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PRIMER

The developing heart: from *The Wizard of Oz* to congenital heart disease

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ABSTRACT

The heart is an essential organ with a fascinating developmental biology. It is also one of the organs that is most often affected in human disease, either during development or in postnatal life. Over the last few decades, insights into the development of the heart have led to fundamental new concepts in gene regulation, but also to genetic and mechanistic insights into congenital heart defects. In more recent years, the lessons learned from studying heart development have been applied to interrogating regeneration of the diseased heart, exemplifying the importance of understanding the mechanistic underpinnings that lead to the development of an organ.

KEY WORDS: Heart development, Gene regulation, Congenital heart disease, Regeneration

Introduction

The heart is an organ that captivates. We feel its incessant beating, it is the beacon of life and it symbolizes love. It is also the first organ to function in the embryo, a role that, in mammals, is essential for embryonic life. Improper development of the heart results in a variety of human diseases, first and foremost congenital heart defects, which are the most common and deadliest birth defects, affecting over 1% of newborns (Bruneau, 2008; Lalani and Belmont, 2014). Other aspects of heart development affect adult-onset heart disease, such as cardiomyopathies and arrhythmias. Of note, genomic association studies have pointed to developmental regulators of heart development as main 'hits' for several adult-onset heart conditions, such as conduction defects (den Hoed et al., 2013; Ellinor et al., 2012; Pfeufer et al., 2010). As such, understanding the mechanisms of heart development is of clear importance to understanding heart disease.

The heart is an organ that is present across evolution, existing as a simple tube in insects such as *Drosophila* and the well-studied tunicate *Ciona intestinalis*, and as increasingly complex structures in vertebrates. In mammals, the heart arises from mesoderm as it has migrated anteriorly (Devine et al., 2014; Lescroart et al., 2014). Its precursors emerge and rapidly coalesce to form the rudiments of the early heart. Then, by integrating as-yet unresolved patterning and morphogenetic cues, the simple heart tube goes through finely coordinated contortions to yield the initial primordia of the heart: two atria, two ventricles, and the connections in between. This initially wild choreography of organogenesis is further refined as these structures acquire more specific identities, add on further specializations such as valves, and mature to support cardiovascular physiology. Details of heart development are not covered here as excellent reviews are already available (Evans et al., 2010; Moorman and Christoffels, 2003; Bruneau and Riley, 2020).

The story of understanding heart development illuminates the power of developmental genetics. From *Drosophila* genetics to vertebrate development, to human disease genetics, discoveries in heart development have led to a rapid revolution in our understanding of organogenesis and gene regulation, and in recent years have provided clear insights into human disease. This is a powerful example of how basic studies of developmental biology have led to important insights into so many fields, including human health, gene regulation, cell signaling and cellular reprogramming.

Tinman and the heart

Amongst the extraordinary set of *Drosophila* mutants isolated in the late 1980s and early 1990s, one mutant in particular stood out for those interested in the heart. Simultaneously discovered by Rolf Bodmer, and by Natalia Azpiazu and Manfred Frasch, the *tinman* mutant – named after *The Wizard of Oz* character with a similar phenotype – did not form any cardiac mesoderm (Fig. 1), and therefore lacked a heart (Azpiazu and Frasch, 1993; Bodmer, 1993). The *tinman* mutations were found to affect the expression of a homeodomain transcription factor. That a single gene mutation could result in the complete absence of the heart was captivating to many developmental biologists, but especially for those studying heart development. A few years before this, Hal Weintraub's lab had shown that a single transcription factor, MyoD, could transform a variety of cells into skeletal muscle (Davis et al., 1987; Lassar et al., 1989). Cardiologists had since been dreaming of finding their 'CardioD', a fabulous master regulator that could be harnessed to regenerate failing hearts.

