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Early Cardiac Development in Vertebrates and Invertebrates: Transcription Factors and Signaling Processes

STÉPHANE ZAFFRAN*
Département de Biologie du Développement, Institut Pasteur, CNRS URA 1947, 25 rue du Dr. Roux, Paris, 75015 France

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The heart is one of the earliest organs to form during embryogenesis and its circulatory function is critical from early stages for the viability of the mammalian embryo. In mammals, the morphological events of heart formation are sensitive to molecular perturbation, leading to congenital heart defects. Hence, it is essential to understand the molecular pathways that control the patterning and morphogenesis of this organ. Due to the evolutionary conservation of many of these processes, major insights have been obtained from the study of a number of animal models, both vertebrate and invertebrate. The fruitfly, *Drosophila,* being highly responsive to genetic dissection, has proved to be a useful tool in the identification of key genes and signaling molecules involved in heart formation that appear to be conserved in vertebrates. More recently, information has emerged about inductive signals that are crucial for heart development, from *in vivo* and *in vitro* studies in chick and *Xenopus*. In this article, I review the molecular and developmental functions of signaling processes during early heart formation that have been defined in both vertebrate and invertebrate models.

Key Words: Heart, Cardiogenesis, *Drosophila*, Vertebrates, Dorsal vessel, Review, Transcription factor, Inductive signals

Introduction

Heart Morphogenesis in Vertebrate and Drosophila Embryos

Heart formation in vertebrates occurs through a complex process, requiring a precisely coordinated series of molecular and morphogenetic events and ending with the formation of a complex organ. In mammals and birds, cardiac precursors arises from the anterior ventral part of the embryonic lateral mesoderm and assemble in a crescent-shaped region called the heart field (DeHaan & Urspurng 1965) (figure 1). More recent studies have identified a second heart field that is located more medially in the splanchnic mesoderm, directly adjacent to the cardiac crescent (reviewed in Kelly & Buckingham 2002). Cells from this bilateral field, termed the anterior or secondary heart-forming field, are fated to generate anterior heart structures of the ventricular outflow tracts (figure 1B,C). Results from explant culture experiments in quail and chick embryos indicate that the specification of cardiomyocytes occurs just prior to the formation of the cardiac crescent (Montgomery et al. 1994, Yatskievych et al. 1997). Soon after their specification, cardiac precursor cells proliferate and converge along the ventral midline of the embryo to form a tubular heart composed of distinct myocardial and endocardial layers (figure 1D). The linear heart tube is organized along the antero-posterior axis to form (from anterior to posterior) the aortic sac, outflow tract (conotruncus), right ventricle, left ventricle, atria and inflow tract of the embryonic heart. In all vertebrates, the tubular heart undergoes a process known as looping. The morphogenetic steps required to achieve looping are guided by molecular asymmetries established in and around the heart by the embryonic left/right axial pathway

**Email:* zaffrans@pasteur.fr; *Tel:* 33-1-4061-3521; *Fax:* 33-1-4061-3452

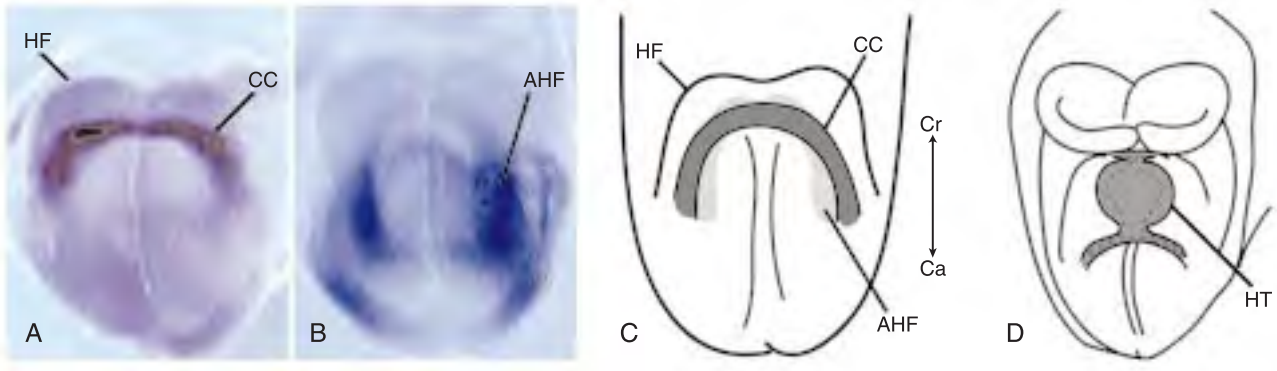

Figure 1 The embryonic heart at the cardiac crescent stage in mouse embryo.
In situ hybridization on whole mount mouse embryos at (E) 7.5-8.0, showing the distribution of α- *cardiac actin* (A) and *Fgf10* (B) transcripts. At this stage α-*cardiac actin* marks differentiated cardiomyocytes within the cardiac crescent while *Fgf10* marks the anterior or secondary heart-forming field (Kelly et al. 2001). (C) Diagram of a ventral view of a mouse embryo at (E) 7.5, showing cardiomyocytes of cardiac crescent (dark grey) and anterior heart-forming field (grey) (D) Diagram of mouse embryo at (E) 8.5, showing the linear heart tube before the rightward looping step. *AHF*, anterior heart forming field; Caudal; *CC*, cardiac crescent; *Cr*, cranial; *Ca*, *HF*, head folding; *HT*, heart tube.

(reviewed in Harvey 1998). During rightward looping, the future right and left ventricles become distinct and the atrial region together with the systemic venous branches are constrained dorsally and cranially. Subsequently, in mammals, the atrial and ventricular chambers become separated and valve formation involves the mesenchymal transition of endothelial cells. This step is essential for the formation of an integrated four-chambered heart with separate venous (or inflow) and arterial (or outflow) poles. A second distinct cell lineage, cardiac neural crest cells that migrate from the neuroectoderm, populate the heart through the outflow channel and contribute to the formation of the great vessels and division of the outflow tract (Farrell et al. 1999, Kirby et al. 1983).

