganized contraction within a chamber also occur in both classes. Other mutant-specific defects include the presence of red blood cells in the pericardial space (suggestive of a loss of endothelial or vascular integrity) and failure of cardiac looping. In some cases, non-cardiac muscle appears to be affected as well, as evidenced by a decreased swim response to touch. As the mutants' heart function deteriorates, over two to five days concomitant changes occur in the shape and size of the heart such that each mutant line exhibits a signature early-to-midstage morphology. However, at late stages, the final morphology of mutants from different classes often merges: embryos develop whole-body oedema (anasarca) and frequently elongated string-like hearts.

4. DISCUSSION

These zebrafish mutations perturb the onset of organotypic function. In all mutants, the heart tube forms initially without obvious defects in global structure. Because the zebrafish can survive for days without cardiovascular function, these mutations can be ascribed to defects in the heart. However, this does not imply cardiac autonomy. Heart cells encounter many tissues which regulate their cell fate, and mutations may well perturb such regulatory signals. Some mutations may primarily affect components of myofibrils. Others might affect signalling between endocardium and myocardium, as noted with targeted mutation of endocardial neuregulin or its receptors ErbB2 and ErbB4 (Meyer & Birchmeier 1995; Lee *et al*. 1995; Gassmann *et al*. 1995).

Many of the previously described cardiac mutations in zebrafish disrupt the form of the heart. Specifically, mutations in at least five genes (*bonnie and clyde*, *miles apart*, *casanova*, *faust* and *natter*) can block the fusion of the cardiac primordia into one tube (Stanier *et al*. 1996; Chen *et al*. 1996); mutations in *cloche* prevent formation of the endothelium (including endocardium) and blood (Stanier *et al*. 1995); ventricle formation requires *pandora* and *lonely atrium* (Stanier *et al*. 1996; Chen *et al*. 1996); and valve development is dependent upon *jekyll* (Stanier *et al*. 1996). Here we show that myocardial contraction appears to be particularly sensitive to genetic perturbation, implying a reliance upon non-redundant multiple critical pathways.

Relevance to human disease awaits cloning of the mutations. Elements of human disease phenotypes may be mirrored in zebrafish. This is especially true of congenital disease. For example, *sauternes* has a mutation in δ-aminolevulinate synthase, resulting in a defect in haem biosynthesis (as occurs in humans with congenital sideroblastic anaemia (Brownlie *et al*. 1998)). Porphyrias, diseases causing accumulation of erythrocyte protoporphyrins, photolysis and ensuing vascular and tissue damage in humans, are modelled by mutations in *yquem* (Wang *et al*. 1998) and *dracula* (Childs *et al*. 2000), which encode a uroporphyrinogen decarboxylase and ferrochelatase, respectively.

What relationship developmental processes bear to complex adult diseases is unknown, but it seems intuitive that abnormalities in growth and repair would have adverse consequences in the adult, especially if combined with other stressors, be they infectious or toxic. There is already a remarkable resemblance between the phenotypes of mutations described here and the spectrum of human disease classified as heart failure. The different dilated group of zebrafish mutants, like human dilated cardiomyopathies, may manifest either global or regional wall motion abnormalities and varying degrees of dilatation and thickness of the chambers. As in hypertrophic human hearts, the zebrafish mutants of the hypertrophic class have small ventricular cavities and areas of wall thickening which may vary from focal to eccentric to concentric. In some cases, as occurs in humans, there is outflow tract obstruction. The mutant ventricles appear compact, unyielding, and unable to fill during diastole. As in humans, other abnormalities, such as arrhythmias, valvular regurgitation and somatic muscle abnormalities can occur in isolation or associated with either class of mutant, and may be secondary to the underlying myopathy.

Interestingly, no autosomal recessive cases of hypertrophic cardiomyopathy have been reported in humans. A minority of human dilated cardiomyopathies are due to X-linked or mitochondrial mutations (estimated at 10 and 16%, respectively), and the rest have autosomal dominant or non-Mendelian undefined genetics (Mestroni *et al*. 1999).

Mutations of consequence similar to those zebrafish mutations described here would be lethal in humans at three weeks of gestation, a stage which is so early that embryonic death would go undetected. In humans or other mammals, detection of the principal underlying contractile defect would be beyond the realm of current technologies, and the window of embryonic survival so brief as to prevent discovery and careful assessment *ex vivo*.

These zebrafish mutations also may shed light on the phenotypic variability of heart failure. The adult heart has, at least as determined with current techniques, a limited repertoire of responses to stress, essentially defined as dilated or hypertrophic. The more fragile embryonic heart can evidently fail in many distinctive ways. Whether these reflect different pathways remains to be seen. However, it is conceivable that the shared phenotype of cardiac dysfunction reflects shared pathways, and heart failure may be parsed in this manner. Furthermore, like human cardiomyopathies, penetrance of failure varies with background and with regard to extent of cardiac dysfunction, dysplasia of heart chambers, age of onset and associated arrhythmias. The genetic and molecular factors which dictate these variations still need to be illuminated.

It is becoming increasingly recognized that there is often an underlying genetic predisposition to human cardiovascular disease, but relatively few responsible genes have been identified. We are currently mapping these zebrafish cardiovascular mutations in order to initiate their cloning. We presume that the corresponding

genes in human will bring candidate disease genes to light. Furthermore, we are assembling gene profile arrays to examine expression patterns from mutant subclasses which share phenotypes. Common and subclass-specific expression profiles may help to identify secondary pathways leading to or modifying disease.

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Nigel Holder, one of the organizers of this conference, was a man of exceptional integrity, courage, intelligence, and warmth, a good friend and outstanding scientist. We salute his memory, with sadness at his absence, but with gratitude for all the positive forces he set in motion.

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