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Advocating developmental biology

This article is part of Development's Advocacy collection – a series of review articles that make compelling arguments for the field's importance. The series is split into two: one set of articles addresses the question 'What has developmental biology ever done for us?' We want to illustrate how discoveries in developmental biology have had a wider scientific and societal impact, and thus both celebrate our field's history and argue for its continuing place as a core biological discipline. In a complementary set of articles, we asked authors to explore 'What are the big open questions in the field?' Together, the articles will provide a collection of case studies that look back on the field's achievements and forwards to its potential, a resource for students, educators, advocates and researchers alike. To see the full collection as it grows, go to: https://dev.biologists.org/content/advocating-developmental-biology.

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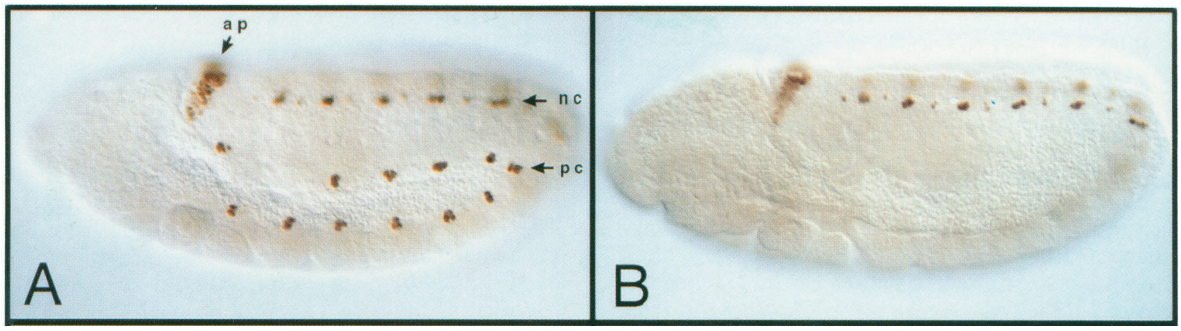

Fig. 1. The *tinman Drosophila* phenotype. (A) A wild-type *Drosophila* embryo stained for *eve*, which marks neural cell (nc) precursors, anal plate (ap) precursors and pericardial cell (pc) precursors. (B) Staining for *eve* in a *tinman* mutant embryo reveals an absence of pericardial cell precursors. This figure was reproduced unmodified with permission from Azpiazu and Frasch (1993), where it was published under a CC-BY-NC 4.0 licence.

It did not take long for several labs to isolate the vertebrate orthologs of *tinman*. More conventionally named (*Nkx2-5* or *Nkx2.5*; from the vertebrate homeodomain screen of Kim and Nirenberg), it was exciting to find that these too were expressed in the early developing hearts of all species examined (Fig. 2) (Komuro and Izumo, 1993; Lints et al., 1993). This suggested the tantalizing notion that the formation of the developing heart and its molecular mechanisms could be conserved from insects to mammals (Bodmer, 1995). Cardiac developmental biologists held their breath. In 1995, Richard Harvey's lab reported the mouse knockout of *Nkx2-5* and the result was, well, not what many expected (Lyons et al., 1995): the knockouts did have a heart. Clearly, *Nkx2-5* in mammals was not the master regulator of heart formation. However, the mutant hearts were severely malformed, with a blurred definition of the segment of the heart that would later form individual chambers. This was later confirmed in an independent knockout from Seigo Izumo's lab (Tanaka et al., 1999).

A flurry of activity surrounded *tinman* and *Nkx2-5*. From this, across many labs, came some of the most informative and pioneering experiments aimed at understanding gene regulation in organogenesis and deciphering biochemical interactions between transcription factors and their target DNA regulatory elements. Indeed, the discovery of *Nkx2-5* launched a 'golden age' in studies of heart development, leveraging molecular and genetic approaches to understand the formation of this essential organ. *Nkx2-5* maybe wasn't 'CardioD', but its discovery shed molecular light on previously occult realms of the molecular regulation of mammalian heart development.

