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Drosophila Model of Congenital Heart Diseases

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1. Introduction

Congenital heart defects (CHD) are the most common birth defects, occurring in about 0.7% of all newborn infants. There are multiple lines of evidence that genetic components are involved in developing CHD pathogenesis. An important aspect in understanding disease mechanisms is that in addition to contributions from a single disease-causing gene (usually seen in many familial cases of CHD), a multitude of other genetically interacting loci can also influence the severity or progression of the disease, often diagnosed as idiopathic CHD. It is likely that such genetic interactions underlie a large proportion of cases of idiopathic CHD, where a direct link to known cardiogenic genes yet to be identified. Recent advances in stem cell research and in the growing field of systems biology provide a tremendous amount of new data leading to new hypotheses and to new heart disease gene candidates that may also have potential roles during heart formation and establishment of cardiac function. Usually, these hypotheses are tested in cell-based assays and eventually in the mouse model, however both systems have their own particular set of limitations. In this article we review recent advancements in using *Drosophila melanogaster* as a model organism to study basic mechanisms of heart development, cardiac function and disease.

2. Comparison between *Drosophila* and vertebrate cardiogenesis

The early development of the *Drosophila* heart shows remarkable similarities with its vertebrate counterparts, both morphologically and genetically (for review, see Bodmer, 1995; Bier and Bodmer, 2004). Our understanding of the regulation of cardiac development by a core cardiac transcription factor network (Venkatesh et al., 2000; Cripps and Olson, 2002; Olson, 2006; Bodmer and Frasch, 2010) began with the identification of the *Drosophila Nkx2.5* homologue *tinman* twenty years ago (Bodmer et al., 1990; Azpiazu and Frasch, 1993; Bodmer, 1993). One decade later, the completion of the sequencing of the *Drosophila*, mouse and human genomes has led to the identification of fly homologues of most cardiac transcription factors. The *Drosophila* model allowed the extensive genetic screening and functional analysis of Tinman (NKX2.5 Yin et al., 1997; Akasaka et al., 2006; Zaffran et al., 2006; Qian et al., 2011; Ryu et al., 2011), Hand (dHAND, eHAND, Han and Olson, 2005; Han

et al., 2006; Lo et al., 2007), tailup/isl-1 (Islet, Tao et al., 2007; Mann et al., 2009), Pannier (GATA4, Alvarez et al., 2003; Fromental-Ramain et al., 2008; Qian et al., 2008; Qian and Bodmer, 2009), Neuromancer-1/-2 (TBX20, Miskolczi-McCallum et al., 2005; Qian et al., 2005a; Reim et al., 2005; Leal et al., 2009), and Dorsocross-1/-2/-3 (TBX5, Reim and Frasch, 2005) and revealed a conserved cardiac transcription factor network responsible for heart specification (Olson, 2006). *Drosophila* and vertebrates also share the same inductive and instructive signaling pathways (Wnt, FGF, BMPs, for review see Frasch, 1999; Cripps and Olson, 2002) during early heart development. This further underscores that the *Drosophila* heart, despite its evolutionary distance from vertebrates, is specified by similar, fundamental mechanisms. This remarkable degree of genetic conservation is paralleled by morphological similarities during early development: the heart originates from a lateral portion in the early mesoderm, and two bilateral regions will eventually fuse and undergo lumen formation. Such genetic and morphological similarities across phyla have led to the conclusion that the cardiovascular system of the fly and vertebrates share true homologies (Hartenstein and Mandal, 2006). Lessons learned from *Drosophila* are likely to translate into a greater understanding of vertebrate heart development and function, and thus will help to understand the pathology and improve the treatment of cardiovascular diseases, as exemplified by Neely (2010) and Qian (2011).

3. Lessons learned from studying *Drosophila* heart morphogenesis

To gain new insights into the role of the cardiac transcriptional network, different groups have begun to analyze the mechanisms underlying heart morphogenesis and heart lumen formation during *Drosophila* embryonic development. After specification, cardiomyocyte precursors (called cardioblasts) migrate towards the dorsal midline of the embryo. These cells will extend filopodia towards their contralateral counterparts to establish a dorsal cell-cell contact. They then undergo cell shape changes, thereby bending around to form a second, ventral contact and enclosing a luminal space (see Figure 1 and Rugendorff et al., 1994). By the end of embryogenesis, these cells will have differentiated into a tubular dorsal vessel, providing the circulation of hemolymph during larval and adult stages. The mechanism by which this migratory behavior is orchestrated is still poorly understood, but recent studies have established a framework of genes involved in heart morphogenesis. An important participating signal transduction pathway is the Slit/Robo pathway, which was originally identified and characterized for its role in axon guidance and in regulation of midline crossing of growing neurons (Dickson and Gilestro, 2006). Slit, an EGF-like ligand, and the Slit-receptor Roundabout (Robo) are both expressed by cardioblasts during morphogenesis and lumen formation, and ChIP data suggest that cardiac genes, such as *tinman* (Liu et al., 2009), directly regulate their expression. Mutants for *slit* or *robo* together with its paralogue *robo2* have distinct defects during these processes (Qian et al., 2005b; MacMullin and Jacobs, 2006; Santiago-Martínez et al., 2006; Medioni et al., 2008; Santiago-Martínez et al., 2008): impaired cardioblasts cell-cell adhesion, which disrupts subsequent heart morphogenesis, and impairment of cell shape changes and lumen formation. In *slit* mutants, polar (or polarly distributed) markers, including the *Drosophila* MAGUK protein Discs-large, are incorrectly localized indicating a loss of overall cardioblast polarity (Qian et al., 2005b). In addition, the cardioblasts fail to correctly change their cell shape in order to enclose a heart lumen (Medioni et al., 2008). This is accompanied by upregulation of the cell adhesion molecule Shotgun/E-Cadherin at the presumptive luminal domain, leading to increased adhesion at the luminal surfaces, which in

