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Towards spatio-temporally resolved developmental cardiac gene regulatory networks in zebrafish

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Abstract

Heart formation in the zebrafish involves a rapid, complex series of morphogenetic events in three-dimensional space that spans cardiac lineage specification through to chamber formation and maturation. This process is tightly orchestrated by a cardiac gene regulatory network (GRN), which ensures the precise spatio-temporal deployment of genes critical for heart formation. Alterations of the timing or spatial localisation of gene expression can have a significant impact in cardiac ontogeny and may lead to heart malformations. Hence, a better understanding of the cellular and molecular basis of congenital heart disease relies on understanding the behaviour of cardiac GRNs with precise spatiotemporal resolution. Here, we review the recent technical advances that have expanded our capacity to interrogate the cardiac GRN in zebrafish. In particular, we focus on studies utilising high-throughput technologies to systematically dissect gene expression patterns, both temporally and spatially during heart development.

Key words: Zebrafish; spatial transcriptomics; gene regulatory network; temporal expression; heart development

Jeannette Hallab has a BMedSc (Hons1, UNSW), 15 years experience as a cell/molecular biologist, and currently leads a project describing the role of Elk1 in heart development and cardiac/skeletal myopathy genetics.

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David Elliott heads the Cardiac Development Laboratory where is focusing on understanding the principles of cardiac development using differentiating hESCs as a model of human heart development.

Enzo Porrello heads the Cardiac Regeneration laboratory which focuses on enhancing our understanding of the transcriptional and epigenetic mechanisms governing cardiomyocyte maturation in the neonatal period.

Mirana Ramialison heads a multidisciplinary team of bioinformaticians and developmental biologists to uncover the gene regulatory networks underlying heart development and congenital heart disease using zebrafish and medaka models.

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Introduction

The deployment of a precise cardiac gene regulatory network (GRN) underpins proper cardiac development. Our knowledge of the cardiac GRN components orchestrating cardiogenesis three-dimensionally is still incomplete, which limits our understanding of heart development and disease. Developmental properties of the zebrafish make it a powerful model for cardiovascular developmental studies. Combining these inherent advantages with cutting-edge techniques for interrogating genetic networks in space and time is rapidly advancing our understanding of cardiac development. Here, we review recent technological advances allowing the systematic investigation of molecular events underpinning cardiogenesis in zebrafish, with spatio-temporal resolution. We first provide a summary of the three-dimensional morphological events leading to the successful formation of the mature zebrafish heart. We highlight the complexity of this process to substantiate the need to apply cutting-edge technologies to decipher the underlying GRNs. We then discuss recent technological advances that have rapidly improved our understanding of the basic molecular mechanisms underpinning cardiogenesis, by allowing systematic GRN investigations at spatio-temporal resolution in zebrafish.

Morphological events underpinning the development of the zebrafish heart

The zebrafish heart develops rapidly: within 5 days, a functional two-chambered, three-layered organ with small valve leaflets is formed from a series of complex three-dimensional patterning and morphogenetic events. Cardiac specification begins early in zebrafish development at five post-fertilisation (hpf), when cardiac progenitors are first identifiable bilaterally, at the ventrolateral regions of the blastula [1, 2] (Figure 1A). Concomitantly, cellular precursors of Kupffer's vesicle (KV), called dorsal forerunner cells, (DFCs) specify at the dorsal margin of the shield stage embryo at approximately 5 hpf [3]. By 10 hpf, KV has formed in the tailbud. KV is essential for cardiac left/right asymmetry development [4]. Asymmetric distribution of cilia in the dorsoanterior region of the KV drives leftward flow across the anterior pole, contributing to establishing the first cardiac asymmetry [5].

Following gastrulation-related involution, cardiac progenitors are located in the anterior lateral plate mesoderm (ALPM) by 12 hpf where they form bilateral rows, with the most medial row comprised of the endocardial progenitors and the central and lateral rows containing myocardial progenitors [1, 6] (Figure 1B). By 15 hpf, the precardiac mesoderm has formed [7] and by 16 hpf, the (central) pre-ventricular row and (lateral) pre-atrial cells become distinguishable [6, 8, 9] (Figure 1C).