From *Tinman* to congenital heart disease

The study of transcription factors in heart development was proceeding at a good clip, with the developmental genetics in mouse together with more conventional developmental biology in other model organisms rapidly providing a wealth of new insights. However, any link to human disease was not apparent. In the late 1990s, Jon and Christine Seidman's lab – which at the time was isolating human mutations in inherited cardiomyopathies – was studying a family with inherited congenital heart disease (CHD) that was considered genetically unmappable. A few had tried and failed. Jean-Jacques Schott, a new postdoc, was handed this family's DNA samples to use with a new mapping technology. To the surprise of some, a clear linkage signal appeared on chromosome 5q35. It was a broad interval, but staring right at Jean-Jacques was *NKX2-5*. One sequencing run later, a damaging mutation that correlated with the disease across the family was found (Schott et al., 1998). Two independent families with a similar type of heart defect were also shown to have mutations in *NKX2-5*. Thus, not only was NKX2-5 important for heart development, but it was a causative gene in CHD. This discovery was very important on many fronts. First, it told us that the genetic basis of CHD, which was unknown until then, was within a developmentally important transcription factor. It also showed that the dosage of these factors is crucial, suggesting a requirement for fine regulation of relative levels of these transcription factors. Further genetic studies identified several other families with *NKX2-5* mutations that provided an additional important insight: the same mutation in several family members or across unrelated individuals was associated with a very diverse clinical presentation (Benson et al., 1999). For example, in one family, carriers could have an atrial septal defect or only an atrioventricular block, whereas another family with the same mutation could have tricuspid atresia, a very severe defect. This showed that the reduced dosage of NKX2-5 could have varied effects on the developing heart, perhaps due to genetic modifiers or environmental influences.

Other transcription factors and CHD

A year before the discovery of *NKX2-5* mutations in human CHD, the Seidman labs and David Brooks' group had identified mutations in an unrelated transcription factor-encoding gene, *TBX5*, as the cause of a rare syndrome named Holt-Oram syndrome (Basson et al., 1997; Li et al., 1997). The main phenotypes of Holt-Oram patients were congenital heart defects similar to those in *NKX2-5* patients, and limb defects. The mutations in *TBX5* were also dominant, again pointing to reduced dosage as a molecular mechanism. Shortly thereafter, dominant *GATA4* mutations were discovered in families with similar heart defects (Garg et al., 2003). There now was a clear pattern emerging from the genetics, with three CHD genetic loci characterized by autosomal dominant inheritance, and three cardiac transcription factors mutated at these loci. The biochemistry that had been carried out on these factors before or subsequent to their human disease association indicated that they function together. Indeed, in the 1980s and 1990s, pioneering work on gene regulation had focused on a cardiac gene, *Nppa*, from which important rules of gene regulation had been obtained, including synergies between heterologous transcription factors (Bruneau et al., 2001; Durocher et al., 1997, 1996). Based on this knowledge, it was immediately obvious that mutations in one transcription factor that interacts with another disease-related transcription factor might affect the function of the partner proteins, and vice versa. A mechanism of combinatorial gene regulation appeared to be at the root of human CHD.

Such a set of interactions had been shown biochemically and genetically as the basis of gene regulatory networks in developmental