The spatial and temporal orchestration of these processes implies a complex program of genetic control. A precise interpretation of different and combinatorial signals is needed to give rise to the mature multi-chambered heart. In this review I summarize our current knowledge of the transcription factors and signals involved in this morphogenetic process. Unexpectedly, genetic and molecular studies of mesodermal development in an invertebrate, the fruitfly *Drosophila*, have been instrumental in the identification of specific genes in cardiogenesis that appear to be conserved in all vertebrates (Bodmer 1995, Scott 1994).

While the *Drosophila* heart is not totally like the heart of vertebrates, it displays a number of features in common with the primary vertebrate heart tube in development and form. The *Drosophila* heart, or dorsal vessel, is a hemolymph-pumping organ composed of a limited number of cells and cell types (reviewed in Bodmer & Frasch 1999). A double row of cardiac cells (expressing muscle-specific proteins) coalesces to form the heart tube. These comprise the contractile cells of the heart (figure 2A). Located in the dorsal midline, the heart is flanked on either side by several types of pericardial cells that are loosely associated with cardiac cells and do not express muscle proteins. The precise roles pericardial cells may play during heart development as well as their physiological function are at present poorly defined (Rizki 1978). Anteriorly, the dorsal vessel is closed by the bilaterally symmetrical lymph glands, endocrine organs of complex cellular origin (figure 2A). The heart tube is covered by a network of extracellular matrix components (Rugendorff et al. 1994, Zaffran et al. 1995). Some of these components, are expressed in pericardial cells and are localized in specialized areas of the heart tube surface to serve as a link with the ectoderm during the dorsal closure (Chartier et al. 2002). The dorsal vessel is attached to the body wall by seven segmentally arranged alary muscles. In the posterior portion, the heart region features three bilateral pairs of inflow tracts, termed "ostiae" (Lo & Frasch 2001, Molina & Cripps 2001, Ponzielli et al. 2002) (figure 2B). In addition to the overall antero-posterior polarity, individual segments of the dorsal vessel also show

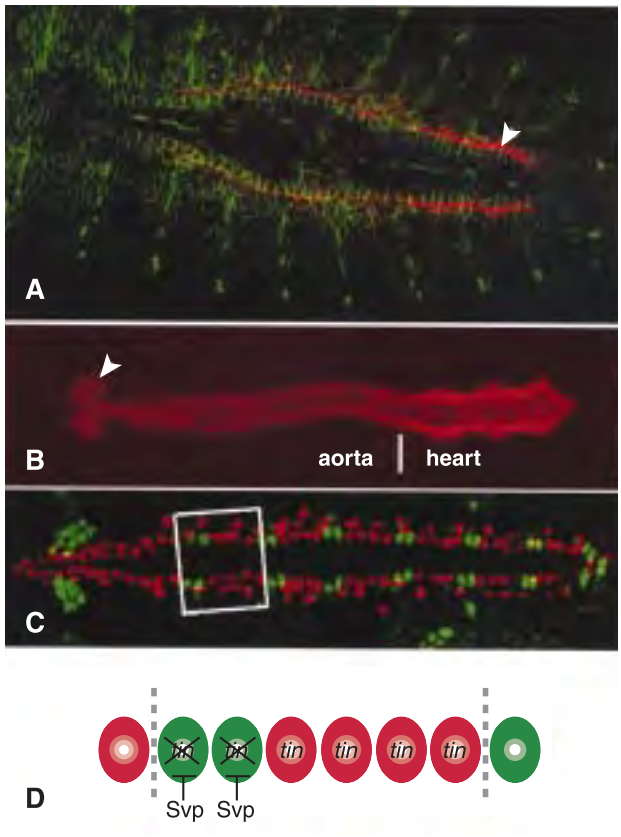

Figure 2 Morphology of the *Drosophila* dorsal vessel. Embryos are shown with anterior to the left. **A,** Dorsal view of an embryo during the dorsal closure stage after staining with anti-α-spectrin (green) and anti-pericardin (red), which marks the basal surface of cardiac cells (Zaffran et al. 1995). α-spectrin shows the baso-lateral surface of cells. The two rows of epithelial cardioblasts will join to form the dorsal vessel beneath the ectodermal leading edge cells (arrowhead); **B,** Dorsal vessel of a late stage embryo stained with anti-pericardin (red). At late stages, the dorsal vessel displays signs of morphological heterogeneity with the aorta and heart at the anterior and posterior regions respectively. A pair of lymph glands closes the anterior most portion of the dorsal vessel (arrowhead); **C,** Larval cardiomyocytes visualized by mCD8GFP. Opening is visible in the heart between the first pair of ostiae cell (arrowhead). The aorta is separated from the heart by a non-muscular cardiovascular valve (cvv); **D,** The developing dorsal vessel in a late stage *svp-lacZ* embryo stained for Tin (red) and βGal (green) showing the intrasegmental heterogeneity of the cardiac cells (Lo and Frasch, 2001). One segment is highlighted in D; **E,** Summary of genetic interactions within one segment of the dorsal vessel. Six cardiac cells, four tin-expressing cells at the posterior and two *svp*-expressing cells at the anterior constitute one segment. The boundary segments are delimited by the ectodermal expression of the pair-rule gene *engrailed* (Fremion et al. 1999).

anteroposterior regionalization. Expression analysis of various molecular markers has revealed that, analogous to the remainder of the insect body, the dorsal vessel is composed of segmental units (figure 2C,D). For example, the NK homeobox gene *tinman* (*tin*), which has an essential early function in cardiogenesis (see below), is expressed in only four of the six bilateral cardioblasts in each segment of the mature dorsal vessel (figure 2C,D). The *tin*-negative cardioblasts are characterized by the expression of other transcription factors, such as *seven-up* (*svp*, figure 2C,D) an orphan nuclear receptor (a member of the steroid receptor super-family) (Gajewski et al. 2000, Lo & Frasch 2001, Ward & Skeath 2000). In the adult, the *tin*-expressing cells form the heart tube while the *svp*-expressing cells form the ostiae (Molina & Cripps 2001, Ponzielli et al. 2002).