At 19 hpf, cellular movements promote convergence of the bilateral cardiac fields at the anterior embryonic midline [10–12]. Here, they fuse to form a bilayered cone with a continuous inner layer comprised of endocardium, an outer layer with an apex and a dorsal region comprised of ventricular progenitors. The later will become the arterial pole, whilst a ventral base comprised of atrial progenitors will become the venous pole [13] (Figure 1D). By 22 hpf, the cardiac cone is orientated with the venous (atrial) pole positioned laterally and the arterial (ventricular) pole positioned medially [6, 14].

Realignment of the cardiac cone is followed by shape changes, as the ventricular bilayer extends into a more tubular form, followed by the atrial bilayer, contributing to the formation of the cardiac tube [15]. By approximately 24 hpf, the heart tube is

formed (Figure 1E) and contraction is initiated [16]. The contractions are unidirectionally propagated along the myocardium, suggestive of sinoatrial (SA) node activity, while the first chamber-specific morphological differences become apparent [17]. Following the initiation of contraction and prior to valve formation, one-way blood flow from the atrial to the ventricular pole is maximised and backflow minimised by contractile, rolling and compressive movements of the heart tube [18].

At 28 hpf, a second wave of cell migration from the second heart field extends the heart at both poles. Following elongation of the tube, cardiac jogging occurs with asymmetric displacement of the venous pole to the left [19]. Cardiac jogging proceeds to robust heart looping at 30 hpf [20] and is regulated by asymmetrical gene expression mediated by directional flow from KV [21]. This results in winding deformations at the arterial pole of the tube [14] with anisotropic growth at outer curvatures of the atrium and ventricle, generating an S-shaped heart by 48 hpf [14] (Figure 1G). Cardiac looping events proceed past this stage and advanced S looping occurs between 42 and 54 hpf when further bending of the heart tube occurs as a result of changes to cell shape in the myocardial wall of the AVC. This results in migration of the atrial chamber cranially, leading to a ventral juxtaposition of both atrial and ventricular chambers [14] (Figure 1H).

During this looping process, within the heart, valvulogenesis is initiated with the formation of the atrioventricular canal (AVC). Earliest features are apparent by 36 hpf in the looped heart when a single squamous endocardial cell (bilaterally) at the border of between atrium and ventricle acquires a cuboidal shape, diverging from the usual squamous endocardial cell morphology [22]. By 48 hpf, the outflow tract (OFT), comprised of the *bulbus arteriosus* (BA) and aorta has formed via developmental events spanning 42–48 hpf where myocardial cells transition into smooth muscle cells followed by further OFT thickening, known to occur past 4 weeks post fertilisation [23]. By 55 hpf, approximately 20 AVC endocardial cells have morphed to the cuboidal shape and overlay the underlying extracellular matrix (ECM) as a single sheet [22]. From 60 to 80 hpf, these cells begin to reach into the ECM between the endocardium and myocardium via cellular extensions [22] (Figure 1H). While earlier studies have suggested that endocardial cushions form in zebrafish by 96 hpf [22], later studies suggest that zebrafish do not develop true endocardial cushions, rather, valve leaflets form by direct invagination of cuboidal endocardium into the underlying ECM in a sheet like manner, not via EMT processes like in other species [18]. Early valve leaflets undergo further maturation involving elongation and shape changes from linear to more cusp-like in a process which has been observed for up to 3 months post-fertilisation [24]. By 105 hpf, small, globular, two cell layer-thick valve leaflets have extended from invaginated endocardium [22] and formation of competent valves is followed by complete prevention of retrograde blood flow by 111 hpf [25] (Figure 1I).

Concomitantly, cardiac trabeculation is initiated at 55 hpf when cardiomyocytes first displace at the luminal interface of the ventricle [26]. Further propagation of trabeculation occurs by 72 hpf including the formation of trabecular ridges across the AVC, where regional atrial contraction and pulsatile flow are thought to promote this process [27]. By 4 dpf (days post-fertilisation) trabecular ridges are prominent throughout the endocardium and between 4 and 5 dpf, interconnections form between trabecular ridges and the trabecular network is elaborated [27] (Figure 1I).