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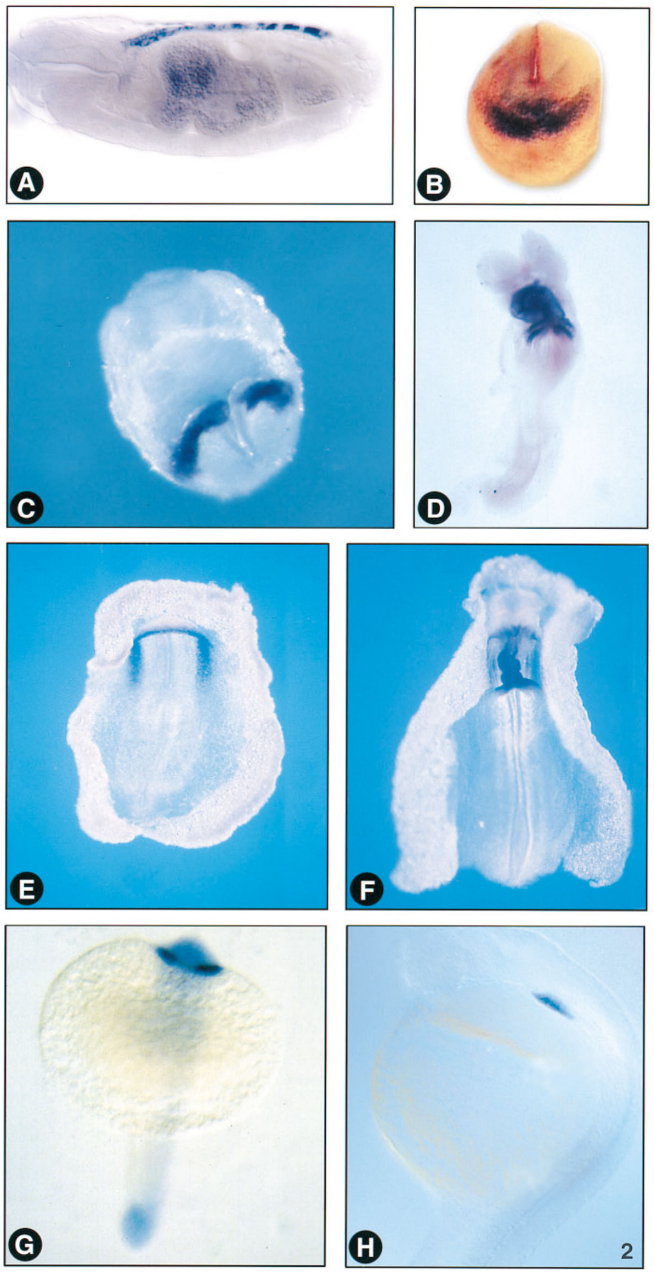

Fig. 2. Conserved expression of tinman and its vertebrate homologs. (A-H) The expression of *tinman* and its vertebrate homologs in heart progenitor cells is shown in *Drosophila* (A), *Xenopus* (B, stage 20 embryo), mouse (E.7.5, C; E8.75, D), chick (stage 6, E; stage 10, F) and zebrafish (19.5 h, G; 24 h, H) embryos. Reproduced with permission from Harvey (1996).

models, most notably the sea urchin. In this simple organism, the late, great Eric Davidson and colleagues had mapped out genetic and genomic connections between transcription factors and signaling molecules on a broad scale to understand the gene regulatory networks (GRNs) that controlled temporal and spatial gene expression in development (Davidson, 2010; Peter and Davidson, 2011). From this, they could generate testable models that unearthed key nodes and important molecules. Mammalian organogenesis was behind in this domain, but with these new links, interactions and phenotypes (i.e. CHD) emerged the beginnings of such a network, but with direct consequences for human disease. The notion was now that CHD-causing mutations lowered the dosage of a transcription factor, thus destabilizing a GRN important for heart development. The extension from this was that reduced transcription factor dosage would have a broader impact than predicted. The important and as yet unresolved question is why there is such fragility in a GRN that is normally considered robust. The answer to this may lie in the finesse of gene regulation that is required for organogenesis, which may lack the buffering mechanisms that other biological processes possess.

Several years later, these same transcription factor genes made an appearance in a surprising setting: genome-wide association studies (GWAS) for adult physiological traits and arrhythmias. Of the several polymorphisms associated with altered function of the condition system of the heart, and of a rhythm disturbance called atrial fibrillation, those near *NKX2-5* and *TBX5* were repeatedly found (den Hoed et al., 2013; Ellinor et al., 2012; Pfeufer et al., 2010). It therefore seemed likely that these genes also had functions, beyond embryonic development, in regulating the physiological function of the heart. Also found in these association studies were mutations that altered the binding of, in one particular case, TBX5 to an enhancer that was under the control of TBX5. It was clear now that a developmental GRN important for development and postnatal heart function was a major node for disease-causing mutations (Fig. 3).