Despite the considerable differences in their anatomy and complexity, there is mounting evidence that hearts of insects and vertebrates share a common evolutionary origin (reviewed in Zaffran & Frasch 2002). Both linear heart tubes share the function of pumping hemolymph or blood, and the cardiomyocytes of insects and vertebrates share ultrastructural features. More recently, is has become apparent that these similarities extend to the mechanisms that regulate early heart development, which involve related signaling molecules and transcription factors. In the next sections I summarize our current knowledge on the different molecules that control heart development in both *Drosophila* and vertebrates, with a particular focus on mammalian cardiogenesis.

Cardiac Transcriptional Regulators

Developmentally, early cardiogenesis in vertebrates and invertebrates shares common feature. Heart specification in both systems requires inductive signals across germ layers, which generate a precardiac region in the lateral mesoderm (Harvey 2002). Endodermal induction generates a complex series of molecular and morphological events within the anterior lateral mesoderm. Events such as specification and differentiation of the precardiac mesoderm are controlled by transcription factors that regulate gene expression. A number of transcription factors of the NK-2, GATA, Hand, Tbx, HRT and MADS box families have been identified among the earliest markers in cardiac cell

differentiation (reviewed in Cripps & Olson 2002). Some of these factors seem to be active only at one specific step, whereas other may be required at a number of different stages. Finally, I discuss what is currently known about how these factors function during heart development and what could be conserved during evolution.

Nk Homeodomain Proteins

Discovery of the essential role of the homeobox gene *tinman* in heart formation has been a major finding in both vertebrate and invertebrate cardiogenesis. *Drosophila* embryos homozygous for loss of function mutation in the *tinman* gene do not form a heart and furthermore have no cardiac precursors (Azpiazu & Frasch 1993, Bodmer 1993). These results indicate that *tinman* function is required during the early steps of heart development. Moreover, *tinman* mutant embryos have other defects including a complete absence of visceral musculature (Azpiazu & Frasch 1993). These results suggest that *tinman* is absolutely required for the specification of all cardiac precursors in the dorsal mesoderm and subsequently for dorsal vessel formation. The spatial pattern of *tinman* is consistent with the proposed developmental function. Expression of *tinman* gene initiates in all mesodermal cells through positive activation by the b-HLH protein Twist which is the first zygotic transcription factor expressed in mesoderm (Baylies & Bate 1996, Yin et al. 1997) (figure 3A). However, after the dorsal spreading of mesoderm along the ectoderm, the expression of *tinman* becomes restricted to the dorsal-most area of the mesoderm while its expression in ventral areas is rapidly lost (Azpiazu et al. 1996). These observations suggest that an inductive signal is necessary to maintain *tinman* expression (see below). Subsequently, *tinman* expression becomes restricted to the progenitor cells of the dorsal vessel. Interestingly, only four of the six cardioblasts per hemisegments (one side of the segment) maintain *tinman* expression (figure 2C), (Jagla et al. 1997, Ward & Skeath 2000). Recent studies have shown that *seven-up*, which is homologous to the vertebrate orphan nuclear receptor COUP-TFII, is expressed in the non-Tinman-expressing cardioblasts and represses *tinman* in these cells (Lo & Frasch 2001, Ponzielli et al. 2002, Ward & Skeath 2000) (figure 2C,D) of

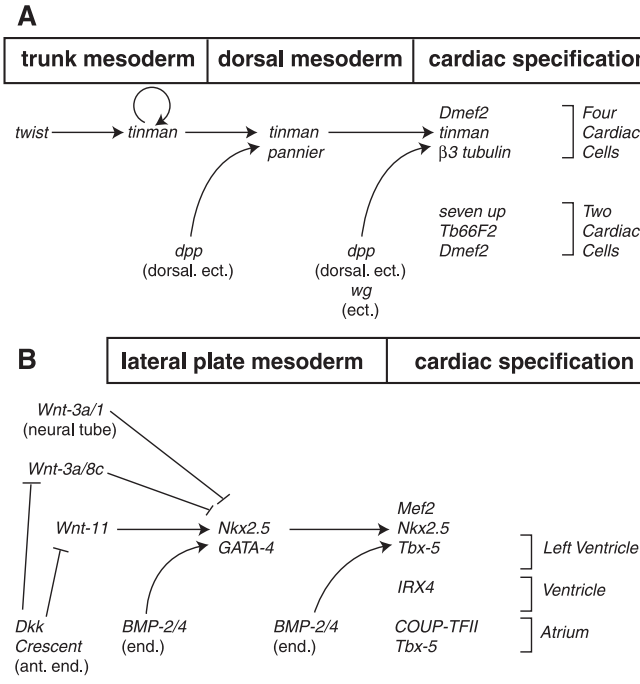

Figure 3 A regulatory network for cardiogenesis. Diagrams showing the major cardiac gene regulatory interactions that been characterized to date. **A,** The regulatory interactions during specification of cardiac mesoderm in *Drosophila* required for the formation of a functional dorsal vessel. See figure 2 for details. An ectodermal *dpp* signal is necessary to maintain *tinman* expression in the dorsal mesoderm; **B,** Studies in different vertebrate systems have led to the present network. Induction of *Nkx2.5* gene in the lateral mesoderm results from a positive activation by BMP-2/4 signals and negative regulation through the secreted molecules Wnt-3a/8c. Wnt inhibitors such as crescent are expressed in the anterior endoderm of the embryo, whereas Wnt-3a and Wnt-8c are expressed in the posterior lateral mesoderm. See text for references.

note, targeted deletion of the COUP-TFII mouse gene results in embryonic lethality with defects in heart development including disruption of common atrium and sinus venosus (Pereira et al. 1999).