turn is likely to prevent further lumen formation (Santiago-Martínez et al., 2008). Both, Slit and Robo are expressed during mouse heart development and expression of Slit3 and Robo2 depend on Tbx20 and Nkx2-5, respectively (Medioni et al., 2010). Functional analysis in zebrafish done by Fish et al. (2011) indicates a role for Slit/Robo signaling during zebrafish heart development. Slit/robo mutant fish hearts show a number of developmental defects, indicating a conserved requirement for this pathway during vertebrate cardiogenesis and thus a role in CHD. Again, the analysis of Slit/Robo in *Drosophila* has paved the way for the subsequent experiments done in vertebrates.

Recent work on fly heart development suggests several possible future research directions. Firstly, genetic screens in *Drosophila* should reveal additional genes involved in cell-cell signaling during development. Among them is the Netrin/Unc-5 pathway that, similar to Slit/Robo, was found to be involved in axon guidance and cell migration. In the heart, the UNC-5 ligand Netrin is also required for heart lumen formation (Albrecht et al., 2011), although with a lesser penetrance than Slit/Robo. This indicates that in fact multiple pathways are required during the formation of the heart, and it would be interesting to see if these two pathways genetically interact, which would indicate a potential cross talk between them. The *Drosophila* model therefore helps pinpoint which pathways may also interact in CHD in humans. Secondly, a more complete and detailed understanding of the signaling pathways themselves during heart development and establishment of cardiac function will be essential for understanding CHD initiation and progression. For example, co-receptors might play an important role in defining pathway sensitivity and downstream activity. In *Drosophila*, cardiac Slit/Robo signaling has been shown to require the activity of the heparan sulfate proteoglycan Syndecan (Knox et al., 2011). Since Syndecan is involved in angiogenesis (through VEGF, Chen et al., 2004) and is also upregulated during cardiac remodeling after myocardial infarction, the *Drosophila* model might help identify important components of Syndecan signaling in these disease-relevant contexts. Thirdly, the cellular machineries through which signaling pathways exert their specific function are largely unknown. Thus, we currently have no clear understanding of the intracellular mechanisms that give rise to the *slit* mutant phenotype.

4. Manipulating the heart and genome of a fly

Parallel pathways and downstream signaling cascades are thought to intersect with a number of cellular effector proteins, such as small GTPases, which in turn may influence cell migration (e.g. changes in the filopodia or lamellipodia dynamics), cell adhesion (e.g. changes in endocytosis of E-Cadherin) or cell contractions (e.g. via Rho-associated kinase activities). The activity of these genes has been studied in great detail in cell-based assays, but experimental evidence on their *in vivo* function is relatively sparse. The lack of available mutants in vertebrate model organisms often prevents such analysis, as has the shortage of tools for tissue-specific manipulations and imaging of single cells in whole animals. Therefore, very few examples of the function these proteins in the context of an entire organ or organ system exist to date (e.g. RhoDF, see Christiaen et al., 2008). In *Drosophila*, small GTPases involved in the above cellular processes have been studied by genetic manipulations during the formation of tissues other than the heart, e.g. during dorsal closure (Jacinto et al., 2002) or wound healing (Stramer et al., 2005). The embryonic *Drosophila* heart is well suited to similar experiments since it is localized just underneath the transparent cell layer of the dorsal epidermis, which allows capture of high quality

fluorescent images *in vivo* (see Figure 1B). At the same time, cardiac cells can easily be manipulated using cardiac-specific Gal4-driver lines (see below) to express GFP-tagged genes, e.g. of the actin cytoskeleton, specifically in the heart. This allows the effects of specific mutations on actin dynamics to be monitored during heart formation (Medioni et al., 2008). Furthermore, a large number of fluorescently labeled genes such as actin^{GFP} that can be overexpressed are readily available from different laboratories and stock centers. Browsing through Flybase (Crosby et al., 2007; Tweedie et al., 2009), a *Drosophila* centered database, allows easy access to the records for any published construct. The power of the *Drosophila* model is the ability to combine mutant alleles of almost any gene with tissue-specific expression of fluorescently labeled markers. Thus, heart development can be studied in great detail at the organ or even the cellular level, permitting the role of individual genes to be examined in the context of a specific cellular function. This approach provides an experimental resolution that is unparalleled in any other model organism.