Within the outer layer of the heart, epicardial development commences at 55 hpf from the proepicardium, a group of mesothelial cells from the pericardium, and located close to

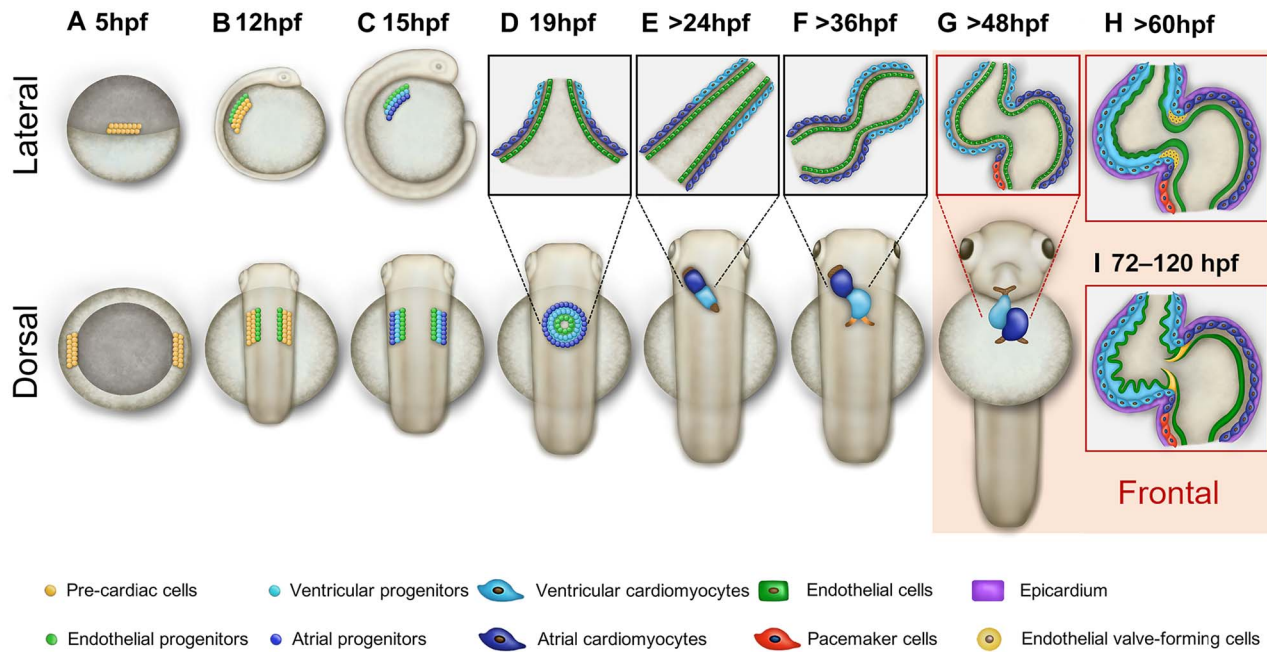


Figure 1. Cardiac cell-types present during critical stages of heart development in zebrafish embryos. (A-F) Upper row, lateral views, anterior is to the right; lower row, dorsal views anterior is to the top. (G-I) Frontal views, anterior is to the top. Hpf: hours post-fertilisation.

the venous pole of the heart [28]. Proepicardial cells are then released into the pericardial cavity and moved to the ventricle through pericardial fluid movements, where they adhere and proliferate to form the epicardial layer by 72 hpf [29, 30] (Figure 1). A subpopulation of epicardial cells then undergoes epithelial-to-mesenchymal transition, forming epicardium-derived cells which in turn aid to give rise to many cell types such as vascular cells or cardiac fibroblasts [31].

Finally, by 100 hpf, the His-purkinje fibres of the fast conduction system have developed and ventricular conduction has evolved from a primitive linear propagation travelling from the AVC to the OFT as observed at 48 hpf, to a more complex conduction pattern propagating from the outer curvature of the ventricle to the base/inner curvature [32].

In summary, zebrafish cardiogenesis is achieved by a series of complex specification, morphogenesis and remodelling events. These events are orchestrated by a tight cardiac GRN [33], where the precise spatio-temporal deployment of this network is crucial for successful cardiogenesis. Decades of developmental studies have uncovered key genes composing the zebrafish cardiac GRN, relying on loss or gain of function models to understand the role of these genes and pathways during heart development [12]. Despite that large body of knowledge, there are still a number of genes expressed in the heart with unknown function that remain to be discovered. Next, we review recent studies and associated technologies that have focused on further decrypting the cardiac GRN, using unbiased systematic approaches, with spatio-temporal resolution.