To date, four cardiac Tinman target genes have been identified: *tinman* itself, the GATA factor gene *pannier*, *DMef-2*, and the structural gene *β3-tubulin* (Cripps et al. 1999, Fossett et al. 2000, Gajewski et al. 1997, Kremser et al. 1999, Xu et al. 1998) (figure 3A). Interestingly, in the enhancers of each of these four genes there are two consensus *tinman*-binding sites, which suggest a potential dimerization of Tinman proteins as recently shown for the vertebrates homologue Nkx2.5 (Zaffran & Frasch unpublished data, Kasahara et al. 2001).

In mice the earliest marker to be detected in cardiac precursor cells prior to differentiation is Nkx2.5, a homeodomain homologue of *Drosophila* Tinman. This gene was originally isolated by two groups (Komuro & Izumo 1993, Lints et al. 1993) and also termed *Csx* (*cardiac-specific homeobox*). It is expressed in myocardial cells from the cardiac crescent stage to adulthood but has not been detected in the endocardium. It is also expressed in endoderm, spleen, tongue and stomach (Lints et al. 1993). Analysis of other species has identified extensive conservation of *tinman/Nkx2.5* genes (Harvey 1996). Like *tinman*, *Nkx2.5* is regulated by a complex series of positive and negative cis acting elements (see below). In the mouse, analysis of embryos that lack *Nkx2.5* activity shows that such loss-of-function does not modify heart tube formation, although no rightward looping is initiated (Lyons et al. 1995). Dominant mutations in the *Nkx2.5* locus have been found in human congenital heart disease, with ventricular and/or atrial septation defects, often accompanied by atrioventricular conduction disease (Goldmuntz et al. 2001, Schott et al. 1998). Thus, human *Nkx2.5* seems to be essential for normal septation during heart development. These data raise the issue of redundancy since *Nkx2.5* is not critical for the initial steps of heart development. In *Xenopus,* overexpression of either X*Nkx2.5* or *XNkx2.3*, another member of *NK-2* family, produces embryos which are morphologically normal except for the fact that they have enlarged hearts, suggesting that both *XNkx-2.3* and *XNkx-2.5* are functional homologues of *tinman* (Cleaver et al. 1996). In mice, another member of the *NK-2* family, *Nkx2.6*, shares some domain of the *Nkx2.5* expression (Biben et al. 1998, Nikolova et al. 1997). In the pharynx, both *Nkx2.5* and *Nkx2.6* are expressed in the ventral region and within the heart, redundant expression of Nkx2.5 and Nkx2.6 has been observed in the sinus venosa and in the outflow tract at early stages of embryogenesis (Biben et al. 1998, Nikolova et al. 1997, Tanaka et al. 2001). Recent analysis of double-mutant *Nkx2.5/Nkx2.6* mice has shown that the pharynx does not form properly (Tanaka et al. 2001). Furthermore, the atrium was less advanced in the double-mutant embryos, indicating that these two genes have essential overlapping functions in cardiac development since neither single mutant shows such a defect (Tanaka et al. 2001).

The mutant phenotypes and the overexpression experiments in *Xenopus* suggest that the cardiogenic function of *Nkx2.5* may depend on interaction with a cardiac restricted factor. Experiments using the yeast two hybrid system revealed a physical interaction between Nkx2.5 and GATA-4 (a zing finger transcription factor, see below) (Durocher et al. 1997, Shiojima et al. 1999). Cotransfection experiments have shown that the promoter of the atrial natriuretic factor (ANF) gene is strongly transactivated by Nkx-2.5 and GATA-4, independently of GATA-4-DNA interactions (Shiojima et al. 1999). Similarly, a T-box transcription factor expressed in heart (see below), Tbx-5, was identified as a physical co-factor of Nkx2.5 (Bruneau et al. 2001, Hiroi et al. 2001). This interaction is consistent with the overlapping expression pattern of *Nkx2.5* with *Tbx5* (Bruneau et al. 1999). Cotransfection experiments with Nkx2.5 and Tbx-5 in different cell lines indicate that these factors synergistically induce cardiac development through their own DNA binding sites (Bruneau et al. 2001, Hiroi et al. 2001). More recently, a physical interaction between another T-box protein, Tbx2, and Nkx2.5 has been identified and characterized as important for the correct patterning of the atrioventricular canal (Habets et al. 2002). This result illustrates the relative divergence of thes proteins.

It is important to mention that despite structural and functional similarities between Tinman and its homologue Nkx2.5, the latter is not able to completely rescue *tinman* function in *Drosophila* (Park et al. 1998, Ranganayakulu et al. 1998). The failure of Nkx2.5 to rescue heart development in absence of *tinman* is due to the presence in Tinman of a unique N-terminal domain (Harvey 1996). When 52-amino acids at its N terminus is transferred to Nkx2.5, complete rescue of the heart phenotype is observed (Ranganayakulu et al. 1998). This result illustrates the relative divergence of these proteins.

Surprisingly the only non-mesodermal domain of *tinman* expression is located at the anterior tip of the embryo, which becomes part of the pharynx and anterior endoderm (Yin et al. 1997). Although this aspect of *tinman* expression has previously received less attention, it is interesting to note that these

territories are also prominent sites of expression of *tinman*-related genes in vertebrates. Indeed, in most species, *Nkx2.5* expression is not restricted to the precardiac region but is also detected, for example, in pharyngeal endoderm and in the future foregut endoderm (Evans et al. 1995, Harvey et al. 1999, Lints et al. 1993, Tonissen et al. 1994). Thus, some of the upstream regulators acting through the early enhancer of *tinman* (Yin et al. 1997) are likely to be evolutionarily conserved as well.