The technological advances in the *Drosophila* model are steadily growing. The Gal4/UAS system (Brand and Perrimon, 1993) had been groundbreaking for tissue-specific genetic manipulations, and continues to be further refined (Osterwalder et al., 2001; McGuire et al., 2004; Pfeiffer et al., 2010; Gohl et al., 2011). Gal4, a transcriptional activator from yeast and without endogenous binding sites in the *Drosophila* genome, is used to trans-activate genes that are engineered to contain Gal4-binding sites (upstream activating sequences, UAS). This heterologous system allows the expression of any UAS-fused gene in any tissue where Gal4 is expressed, which itself is driven by tissue-specific promoters. Gal4 and UAS-lines can be created either by random insertion of transposable elements into the fly's genome (Cooley et al., 1988) or targeted insertion at particular "landing sites" (Fish et al., 2007). The first method has been extensively used to create a vast amount of "enhancer trap" lines that express the Gal4 driver in many different, tissue-specific patterns including the heart. A recent technique by Gohl et al. (2011) has further increased the versatility of such Gal4-enhancer trap lines by developing a method to replace the Gal4 driver with any other reporter (e.g. GFP) or effector gene (e.g. the Gal4 repressor Gal80, which will inhibit the Gal4 activity of a different line in the intersecting cells). The Gal4/UAS system not only allows selective expression of marker genes in specific tissues (e.g. Figure 1B, expression in cardiac tissue using tinCΔ4-Gal4, Lo and Frasch, 2001), but also permits genetic manipulations by ectopic or overexpression of genes or by reducing their expression levels using RNA interference (RNAi, see below). In combination with lines that express the Gal80 repressor in a subset of Gal4-positive cells, the Gal4 expression pattern can be further spatially refined. In addition, use of a temperature-sensitive version of Gal80 (Gal80-TS, McGuire et al., 2004) gives temporal control over Gal4 expression, which then becomes active only under the permissive temperature.

One limitation of the Gal4/UAS system is that all transgenes that carry UAS sites respond at the same time. Therefore, different tissues or cells cannot be manipulated individually, although this could be a useful approach to study their interaction. The recent invention of the Q system (Potter et al., 2010) is a novel approach to circumvent this limitation. It works in a similar manner as Gal4/UAS but uses the *Neurospora* transcriptional activator QF, which recognizes its own specific binding sequence (QUAS). Just like Gal4, a fly line that expresses QF in a certain tissue or cell will drive expression of a gene that contains the QUAS binding sites. Similar to Gal80, the activity of QF can be suppressed by expression of QS (allowing further refinement of QF expression), and feeding flies quinic acid releases this suppression in a dose-dependent manner. Thus, QS gives both, spatial and temporal control

over the activity of QF, just like Gal80 and Gal80^{TS} for Gal4. A combination of both the Gal4 and QF systems therefore would allow distinct expression of multiple transgenes in a precise tissue-specific and temporal-specific manner in an otherwise unchanged genetic background.

Recent advances in RNA interference (RNAi) technology, combined with the spatio-temporal control of the Gal4/UAS system, have allowed tissue-specific studies of gene function during almost any developmental stage. RNAi has therefore been useful to analyze genes that when mutated would cause early lethality or pleiotropic effects, but it is also the only method currently available to study gene function when no mutant alleles for a particular gene are available.

Systematic analysis to determine optimal hairpin formation and careful analysis of insertion sites have greatly increased the efficacy of RNAi (Ni et al., 2008; 2009; 2011). The Transgenic RNAi project (TRiP) is currently generating these optimized RNAi lines for all *Drosophila* genes, which complements other RNAi resources (like VDRC, Dietzl et al., 2007). As an alternative reverse genetic approach, the directed mutation or knockout of a particular gene of interest by homologous recombination (reviewed in Maggert et al., 2008) has also been developed further. In addition to generating a knockout allele, Huang et al. (2009) have added a recombinase-based feature that allows modification of the deleted locus by inserting virtually any sequence ("genomic engineering"). Of note, this permits modification of gene function in an otherwise unaltered genetic background. Furthermore, efforts to create genomic duplications for regions of the X chromosome have resulted in the creation of two independent sets of fly lines, one set with a duplication located on the Y chromosome (Cook et al., 2010) and one set on the 3rd chromosome (Venken et al., 2010). This will facilitate the recovery and identification of X-linked mutations and also allow assessment of the fly's susceptibility to increased gene dosages. These improved techniques of Gal4/UAS transactivation, paired with the expression of fluorescently tagged reporters and RNAi lines as well novel forward and reverse genetic techniques and resources are likely to unravel new, previously unnoticed gene functions in different tissues and under different developmental contexts.