Towards a spatially-resolved zebrafish cardiac gene regulatory network

The first step in systematically investigating the cardiac GRN is to identify, in an unbiased manner, the GRN's components that are specifically expressed in well-defined cardiac regions. Traditional approaches such as *in situ* hybridization have provided vital examples of how expression patterns differ across the heart

(Singh et al., 2016). RNA-seq studies facilitate far more detailed gene expression profiles to be established and provided new discoveries regarding the gene network architecture underlying cardiac disease and development [34]. Emerging technologies in the spatial transcriptomics field provide the most comprehensive analysis of heart structure and development to date, by combining spatial visualisation and understanding of transcriptional networks [35, 36].

One such technology is RNA tomography sequencing (Tomoseq), which relies on the sectioning of the tissue of interest in three directions followed by RNA-sequencing and mathematical triangulation to retrieve precise gene expression at the intersection [37]. This technology has been applied to generate the first spatially resolved transcriptome map of the developing zebrafish heart at 48 hpf [38]. In that study, the whole heart was cryosectioned at 10 μ m thickness which allowed the capture of gene expression even in rare cell types. This dataset provided an unprecedented platform to first identify global synexpression domains in the developing heart. Hierarchical clustering was used to find gene clusters according to peak expression and four large clusters showed anterior-posterior region-specific expression differences. These clusters were identified as the outflow tract, ventricle, AVC and atrium based on gene profiling of the clusters. Separation between these tissues was confirmed using known tissue markers such as *myl7* and *vmhc*. Small regions such as the sinoatrial region could only be identified using marker genes, not cluster analysis due to thresholding limits within the cluster analysis.

Most importantly, differential gene expression analysis provided an unbiased overview of the molecular pathways defining these different cardiac compartments. Indeed, when compared to other compartments, 502 genes were found differentially expressed in the sinoatrial region, 346 in the AV canal, 196 in the ventricle and 99 in the atrium, including many previously uncharacterised genes. Based on these differentially expressed genes, sub-compartment specific gene expression was discovered in all four regions. *In situ* hybridisation at 48 hpf

– 52 hpf validated sub-compartment specific expression and, in general, good correlation was found between the Tomo-seq gene expression profiles and the *in situ* hybridisation gene expression patterns. Gene ontology analysis revealed enrichment of oxygen transport and tricarboxylic acid processes in the ventricle indicating the initiation of the phosphorylation shift expected during cardiac looping. Interestingly, the sinoatrial region was enriched for Hippo and Wnt signalling factors. Wnt/ β -catenin signalling is known to be important in cardiomyocyte differentiation [39]; however, no role in pacemaker development had previously been described. This study revealed that differential Wnt/ β -catenin signalling was detected in the pacemaker cells, with a different role in the AVC and sinoatrial region. Indeed Wnt/ β signalling regulates the expression of *bmp4* and *tbx2* in the AVC but not in the sinoatrial region (where *bmp4* and *tbx2* are also expressed). Wnt/ β signalling in the sinoatrial region was further demonstrated to be *Isl1*-dependant in order to control the autonomic heart rate. Further, enrichment analysis showed evidence that the cardiac lymphatics develop from the *sinus venosus* as observed in the mammalian heart [40].

Other spatial technologies combining *in situ* visualisation with single-cell transcriptomics were employed to dissect the molecular interactions occurring within the epicardium [31]. During the development of the zebrafish heart the epicardium is formed by three distinct subpopulations, each with their own set of gene specific markers and distinct programs, exemplified by the heterogeneous expression of *tcf21*, *tbx18* and *wt1b* at 3, 5 and 7 dpf [31]. In order to systematically understand the molecular basis of this heterogeneity, single-cell RNA-seq (scRNA-seq) was performed on epicardial cells, myocardium and blood in BAC transgenic reporter (*tcf21*, *tbx18* and *wt1b*) lines and wild-types. This led to the identification of three distinct populations in the epicardium, Epi1, Epi2 and Epi3, which each express a different subset of the epicardial markers *tcf21*, *tbx18* and *wt1b*. In order to functionally assess Epi1, Epi2 and Epi3, the CRISPR/Cas9 system was used to knock out genes highly enriched in each population. Findings suggested that *tgm2b* in Epi1 is crucial in maintaining a cohesive epithelial sheet, *sema3fb* in Epi2 may be important in controlling the number of Epi2 cells in the outflow tract and *cxcl12a* in Epi3 may attract leukocytes in the heart. Despite the relatively low cell numbers analysed which might have hindered the identification of other subpopulations in the epicardium, this study demonstrates that the combination of single-cell sequencing in restricted tissues offers a powerful approach to unravel spatially restricted gene GRNs.