GATA Factors and Cardiogenesis

The cardiac *GATA* factors are zinc-finger proteins, which binds to the WGATAR motif in the promoter regions of cardiac- and gut-specific genes (Molkentin 2000). The importance of *GATA-4* in heart development has been suggested based on its expression in the precardiac mesoderm, its presence in the differentiated myocardium throughout embryogenesis and into adulthood, similar to the homeobox gene *Nkx2.5* (Grepin et al. 1994, Heikinheimo et al. 1994, Molkentin et al. 1994). To assess the role of GATA factors in heart development, mice carrying a targeted deletion of the *GATA-4* gene were generated and embryos lacking *GATA-4* present a bilateral heart defect with a reduced number of cardiomyocytes (Kuo et al. 1997, Molkentin et al. 1997). However, chimera experiments using *GATA-4*$^{-/-}$ ES cells demonstrate that *GATA-4* deficient cardiac myocytes can populate the embryonic heart (Kuo et al. 1997, Narita et al. 1997). This finding suggests that the heart defect seen in the absence of *GATA-4* is not cell autonomous. Two other GATA factors, GATA-5 and -6 are also expressed during early heart development (Morrisey et al. 1996, Morrisey et al. 1997). Thus, it was important to study the pattern of *GATA-5* and *-6* expression in *GATA-4* deficient mice embryos. In such embryos, *GATA-5* expression appeared normal while a higher level of *GATA-6* is observed in cardiomyocytes suggesting that *GATA-4* and *-6* may act in a common pathway. Moreover, *GATA-4* and *-6* were found to interact functionally and physically and to provide cooperative activation of the ANF promoter (Charron et al. 1999). An important experiment reveals that wild-type endodermal cells rescue the heart phenotype of *GATA-4*$^{-/-}$ mutant embryos (Narita et al. 1997). It appears clear that the cardiac *bifida* phenotype observed in mutant mice results from the absence of *GATA-4* in the endoderm. In contrast, *GATA-5*$^{-/-}$ mice do not display abnormal heart morphology, which suggests that other *GATA* factors may have functions overlapping those of *GATA-5* in other tissues (Molkentin et al. 2000). Interestingly, experiments in chick (*Gallus*), *Xenopus* and zebrafish (*Danio*) provide further in vivo evidence for the role of *GATA* factors in heart development. Inhibition of GATA activity using antisense oligonucleotides complementary to *GATA-4*, *-5* and *-6* produces variable abnormalities in avian cardiac morphogenesis (Jiang et al. 1998). Overexpression of *GATA-4* and *-5* in *Xenopus* embryos leads to the premature expression of the myocardial genes such cardiac actin and Myosin Heavy Chain (MHC) (Jiang & Evans 1996). More recently, analysis of the zebrafish *faust* mutant, in which *GATA-5* gene is mutated, indicates that early in embryogenesis *GATA-5* is required for the normal expression of *Nkx2.5* and later for elaboration of ventricular tissue (Reiter et al. 1999). Consistently, overexpression experiments in zebrafish show that *GATA-5* can ectopically activate several myocardial genes including Nkx2.5 (Reiter et al. 1999). These observations demonstrate clearly that *GATA-5* could participate in heart development in zebrafish.

Clear evidence for a requirement for cardiac GATA in cardiomyocyte specification has also been obtained in *Drosophila* through the study of the gene *pannier* (Gajewski et al. 1999). The function of *pannier* has been well studied in the ectoderm but ignored in the mesoderm (Ramain et al. 1993). A precise study of embryos carrying-out a null mutation for *pannier*, has revealed the absence of all cardiomyocytes and supernumerary pericardial cells of the dorsal vessel (Gajewski et al. 1999). While *tinman* is expressed in all dorsal mesoderm, *pannier* is detected within a narrower dorsal domain. However, Pannier and Tinman appear to act synergistically during the specification of the cardiac precursors through their common target genes (Gajewski et al. 1997, Gajewski et al. 2001). As expected for their corresponding vertebrate homologues, both proteins Tinman and Pannier interact physically to modulate their activity

(Gajewski et al. 2001). The two factors are also part of a cross-regulatory loop, since the early expression of *pannier* is directly controlled by *tinman* while the late cardioblast expression of *tinman* (perhaps indirectly) requires *pannier* (Gajewski et al. 2001).

The activity of cardiac GATA factors is modulated by association with the zinc-finger protein *Friend of GATA-2* (*FOG-2*) and orthologue of *Drosophila* U-shaped (Ush) (Lu et al. 1999). Both *FOG-2* and *Ush* are expressed in the developing myocardium and have been characterized as essential for its correct development (reviewed in Fossett & Schulz 2001). Again, these similarities provide further evidence for the conservation of gene functions during cardiogenesis in *Drosophila* and mammals.

T-box Factors and Cardiac Development

A recently identified family of genes, characterized by the presence of a region of homology to the DNA-binding domain of the *Brachyury* gene (*T*) named the T- box, has been studied for its role in the regulation of early embryogenesis (reviewed in Smith 1999). The involvement of T-box genes in heart development is accentuated by the phenotypic analysis of mice and humans who lack *Tbx1* or *Tbx5,* which have been associated with the human DiGeorge and Holt-Oram syndromes, respectively (Basson et al. 1997, Bruneau et al. 2001, Jerome & Papaioannou 2001, Lindsay et al. 2001). Holt-Oram syndrome is an haploinsufficient syndrome characterized by heart and forelimb defects, including ventricular and atrial septa defects and atrioventricular-canal defects (Basson et al. 1994). Mutations in human *Tbx5* were identified as a cause of the Holt-Oram syndrome (Basson et al. 1997, Li et al. 1997). More recently, heterozygous null *Tbx5* mice have been shown to exhibit the congenital heart malformations of Holt-Oram syndrome, associated with a reduction of cardiac gene expression including that of *ANF* and *cx40* (Bruneau et al. 2001). In contrast, the right ventricle and outflow tract appear to be *Tbx5* independent since *Tbx5* is not expressed in these regions and that they are not affected in *Tbx5* mutant mice (Bruneau et al. 2001). Those cardiac defects are consistent with the localized pattern of *Tbx5* expression (Basson et al. 1997, Bruneau et al. 1999, Bruneau et al. 2001). Indeed, Tbx5 is initially expressed throughout the cardiogenic region and is subsequently restricted to the sinoatrial segments of the heart, consistent with the timing of atrial chamber determination (Bruneau et al. 1999). In contrast, forced expression of *Tbx5* in the anterior region of the cardiac crescent results in aberrant ventricular morphogenesis associated with a reduction of *Mlc2v* expression (Liberatore et al. 2000). Together these data indicate a role of *Tbx5* in the initial diversification of atrial and ventricular chambers, of note the *heartstrings* mutation has been recently mapped and find it to encode the zebrafish orthologue of the *Tbx5* gene (Garrity et al. 2002). The heart of *heartstrings* mutant embryos appears to form and function normally through the early heart tube stage. However, the heart fails to loop and then degenerates, a process affecting the ventricle as well as the atrium, which are smaller and thinner respectively (Garrity et al. 2002). Further these data suggest that relative to mammals, fish required lower level of *Tbx5* to produce malformations and display whole-heart rather than atrial-predominant cardiac defects.