5. Exploring fly heart function to understand CHD-related cardiomyopathies

By definition, congenital heart disease refers to the presence of structural heart and large vessel defects at the time of birth. Many of these can be repaired by surgical intervention, but depending on the severity of the defect, patients may require life-long medical follow-ups to monitor cardiac performance and to detect signs of functional decline. Furthermore, late onset complications either due to persistent impact of structural defects or related to the applied intervention are often linked to lethal arrhythmias or progressive congestive heart failure. Such secondary complications might not be caused directly by CHD genes, but due to a maladaptive response of the cardiac tissue. In the light of these considerations it is therefore necessary to understand how cardiac tissues respond to these interventions and which other genes might contribute to arrhythmias and cardiomyopathies. The *Drosophila* model has helped to identify a number of novel genes and pathways required to maintain myofibrillar organization and overall heart structure and function. Because similarities between the *Drosophila* and murine heart are found both at the molecular and functional level it is therefore likely that new risk factors of cardiac disease can be identified using *Drosophila*. The cardiac proteasomes of the mouse and the fly have been shown to be

comparable with respect to their overall composition (Cammarato et al., 2011), and functional analysis revealed further evidence of the conserved cardiac characteristics of the fly heart (e.g. Ocorr et al., 2007a; Buechling et al., 2009; Choma et al., 2010). In particular, the contractile and electrical properties have been investigated in the *Drosophila* heart and found to be remarkably similar in fundamental aspects, such as the contribution of ion channels to heart contraction or the effects of mutations of genes of the myofibrillar apparatus (K. Ocorr, unpublished and Lalevée et al., 2006; Wolf et al., 2006; Ocorr et al., 2007b; Cammarato et al., 2008b; Mery et al., 2008). In addition to determining cardiac parameters in vivo and in situ (see below and Fig. 2), electrical properties can be measured using the fluorescent Ca^{2+}-sensor GCaMP (Nakai et al., 2001), where Ca^{2+}-transients are monitored *in vivo* (Lin et al., 2011).

These techniques have set the stage for using the *Drosophila* model to identify new cardiac genes involved in CHD and cardiac disease. In a recent screen for genes affecting *Drosophila* heart function under stress conditions, components of the CCR4/NOT complex, which is a regulator of gene transcription and mRNA degradation, have been shown to play a pivotal role in maintaining heart function in flies, but also in mice and possibly humans (Neely et al., 2010). In this particular study, hearts in mutant flies were functionally analyzed as semi-intact preparations (using the methods described in Ocorr et al., 2007b; 2009; Vogler and Ocorr, 2009) and subsequently analyzed for structural defects (as described in Alayari et al., 2009; Fink et al., 2009b). These methods allow the assessment of numerous fly heart parameters (such as diastolic and systolic diameters and intervals, intrinsic heart rate of the denervated heart and estimates on the degree of arrhythmias; see Fig. 2). For CCR4/NOT complex mutants, it was shown that these flies have hallmarks of dilated cardiomyopathy. Remarkably, *not3*$^{+/-}$ heterozygous mice are haploinsufficient and exhibit less resistance to cardiac stress, indicating that this pathway is required for maintaining proper heart function. Administration of HDAC inhibitors ameliorates these phenotypes, indicating that changes in chromatin remodeling are likely to play a major role. Lastly, the authors showed that a single-nucleotide polymorphism (SNP) near the human NOT3 genes is associated with prolonged long QT intervals. Together, these data show that the cardiac role of the CCR4/NOT complex is highly conserved, and that *Drosophila* indeed is useful for identifying novel genes and pathways involved in cardiac disease. Due to the broad role of the CCR4/NOT complex in regulating both, gene transcription and posttranslational modification, it remains unclear by which mechanisms and genes the cardiac phenotypes become manifest. Further analysis of the CCR4/NOT complex is therefore required, and the fly heart is likely to provide further insights. Importantly, this approach showed that RNAi-mediated genetic screening is a promising approach to identify new cardiac risk genes in humans.

In *Drosophila*, as well as in higher organisms, transheterozygous mutations can unravel a hidden link between two genes (genetic interaction). *Drosophila* is well suited for such experimental screening approaches since most genes are not duplicated, thus interactions are less likely to be masked by compensatory mechanisms. In a recent study, this particular strength of the *Drosophila* model has been successfully exploited to identify a novel link between the cardiac transcription factor *Nkx2.5/tinman* and the small GTPase *Cdc42* (Qian et al., 2011). In the aforementioned study, flies that were double heterozygous for *Cdc42* and *tinman* showed altered cardiac function and also showed structural defects, something not observed in the single heterozygous animals. The subsequent analysis of double

heterozygous *Cdc42;Nkx2.5* mouse hearts also revealed an impaired heart function when compared to single heterozygous animals, again indicating that this genetic link between *Cdc42* and *tinman/Nkx2*.5 is conserved. Such complex *in vivo* screens to unravel genetic interactions in higher eukaryotes are currently only feasible in the fly model organism. With respect to understanding the genetics of heart development and disease, *Drosophila* is the simplest genetic model with a heart (Bier and Bodmer, 2004). Moreover, from a systems biology point of view, the fly is a perfect model organism to rapidly test genetic interactions that are predicted from networks based on genetic information, bioinformatics and the integration of other data obtained from many different model organisms and patients (for the fly, such data can be accessed through DroID, the *Drosophila* interactions database, see Yu et al., 2008; Murali et al., 2010).

6. A genetic model for heart diseases

The knowledge of molecular mechanisms underlying important biological processes gained from *Drosophila* has been successfully extended to studies of human diseases especially in the field of neural degenerative diseases (Bilen and Bonini, 2005; Marsh and Thompson, 2006). Recent studies in flies have been directed towards understanding more complex and multifactorial diseases such as heart disease. In this section we specifically demonstrate how *Drosophila* can be used as a model to elucidate the molecular mechanisms of CHD and cardiomyopathies. As we mentioned above, both anatomical and molecular features of *Drosophila* heart development (as outlined in Figure 1) and aspects of adult structure and function (see Figure 2) are similar to those observed in the human heart, making *Drosophila* a useful model system with the advantage of a much simpler genetic and tissue organization.