Chromatin factors in heart development and CHD

The biochemistry and developmental biology of gene regulation was moving quickly into the field of chromatin-level control. Chromatin remodeling factors and histone modifying proteins, well studied biochemically, were being shown to be important for several developmental processes, including heart development. Considered for a long time to be ‘general’ regulators of gene expression, genetic loss-of-function experiments revealed surprisingly specific functions for chromatin remodelers. For example, some were found to be cardiac-specific, and could functionally and genetically interact with cardiac DNA-binding transcription factors (Ho and Crabtree, 2010; Hota and Bruneau, 2016). One factor, *BRG1* (also known as *SMARCA4*), was found to be haploinsufficient in the heart, and genetically interacted with CHD-associated transcription factors, including Nkx2-*5* and Tbx5 (Takeuchi et al., 2011). In some sense, studies in the developing heart had revealed an additional layer of tissue-specific gene regulation, bridging transcription factors and chromatin remodeling factors.

Genetic linkage studies continued to reveal roles for cardiac transcription factors in inherited CHD, but few new discoveries were being made, largely owing to the rarity of large genetically mappable families with CHD. Enter exome sequencing – an approach that employs high-throughput short-read sequencing of all coding exons of the genome. This approach did not require large families, and a group of pediatric cardiologists and geneticists set out to use this approach to discover *de novo* variants in children with CHD. The results were fascinating: of the dozen or so likely causative mutations, almost all were in genes encoding proteins associated with chromatin, be it readers, writers or erasers of chromatin marks (Homsy et al., 2015; Jin et al., 2017; Zaidi et al., 2013). This was another layer of gene regulation that had been studied in cellular contexts, but less so in developmental contexts, that was now clearly associated with heart development and human CHD. Along with the developmentally important transcription factors, this cemented the notion that broad GRNs that operative in heart development were the nexus for human disease. Now, the developmental biologists had to go back and figure out the nature of these networks.

The conceptual issue with the implication of chromatin-modifying proteins in CHDs is that they are broadly expressed

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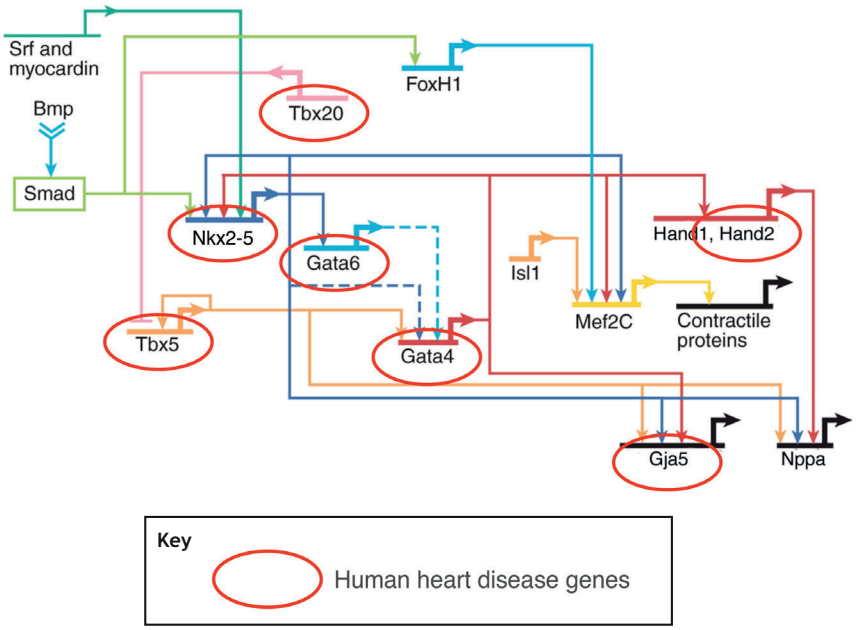

Fig. 3. Transcriptional network underlying heart development. The gene regulatory network underlying heart development is shown. Genes that have been implicated in human CHD are highlighted (red ovals). Adapted from Davidson (2010).

and regulate thousands of genes. So why are their mutations predominantly causing CHD? To date, this mystery has not been solved, but some hints from careful clinical evaluation of patients indicate that these CHD-causing mutations are also associated with cognitive and learning defects, suggesting that congenital heart defects are perhaps a more obvious manifestation of multi-organ developmental defects. It will be fascinating to determine which developmental pathways are affected by these mutations, and if they are shared across organs.