Interestingly, in mammals a second T-box gene, *Tbx1,* has also been identified as a candidate for involvement in a common human syndrome, DiGeorge syndrome (Jerome & Papaioannou 2001, Lindsay et al. 2001, Merscher et al. 2001). Mice heterozygous for a null mutation in *Tbx1* develop conotruncal defects, with abnormalities of patterning of the great vessels, phenotypic features of the DiGeorge syndrome (Jerome & Papaioannou 2001, Lindsay et al. 2001, Merscher et al. 2001). *Tbx1* is expressed in pharyngeal endoderm and the core mesoderm of the pharyngeal arches surrounding the aortic arch arteries, which give rise to the great vessels (Chapman et al. 1996). These results, together with the expression patterns of *Tbx1*, suggest a major role for this gene in the molecular pathogenesis of DiGeorge syndrome.

In *Drosophila*, two *T-box* containing genes have been identified which are expressed in the dorsal vessel. Based on their expression pattern two related *H-15* genes are detected in the primordia of the dorsal vessel but to date there is no evidence of their involvement in development of the dorsal vessel (Zaffran & Frasch unpublished data, Griffin

et al. 2000). *Tb66F2*, also named *Dorsocross* (*Doc*) has recently been identified which is normally expressed in the mature dorsal vessel in small segmentally repeated clusters of cardioblasts (Lo & Frasch 2001). Interestingly, *in situ* hybridization reveals that *Tb66F2* expression is specific to the *tinman*-negative cardioblast cells, which expressed the *seven up* marker. In *seven up* null mutant embryos, the transformation of the *seven up* cardioblasts into *tinman* cardioblasts results in the total loss of *Tb66F2*. Moreover, ectopic expression of *seven up* in all cardioblasts leads to uniform expression of *Tb66F2* in the dorsal vessel (Lo & Frasch 2001). The hypothesis that *Tb66F2* may be direct repressor of *tinman* during the formation of the heart tube could be easily validated using *Drosophila* genetics.

Inductive Signals and Precardiac Mesoderm

Dpp/BMP Signaling

Studies in *Drosophila* led to the implication of BMP signaling in early cardiogenesis. Genetic studies have shown that Dpp, a member of the BMP family of TGF-β proteins, plays a major role in the transmission of a positional information from ectoderm to mesoderm (Frasch 1995, Staehling-Hampton et al. 1994). Notably, *Drosophila* mutant embryos that lack Dpp activity form neither a dorsal vessel nor any of its precursor cells (Frasch 1995). Conversely, upon ectopic expression of Dpp in the ventral ectoderm or forced mesodermal expression of a constitutively active type I Dpp receptor, Tkv^{act}, the underlying *tinman* domains expand accordingly to the ventral mesoderm (Frasch 1995, Yin & Frasch 1998). These data suggest that ectodermal Dpp signal serves as signal to induce *tinman* expression in the mesoderm. A *tinman* enhancer, termed Tin-D element, that was identified based on its activity in the dorsal mesoderm serves as a Dpp-response element of the *tinman* gene (Xu et al. 1998, Yin et al. 1997). Mutation analysis on the Tin-D element has identified four Smad (Mad and Medea) binding sites as essential for its activation in the dorsal mesoderm. Indeed, mutant embryos that lack Medea activity fail to induce expression of *tinman* transcript in the dorsal mesoderm (Xu et al. 1998). Interestingly, Tinman activity itself is required for the Dpp-dependent induction of its own gene (Xu et al. 1998). Thus the homeodomain protein Tinman and the Dpp signals, through Mad and Medea, act synergistically during this process to maintain the expression of *tinman* in the dorsal mesoderm, which is required for correct heart precursor specification. It will be interesting to determine whether similar synergistic mechanisms are used later to activate specific target genes that control downstream events in the differentiation of cardiac cells.

There are several similarities between the specification of the dorsal mesoderm in *Drosophila* and that of anterior mesoderm in vertebrates. Early in chick development, cardiac precursors are located on either side of Hensen's node in the anterior mesoderm (DeHaan & Urspurng 1965) and become specified in response to inductive signals from endodermal tissue (Schultheiss et al. 1995, Schultheiss et al. 1997). Studies in *Xenopus* and mice have shown that anterior endoderm is required for cardiac specification during early gastrulation (Nascone & Mercola 1995, Tam et al. 1997). *In situ* hybridization reveals that at least three *dpp* like genes, BMP-2, -4 and -7 are expressed in the anterior lateral region of the embryo, overlapping the precardiac region (Schultheiss et al. 1997). Recent evidence that BMP signal is required for heart formation has been obtained from studying BMP receptors. Ectopic expression of dominant negative Type I (tALK3) or Type II (tBMPRII) BMP receptors in developing *Xenopus* embryos results in the reduction or absence of heart formation (Shi et al. 2000). Furthermore, *in vitro* experiments using an inhibitor of BMP, *noggin*, demonstrated that interference with BMP signaling prevents cardiogenesis in cultured explants of chick precardiac mesoderm and blocks cardiogenesis in vivo when ectopically expressed (Schultheiss et al. 1997, Schultheiss & Lassar 1997). Consistent with this, the anterior paraxial mesoderm (non-precardiac mesoderm), can be induced to express cardiac genes in explant culture by exposure to BMP-2 (Schultheiss et al. 1997). However, a limited effect of BMP-2 was observed when the neural tube and notochord was added to these explants (Schultheiss et al. 1997). Interestingly, BMP signals cannot activate cardiac genes in posterior mesoderm suggesting the existence of a field of

cardiogenic competence (see below, Marvin et al. 2001). BMP signaling is also likely to be involved in the later processes of septation and valve formation (Allen et al. 2001, Eisenberg & Markwald 1995). Recent studies have shown similarities in the molecular genetic mechanism that controls early induction of heart precardiac mesoderm in vertebrates and insect embryos. Significantly, one of the early *Nkx2.5* cardiac enhancers is activated in response to BMP signaling (Liberatore et al. 2002). Point mutations within this cardiac enhancer identify multiple Smad binding sites that appear to direct expression of *Nkx2.5* during several stages of cardiac development in mouse embryos (Liberatore et al. 2002, Lien et al. 2002). These findings in *Drosophila* and vertebrates firmly establish the importance of BMP signaling in early cardiogenesis.