In summary, these examples illustrate the rapid advances gained through the application of spatial transcriptomics during zebrafish cardiac development. Both studies however were limited to one developmental stage 40 hpf [38] and 5 dpf [31] respectively. Parallel investigation of gene expression dynamics would provide a complete understanding of the networks at play during cardiogenesis.

Towards a temporally resolved zebrafish cardiac gene regulatory network

Following efforts to capture spatially-resolved data, the next step in systematically investigating the cardiac GRN is to decipher *when* genes are expressed during the course of development. Developmental time series data are a powerful resource for identifying genes that are potentially coregulated and/or involved in the same biological processes. While conventional

transcriptomics performed under different conditions can reveal differentially expressed genes that underlie condition-specific responses, transcriptomes from time-course experiments can provide data that inform the dynamic regulation of developmental processes. To deconvolve time-course data, a class of computational network analysis methods helps construct GRNs as well as the dynamics of biological processes underlying heart development.

While temporal transcriptomics in the heart have been explored in mice and in cell culture [41–43], fewer studies have systematically investigated zebrafish heart development using time-course datasets [44, 45]. One approach combining transcriptional information (obtained by RNA-seq) and genome-wide chromatin accessibility (obtained by ATAC-seq) revealed heart-specific temporally co-expressed network modules [44]. The datasets were generated in zebrafish isolated cardiomyocytes at three different stages of heart development: tube morphogenesis (24 hpf), looping (48 hpf) and maturation (72 hpf) (Figure 1E, G, I) [44]. This study revealed that the major gene expression profile and chromatin landscape changes in developing cardiomyocytes occur between tube formation and looping. These findings are supported by a strong correlation between gene expression levels and chromatin accessibility, demonstrated by the nucleosome-free regions in their respective promoters at 24 and 48 hpf. The specific GRNs deployed during this period were further resolved by hierarchical clustering-based weighted gene co-expression network analysis (WGCNA), which is routinely used in network analysis to identify clusters of genes with highly correlated expression patterns [46]. Instead of focusing on individual genes, the WGCNA method focus on the connectivity between genes, and the strength of these connections is measured based the correlation of gene expression over time. Using WGCNA analysis, cardiac modules enriched in genes involved in embryonic heart tube development, cardioblast differentiation, heart valve development and heart formation were identified. These clusters are proposed to be under the direct control key cardiac transcription factors (TFs) as demonstrated by the presence of enriched TF-binding sites including those of the *Nkx* family and *T-box* family. Hence, this study demonstrates that the combinatorial integration of temporal gene expression datasets with chromatin accessibility is a promising approach to uncover the temporally resolved network modules underlying cardiac development.

Similar results were obtained from a different study analysing transcriptional profiling datasets of the whole heart every 6 h from 30 to 72 hpf obtained by RNA-sequencing [45]. Co-expressed genes were identified by clustering the temporal gene expression datasets using self-organising map (SOM) analysis [47]. SOM is an artificial neural network learning algorithm that fits a grid of nodes to high-dimensional data – gene expression patterns for individual genes in this case – and then assigns each pattern to the nearest node. Thus, it can be thought of as a non-linear principal component analysis (PCA), except each gene expression pattern is assigned to a specific cluster instead of merely generating loading values. SOM analysis enables human-driven gene pattern recognition by allowing the expression patterns of genes in different clusters to be compared visually to determine whether they are largely similar or are largely contrasting [47]. Using SOM analysis, clusters of genes with similar temporal gene expression patterns during zebrafish cardiac development were systematically retrieved [45]. These clusters contain cohorts of co-regulated genes, as confirmed by analysing the frequency of known gene regulatory interactions within the clusters and by the presence of shared TF binding

motifs in the genes' proximal cis-regulatory regions including Foxa-, Sox-, Klf- and Tbx-binding sites [45].