Back to the issue of regeneration

The initial excitement in 1993 over the discovery of *tinman* was that the instructive nature of a master regulator could be used to make new heart cells and regenerate a diseased heart. Heart muscle has the notable property that it cannot regenerate after injury, so an attractive approach to treating heart disease would be to make new heart cells. And what better way than 'simply' reprogramming non-cardiac cells the MyoD way! Many tried, but no single factor could do it. Some of our own efforts were aimed at using combinations of transcription factors and chromatin remodeling factors, and this indeed was potent enough to drive non-cardiac mesoderm to a cardiac fate (Takeuchi and Bruneau, 2009). Then came the approach used by Kazu Takahashi and Shinya Yamanaka for discovering the factors that could reprogram somatic cells to pluripotency: instead of one factor at a time, take as many transcription factors as possible, based on knowledge of their function in the desired cell type, and add them together. The approach worked, and was refined by removal of individual factors from the pool to narrow down the minimal set of reprogramming factors. This led to the discovery of induced pluripotent stem cells (iPSCs) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006). The developmental insight from this discovery was that a somatic cell could receive a minimal set of instructions and revert back to a pluripotent state.

Inspired by this, Masaki Ieda in Deepak Srivastava's lab took a similar approach, this time with cardiac factors. He revealed that, as with the iPSC experiments, a defined set of transcription factors – GATA4, MEF2C and TBX5 (but not NKX2-5!) – could transform fibroblasts into functional cardiomyocytes (Ieda et al., 2010). A key insight in this discovery was that fibroblasts were directly transformed into cardiomyocytes, without passing through a pluripotent or precursor intermediate. Along with contemporaneous neural reprogramming efforts, this led to the inescapable conclusion that a supposedly stable differentiated state could be radically altered by a simple set of instructive factors. The concept of 'terminal differentiation', which John Gurdon had put into question with his frog cloning experiments, was clearly now shown to be much more plastic than many had previously considered.

The reprogramming, which was successful *in vitro*, needed to be attempted in an *in vivo* disease context to fulfill its therapeutic promise. Two groups independently succeeded in using the cardiac reprogramming factors (and other factors) to reprogram endogenous fibroblasts into cardiomyocytes and improve heart function after an infarct (Qian et al., 2012; Song et al., 2012). An important aspect of these mouse experiments was that a lineage-tracing strategy was used to prove that the newly created cardiomyocytes were derived from fibroblasts. Moreover, an intriguing facet of these *in vivo* reprogramming experiments was that the cardiomyocytes appeared more mature compared with their *in vitro*-derived cousins, suggesting that tissue context could be providing some enhancing cues. Indeed, over several years, a number of signaling pathways and other mechanisms have been shown to be permissive or additive in cardiac reprogramming (Farber and Qian, 2020). Of note, the molecular paths taken by reprogrammed fibroblasts as they become cardiomyocytes appear to be quite different to those taken during endogenous cardiogenesis (Stone et al., 2019; Zhou et al., 2019). Although much has to be achieved before *in vivo* reprogramming can be used to treat diseased hearts in the clinic, the promise of 'CardioD', stemming from the discovery of *tinman* almost 20 years earlier, and bolstered by two decades of developmental biology, is coming to fruition.

Concluding remarks

The path from *Drosophila* genetics to mammalian orthologs, to human disease and heart regeneration illustrates the importance of developmental biology and the need to understand basic concepts underlying gene regulation. Developmental biologists have often

been asked why one needs to understand organogenesis and its mechanisms. Other than the obvious desire to learn how we come to be, we need to understand these processes to learn about congenital defects. The heart is used as an example here, but myriad other examples exist for various organs. We developmental biologists have often said that if we understand nature's instructions for making an organ or cell type, that we could harness this to help devise therapies for diseased adult organs. In the case of the heart, this has been borne out, with clear potential for real therapies. So what developmental biology has done for us is to allow us to understand the magic of embryology, the careful choreography of organogenesis, and important insights into human disease. There is so much more to be discovered and learned, and the heart's mysteries will certainly continue to amaze us.

Competing interests

B.G.B. is a co-founder of Tenaya Therapeutics, which aims to use developmentally important factors as therapies for heart failure.

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