Primary cardiomyopathies are contractile disorders of the myocardium. The majority of cases of cardiomyopathy are classified into two disease types: hypertrophic cardiomyopathy (HCM) and dilated cardiomyopathy (DCM). HCM, defined as a hypertrophic ventricle with myofibrillar disarrays, can sometimes lead to sudden death in young subjects, but many cases of HCM maintain stable hemodynamics until late stages. DCM, defined as dilated ventricle with systolic dysfunction, also shows myofibrillar disarrays and clinically exhibits refractory arrhythmias and severe heart failure. In both disease types genetic causes have been found. The first case of a male-sibling DCM with X-linked inheritance had a mutation in *dystrophin* (*dys*), a gene that plays an important role in the anchoring of muscle cells (Towbin et al., 1993). Mutations in *dys* are a cause of Duchenne-type (null function of *dys*) and Becker-type (hypomorphic function of *dys*) muscular dystrophy, whose clinical entity is characterized by progressive muscle weakness and degeneration of muscle fibers (Koenig et al., 1988). In both types late-onset cardiac dysfunction is frequently observed, and improvement of heart function is an important therapeutic target for improving life prognosis. As in humans, fly Dys is associated with the plasma membrane at the sarcomeric Z-line and is already present during early embryogenesis of the *Drosophila* heart (Taghli-Lamallem et al., 2008). In *dys* deficiency flies the myofibrillar structure of the heart cells is disorganized in that myofibrils are not tightly packed and appear sparse. This phenotype worsens with age, consistent with the late onset of cardiac dysfunction in muscular dystrophy patients. Real-time imaging of heart movements using high-speed digital video

recording system allows a detailed analysis of the heart's performance and pathology, with quantitative measurements of heart period, rhythmicity, size, and fractional shortening (an index of contractility, Akasaka and Ocorr, 2009; Fink et al., 2009a). Using this methodology, it was observed that *dys* mutants exhibited a significantly wider diastolic (80-90μm) and systolic (60μm) diameter compared to laboratory wild-type strains (diastolic diameter 60μM, systolic diameter 40μm), suggesting that the *dys* mutant produces a dilated, cardiomegaly-type phenotype. In addition, fractional shortening in the mutants is reduced to 25-30% (compared to 35-40% in wild type). Those features are reminiscent of DCM in humans and a Duchenne-type mouse model (*mdx* mouse) (Quinlan et al., 2004; Wehling-Henricks et al., 2005). Interestingly, a short C-terminal form of human *dys* (Dp116, Judge et al., 2006) rescued the DCM phenotype of *dys* mutant flies (Taghli-Lamallem et al., 2008), but this micro-*dys* could not improve skeletal muscle function in the *mdx* mice model. Because this isoform is incorporated into the dystrophin glycoprotein complex (DGC) but is not capable of binding to the actin skeleton, successful DGC formation may be a critical characteristic. Failure to form this complex may then lead to the observed pathogenesis in the heart, which potentially may be due to dysfunction in force transmission and/or impairment of the signal transduction through DGC. This study is just one example of the use of *Drosophila* as a model for comprehensive human cardiac disease, and which may also allow testing the potential of therapeutic strategies such as the introduction of micro-*dys* to the heart.

Molecular and genetic examinations of cardiomyopathy populations have produced data indicating that mutations in sarcomere-related proteins are involved in the cardiomyopathy phenotype (Hershberger and Siegfried, 2011; Seidman and Seidman, 2011). Myosin is a molecular motor composed of two myosin heavy chains (MHC) and four light chains. This hexameric myosin is a major component of the thick filament and allows them to slide along the thin actin filaments in an ATP-dependent manner. Two mutant alleles of myosin, D45 (A261T) and Mhc^5 (G200D), have missense mutations occurring close to the ATP catalytic site, and it was postulated that those amino acid substitutions would affect ATPase activity (Kronert et al., 1999). In fact, ATPase activities of both D45 and Mhc^5 mutant myosin were depressed compared to wild-type myosin; however, *in vivo* motility of F-actin on a myosin coated slide showed a reduced velocity for D45 myosin to almost half of that of wild-type and an increased velocity in Mhc^5 myosin to about 115% of wild-type (Cammarato et al., 2008a). Interestingly, these myosin mutants showed different pathologies in the heart. Compared to wild-type, D45 mutant hearts are dilated exhibiting an increased systolic and diastolic diameter, whereas Mhc^5 mutants appear restricted showing a decreased diameter only during diastolic phase (Cammarato et al., 2008a). The depressed motor function and dilation in D45 myosin is evocative of DCM in humans, whereas the increased motor function and reduced diastolic function in Mhc^5 is similar to human restricted cardiomyopathy (RCM, Cammarato et al., 2008a), a rare type of cardiomyopathy in which decreased myocardium elasticity affects the ventricular blood filling during the diastolic phase. Unlike the fly D45 and Mhc^5 pathogenesis, biochemical and structural investigations in vertebrates are not always able to reveal how mutations contribute to cardiac pathologies. Instead, the role of a particular gene is primarily obtained from the phenotype and/or symptoms of a patient carrying a mutation in this gene. However, even this clinical approach requires costly and labor-intensive efforts in order to first identify these patients. In addition, clinical studies often require supplemental tests, which are sometimes difficult

to perform. Reverse genetics may be able to compensate for these disadvantages of clinical studies, and especially in the fly system the availability of genetic tools for the entire genome is very useful for a systematic approach to test gene functions. For example in the fly, the *MHC* is encoded by a single gene, thus the analysis of specific mutations in this gene can inform us how alterations in myosin structure directly contribute to alterations in function and the pathophysiological consequences.