Wingless/Wnt Signaling

As discussed above the spatial domains of BMP signaling alone is not sufficient to define cardiogenic mesoderm either in vertebrate or in *Drosophila* embryos. In *Drosophila* and vertebrates, Wnt signaling has been shown to play a major role in further restricting the domains in which BMP acts as inductive signal to the lateral mesoderm. Despite some similarities, the mechanism through which this effect is achieved differs between *Drosophila* and vertebrates.

Although *tinman* and *dpp* are necessary, they are not sufficient to control heart formation. This is evident from ectopic expression of *dpp*, and subsequently *tinman*, in ventral mesoderm, which does not cause expansion of cardiac precursors (Frasch 1995). In addition, in early *Drosophila* embryos, the cardiac precursors form sequentially repeated clusters before formation of the dorsal vessel. These observations suggest that regulator(s) with segmental modulated activities are required in addition to Tinman and Dpp. *wingless* (*wg*), which encodes a secreted molecule of the Wnt family is expressed in the corresponding compartments, but in the ectodermal layer. Heart precursors are actually specified at the extreme dorsal edge of the wg/dpp intersect. Heart precursors are missing in embryos lacking *wingless* activity or components of the Wingless signaling pathway (Baylies et al. 1995, Park et al. 1996, Wu et al. 1995). Genetic experiments using mosaic clones for *wg*, have determined that *wg* can induce heart precursors from either mesoderm or ectoderm tissues (Lawrence et al. 1995). Recent studies have shown that *wg* acts as negative regulator of non-cardiac cells in the cardiac compartment, by activating the winged-helix domain protein Sloppy Paired which is expressed in stripes within the mesoderm (Lee & Frasch 2000, Riechmann et al. 1998). Recently, experiments of mis-expression of *wg* and/or *dpp* have demonstrated that ectopic heart tissue can be generated by altering patterns of *wg* and *dpp* within the *tinman* expressing mesoderm (Lockwood & Bodmer 2002). Future studies may reveal a direct role for Wingless during heart differentiation. For example, *wg* may be directly involved in generating heterogeneity of the cardioblasts, which are characterized by the expression of several marker genes (see above).

In contrast to *Drosophila*, where Wingless is essential for the induction of cardiogenesis, studies in vertebrate systems have found a major role of Wnt signaling in inhibition of cardiogenesis (except for Wnt-11 which is required for activation, see below).

Strong candidates for endogenous Wnt antagonists that function to de-repress cardiac induction have been identified in both chick and *Xenopus* (Marvin et al. 2001, Schneider & Mercola 2001). These are the secreted factors Crescent and Dkk-1, which are expressed in anterior endoderm and can inhibit Wnt ligands, including Wnt3A and Wnt-8c (Glinka et al. 1998, Pfeffer et al. 1997) (figure 3B). Ectopic expression of Crescent and Dkk-1 in the non-cardiogenic ventral marginal zone mesoderm is sufficient to induce heart formation (Marvin et al. 2001, Schneider & Mercola 2001). Conversely, infection of either Wnt-8c or Wnt-3a in precardiac mesoderm blocks cardiogenesis (Marvin et al. 2001). Subsequent experiments revealed that ectopic expression of Wnt-3a or Wnt-8c, but not Wnt-5 or Wnt-11, in explant culture of precardiac mesoderm can block cardiogenic differentiation in agreement with their endogenous expression pattern (Marvin et al. 2001, Schneider & Mercola 2001). Thus, these findings show that inhibition of Wnt signaling promotes heart formation in the anterior lateral mesoderm. Tzahor and Lassar (2001) have shown

that a second Wnt signal from the neural tube may block ectopic cardiogenesis in chick embryos. Indeed, previous studies have indicated that signals from the neural tube suppress heart formation in adjacent tissue (Climent et al. 1995, Schultheiss et al. 1997). Since Wnt-1 and Wnt-3a are expressed in the neural tube that lies adjacent to the anterior paraxial mesendoderm, these authors tested whether these factors could mimic an inhibitory effect on cardiogenesis by using explant cultures. Co-culture of anterior paraxial mesendoderm explants with fibroblasts expressing either Wnt-3a or Wnt-1 inhibits expression of cardiac markers from this tissue (Tzahor & Lassar 2001). Although expression of the above Wnts in precardiac tissues apparently blocks heart formation, recent findings demonstrate that a related Wnt (Wnt-11) which is expressed along the posterior edge of the precardiac mesoderm (Eisenberg et al. 1997) can induce cardiogenesis in posterior non-precardiac mesoderm (Eisenberg & Eisenberg 1999). Wnt-11 mediates this effect through a non-canonical Wnt signaling pathway. Recent loss-of-function experiments in *Xenopus* using a dominant negative XWnt-11 construct introduced into the presumptive cardiac region revealed a strong decrease in expression of several cardiac markers, such as *XNkx2.5* (Pandur et al. 2002). Moreover, overexpression of *XWnt-11* in the ventral marginal zone was sufficient to convert non-cardiogenic explants to a contractile tissue fate suggesting an inducing role of Wnt-11 (Pandur et al. 2002). Despite the previous known role of *Wnt-11* as antagonist of Wnt signaling, these observations suggest that Wnt-11 is required to initiate the cardiac development program.