Both, dystrophin and myosin-related cardiomyopathies are caused by a dysfunction from within cardiomyocytes, and therefore are not necessarily linked to defects in heart formation, which are the basis of CHD. But can the fly model be used to investigate CHD, even though it lacks higher-order structures, such as looping, septation, and chamber formation? There are many cases of CHD where cardiomyocyte function is still far from normal, regardless of the success of corrective surgical procedures. This suggests that those cases could have primary defects within cardiomyocytes in addition to the overall morphological defects in the heart's architecture. Investigations of these questions may also benefit from the cardiomyopathy models in *Drosophila* mentioned above. Taking advantage of the *Drosophila* model we recently performed a study using data obtained from a patient with hypoplastic left heart syndrome (HLHS). HLHS is the most severe type of left-sided heart defect, and occurs in 2-4% of all infants born with congenital heart disease (Loffredo, 2000). We found that this patient had a balanced chromosomal translocation whose breakpoint is in close proximity to a member of the kinesin family (Akasaka, Grosfeld, et al., unpubl.). Heart-specific over expression of kinesin in the fly model disrupts the contractile muscle structure and reduces the quantity myofibrils. Those phenotypes resemble what is observed in micrographs of heart tissue from HLHS patients; cardiomyocytes with scant cytoplasm and myofibrillar disarray (Bohlmeyer et al., 2003). Therefore, despite the differences in the fly heart's gross anatomy this system can provide insights into CHD pathogenesis and this information can be applied to the development of both preventive and therapeutic strategies in the future.

7. Conclusions

To understand the complex etiology and genetics of congenital heart disease, synergistic efforts from all fields of medical and biological sciences are required. For many decades, the invertebrate model organism *Drosophila* has provided exciting new insights into the genetics, development and function of multi-cellular organisms. In this review, we have highlighted some of the recent advances and findings gained from a *Drosophila* model for CHD. Despite its evolutionary distance from vertebrates there is a remarkable conservation of genetics and function. The development of technologies such as time-lapse analysis of heart formation and optical techniques to study function suggest that further studies using this system will provide insights into fundamental cellular mechanisms underlying heart function and disease. The fly has been shown to be a useful model that is able to complement the shortcomings of other model systems. Its simpler genetic architecture allows researchers to dissect the basic networks involved in organ formation and by extension to gain insights into the genetics underlying CHD and cardiac diseases in the same way that the *Drosophila* model has advanced our understanding of human genetics and embryonic development.