More generally, signals from the endoderm "organizer" initiate cardiogenesis in adjacent mesoderm by establishing a zone of reduced Wnt/β-catenin signaling activity, but also high levels of Wnt/JNK activity in order to establish a competence to the BMP signal. In parallel, the neural tube restricts the zone of competence to the lateral mesoderm by inhibiting the effect of BMP signals on the anterior paraxial mesendoderm (figure 3B).

Fgf Signaling

There is some evidence that Fgf (fibroblast growth factor) signaling participates in signal induction of the precardiac tissue in both *Drosophila* and vertebrates. However our understanding of the specific role of Fgf signal in this pathways is difficult due to their involvement in early mesoderm induction and cell migration during gastrulation (reviewed in Rossant et al. 1997).

In *Drosophila* one of the two Fgf receptors, named Heartless (Htl) is expressed in mesoderm just prior to gastrulation until differentiation. The observed absence of a dorsal vessel in *heartless* mutant embryos is, at least in part, an indirect consequence of the requirement of the Fgf signaling in the migration of the early mesoderm (Beiman et al. 1996, Gisselbrecht et al. 1996). Hence, the majority of the mesodermal cells cannot reach the dorsal ectoderm and fail to respond to the inductive signal from Dpp and other factors (see above). An additional, a more direct role of FGF signaling in *Drosophila* heart development has been demonstrated by experiments which inhibit Fgf signaling only after the migration of the mesoderm is completed. Reduction of cardiac cells is observed when a dominant negative version of Heartless is expressed in dorsal mesoderm or ectoderm (Michelson et al. 1998). Despite clear evidence of a requirement for FGF signaling in *Drosophila* heart development the identity and distribution of the ligand of the Fgf receptor Heartless are not yet known.

In the chick system *in vitro* experiments demonstrated that the combined action of BMP-2 and Fgf-4, but neither factor alone, promotes cardiogenesis in non-precardiac mesoderm explants (Lough et al. 1996). Although Fgfs are expressed in the early chick embryo at the correct place to be able to function in cardiac specification, we can not distinguish between direct or indirect effects of FGF signals. In vertebrates, the clearest data on the *in vivo* function of Fgf signaling in cardiac induction has been obtained in the zebrafish. Zebrafish *fgf8* is expressed in the cardiogenic fields of the lateral plate, as well as in specific areas of the neural tube. Homozygous *fgf8* (*acerebellar*) mutants fail to initiate proper gene expression of the cardiac transcription factors *Nkx2.5* and *GATA-4*, resulting in a severely malformed heart with a particular loss of ventricular structures (Reifers et al. 1998, Reifers

et al. 2000). Importantly, inhibitor treatment during early embryogenesis with SU5402, which probably blocks all Fgf signals, results in a phenocopy of the *acerebellar* heart phenotype, including failure to initiate cardiac factors (Reifers et al. 2000). Together, these data provide strong evidence that Fgf signals act during induction of cardiogenic transcription factors. It is possible that different members of the Fgf family have partially redundant activities during this process, which may explain the normal expression of *GATA-6* and the formation of residual heart tissue in *acerebellar* mutants (Reifers et al. 2000). As described in the introduction, a population of myocardial precursor cells in chick and mouse embryos has been identified in pharyngeal mesoderm anterior to the early heart tube. This anterior or secondary heart-forming field gives rise to myocardium of the outflow region or arterial pole of the heart (reviewed in Kelly & Buckingham 2002). In the mouse, the cells of the anterior or secondary heart-forming field already express Fgf-10 at the cardiac crescent stage (Kelly et al. 2001). Therefore, it is tempting to speculate that apart from a possible role in the anterior migration of these cells, Fgf-10 may also be involved, perhaps in combination with a BMP signal, in the induction of factors that control arterial pole development. In the chick Fgf-8 and BMP-2 are present in the ventral pharynx and anterior or secondary heart-forming field, respectively, and appear to affect induction of the cells in a manner that mimics induction of the primary myocardium from the primary heart fields (Waldo et al. 2001).

Conclusion

Comparison of the cardiac factors that are involved in heart development in *Drosophila* and vertebrates makes it clear that there are several analogies in the molecules and mechanisms that control the identity of cardiac cells, as well as in the signals that mediate cardiogenic induction.

As discussed in this review the first cardiac factor, which was found to show similarity between both *Drosophila* and vertebrates, was the homedomain protein, Tinman (or Nkx2.5). The conservation of Tinman and Nkx2.5 proteins is not restricted to their sequence structure but also to their partners such as the cardiac GATA factors and T-box proteins. This observation may reflect functional homology of these proteins, and common ancestry of this regulatory network. As described above, the expression of *tinman* is modulated during development of the *Drosophila* embryo and persists only in cardiac cells of the dorsal vessel. It will be interesting to investigate the role of *tinman* in diversification of the cardiac cells as compared to the function of its homologue *Nkx2.5*. Indeed, *Nkx2.5* is not involved in the specification but rather in the differentiation of cardiac cells, which suggests a partial conservation of *tinman* function. This divergence is also illustrated by the differential rescue of the visceral mesoderm versus heart development in the trans-complementation experiment.

I have focused in this review on our current knowledge concerning these molecules and have summarized their roles during both *Drosophila* and vertebrate cardiogenesis. However, it should not be forgotten that still unknown factors are certainly involved in cardiac development in *Drosophila* and vertebrates. Indeed, an accurate fate map of the heart-forming region in avian embryos has shown that *Nkx2.5* is not expressed in the entire precardiac region as suggested previously (Redkar et al. 2001). This observation suggests that *Nkx2*.5 alone is insufficient to specify the entire precardiac region and that other factors play a role in specification.

Finally, it is clear that despite evident morphological divergence the insights gained from *Drosophila* have been useful in the study of vertebrate cardiogenesis. Recent work shows that a newly initiated genetic approach in zebrafish is already making significant contributions to understanding the development of the vertebrate heart.

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