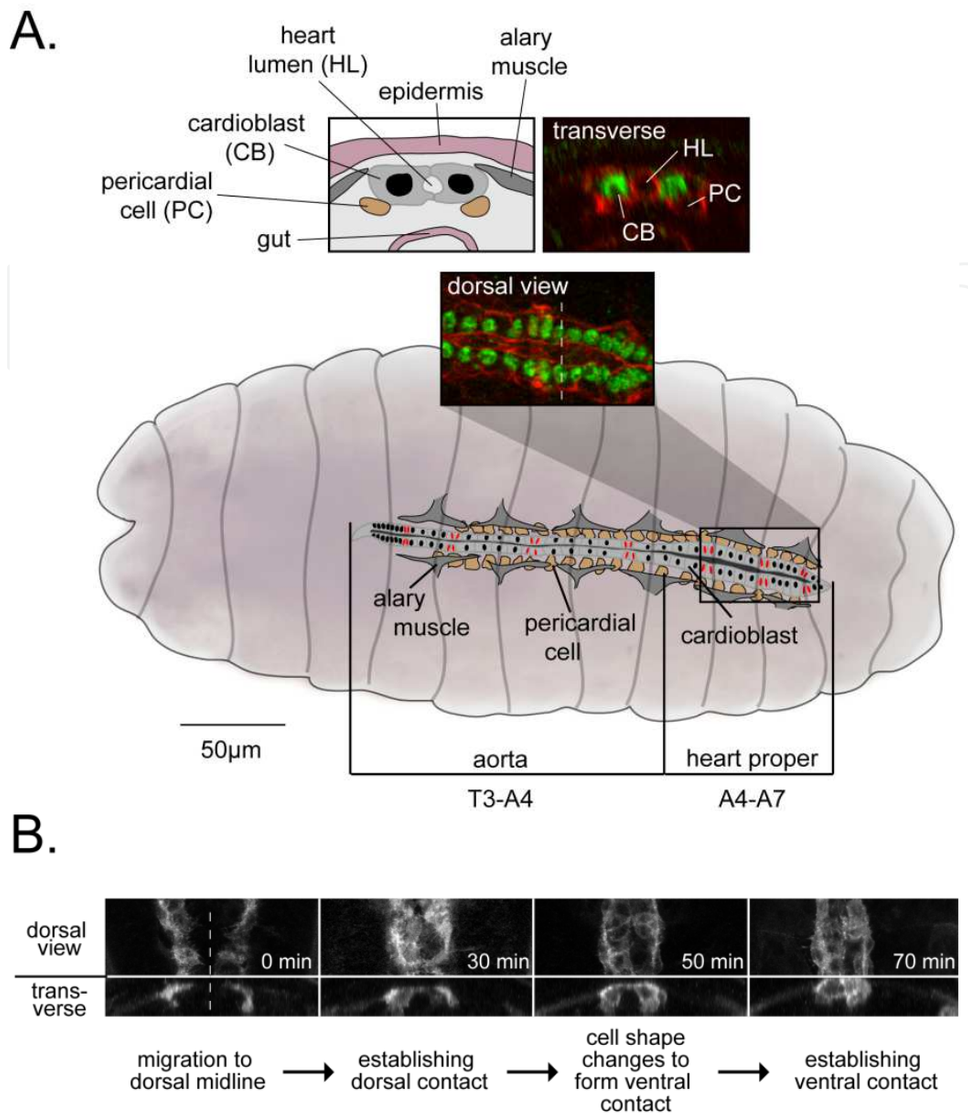

Fig. 1. **A.** Morphology of the late embryonic heart of *Drosophila*. After 17 hours of development, the cardiac precursor cells have completed migration and heart assembly. The heart is located underneath the epidermis along the dorsal midline. It consists of two morphologically different portions, the anterior aorta (spanning segments T3-A4) and the posterior heart proper (segment A4-A7), which is characterized by a much wider lumen. The two major cell types are cardioblasts (CBs), which will differentiate into cardiomyocytes, and pericardial cells (PCs), which will become nephrocyte-like cardiac support cells. The heart is also connected to specialized lateral body wall muscles, named alary muscles. The cardioblast nuclei can be specifically labeled (e.g. by anti-Nmr1 antibody, green) to assess CB alignment. Cell surfaces are stained using Dystroglycan antibody (red). The aorta and heart contain a central (HL). **B.** Heart assembly, visualized by a time-lapse movie of cardiac cells expressing actin^{GFP}. Before alignment, two lateral rows of CBs and PCs migrate towards the dorsal midline (indicated as hatched line). The CBs elongate at the dorsal side and extend filopodia towards the contralateral side to form the dorsal contact. Following this contact, the cells change in shape to contact ventrally, thereby enclosing a luminal space.

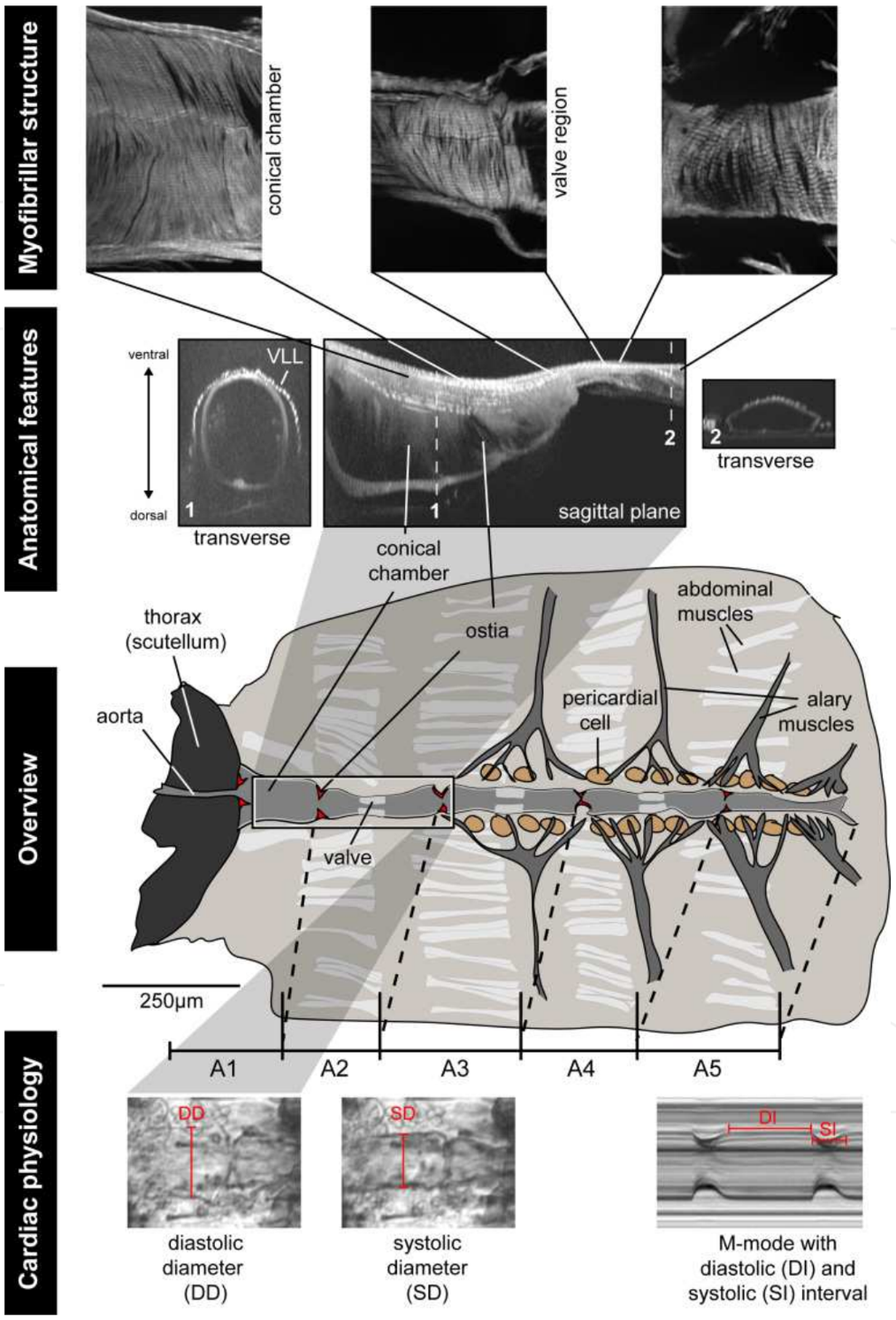

Fig. 2. Morphological features of the adult *Drosophila* heart and determination of structural and functional parameters for phenotypic analysis. Overview: The adult *Drosophila* heart is a contractile tube located at the dorsal midline of the abdomen. Along the heart, specialized cells and structures can be identified: 5 pairs of inflow valves (ostia, red) and 3 pairs of valves inside

the tube. The anterior portion of the heart shows a prominent specialization (the conical chamber, which is larger in size) and anastomoses into the aorta that runs from the posterior end of the thorax into the head capsule. Several pairs of alary muscles are attached to the heart tube, which are likely to help to maintain heart position. The pericardial cells are found alongside the heart and have a nephrocyte-like as well as other cardiac support functions. The ventral longitudinal layer consists of several multi-nucleated muscle cells that ensheath the heart from A1 to about mid-A5 (indicated as VLL in transverse section of the conical chamber). Anatomical features: The conical chamber is the largest chamber of the adult fly heart (compare transverse sections 1+2). The VLL that ventrally and laterally covers the heart can be seen in transverse section 1. Myofibrillar structure: The cardiomyocytes of the conical chamber are much larger in size and have a higher acto-myosin content compared to other regions of the heart. The cardiomyocytes of the valves show a very dense packaging of myofibrils compared to regular cardiomyocytes of the heart. Cardiac physiology: High-speed image capturing from semi-dissected fly hearts allows determination of several parameters, which are indicative for fly heart morphology and function: the diameters of the heart during diastole (DD) and systole (SD) are determined from the original movies. M-modes are generated by aligning a 1-pixel wide strip from each frame showing the location of the heart walls (Y axis) over time (X-axis) (Ocorr et al., 2007). Heart rate, durations of diastoles and systoles (DI and SI) and rhythmicity are determined by semi-automated analysis using MATlab-based software (Fink et al. 2009).

8. Acknowledgment

We are grateful to Drs. Karen Ocorr and Paul D. Grossfeld for helpful discussion and critical reading of the manuscript. This work is supported by grants from AHA to G.V., from NIH (NHLBI, NIA and NIDDK) and The Ellison Medical Foundation to R.B., and from Sanford Children's Hospital Research Center to T.A.

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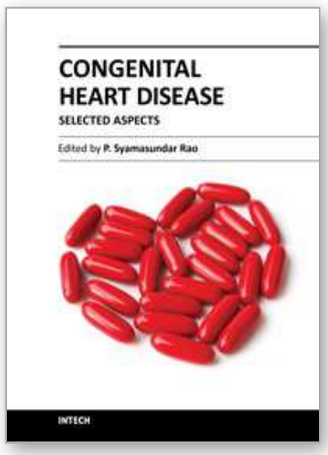

Congenital Heart Disease - Selected Aspects

Edited by Prof. P. Syamasundar Rao

ISBN 978-953-307-472-6
Hard cover, 348 pages
Publisher InTech
Published online 18, January, 2012
Published in print edition January, 2012

There are significant advances in the understanding of the molecular mechanisms of cardiac development and the etiology of congenital heart disease (CHD). However, these have not yet evolved to such a degree so as to be useful in preventing CHD at this time. Developments such as early detection of the neonates with serious heart disease and their rapid transport to tertiary care centers, availability of highly sensitive noninvasive diagnostic tools, advances in neonatal care and anesthesia, progress in transcatheter interventional procedures and extension of complicated surgical procedures to the neonate and infant have advanced to such a degree that almost all congenital cardiac defects can be diagnosed and "corrected". Treatment of the majority of acyanotic and simpler cyanotic heart defects with currently available transcatheter and surgical techniques is feasible, effective and safe. The application of staged total cavo-pulmonary connection (Fontan) has markedly improved the long-term outlook of children who have one functioning ventricle. This book, I hope, will serve as a rich source of information to the physician caring for infants, children and adults with CHD which may help them provide optimal care for their patients.

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