Altogether, these studies demonstrate that temporal GRN models directing cardiogenesis can be retrieved from temporally resolved transcriptomics and genomics datasets. Both studies substantially diverge in sample preparation (cardiomyocytes versus whole hearts) and computational techniques (WGCNA versus SOM). Nonetheless, the findings concur that GRNs identified contained several groups of genes with similar temporal expression patterns regulated by shared sets of multiple cardiac transcription factors.

Conclusions and future directions

The heart is a highly heterogeneous organ, with a vast array of different structures (e.g. chambers, valves, vessels), each of these further varied in cell-type composition. Elucidating the components of the cardiac GRN driving this complexity, and deciphering the interactions between these components, are paramount to our understanding of cardiogenesis. Despite the number of genes that have been uncovered to be crucial to proper heart formation, the precise spatio-temporal transcriptional programs that ensure the proper deployment of these genes at the right time and in the right place in the embryo are not yet fully understood. Advances in high-throughput sequencing technologies have rapidly enabled the systematic investigation of these networks. Here, we reviewed recent applications of these technologies that gave insight into the spatial deployment and dynamic recruitment of GRN modules involved in the development the zebrafish heart.

Spatially resolved technologies such as Tomo-seq, have systematically uncovered cardiac gene expression in the just fully formed embryonic zebrafish heart [38]. Similar studies in mice, employing microdissection, risk loss and damage of tissues during sectioning, and may miss rare cell types, as not all cells are sequenced [42, 43]. Although the Tomo-seq approach is not cell-type specific, additional analysis combining *in situ* and scRNA-seq data can overcome this limitation. scRNA-seq was used to successfully identify heterogeneous subpopulations in the epicardium of the developing zebrafish heart. Similarly, Tomo-seq [38, 48] and other spatial technologies such as *in situ* sequencing [35] would complement our understanding of the heterogeneity and spatio-temporal gene expression in these epicardial subpopulations.

Combination of time-series gene expression datasets with systems-biology integration is a powerful approach to define significant genes at any developmental stage and to deduce underlying molecular relationships within the regulatory circuitry. Such studies obtained by bulk RNA-sequencing of either a specific cell-type (cardiomyocytes) [44] or the whole heart [45] have systematically identified the temporal clusters of genes and the associated key transcriptional networks that are deployed during cardiogenesis. Interestingly, a large number of genes expressed in the heart could still not be assigned to a cluster, suggesting that there are further improvements to be performed in either data acquisition or integration. Single-cell temporal data analysis on whole hearts will provide a platform to further address the remaining unresolved interactions that may due to cell-type specific differences. Indeed, new research is revealing a greater degree of assortment among these cells than previously predicted. Single-cell sequencing in the human heart has revealed high transcriptional diversity, and new targets for cell-type specific therapeutics [36, 49]. Similar approaches in

zebrafish will provide a broader molecular understanding of the developmental cardiac GRN.

In vivo validation of novel GRN components and assessment of their contribution on cardiogenesis, spatially and temporally, can be rapidly and easily performed in zebrafish. Indeed, external development and the transparency of the embryos, combined with progress in high-throughput imaging and gene editing, render zebrafish as an excellent model for further exploring spatio-temporally resolved GRNs compared to the more complex mammalian animal models [50].

Altogether, these studies have informed us that the most promising approach to studying the complex arrangement of cardiac tissues is to integrate a combination of datasets and employ powerful visualisation techniques. New technologies which study chromatin architecture and interaction of non-coding sequences and promoters have paved the way for understanding how these cardiac genes are actually regulated [44, 51, 52]. To date, there are no studies in zebrafish which combine these chromatin structure experiments with *in situ* visualisation in the heart. Studies in other models, however, have already used these modern technologies in a spatial context [53, 54]. Application of these techniques during heart development in zebrafish has significant potential to improve our understanding of basic heart developmental dynamics across species. However, significant technical challenges still impede this vision and further developments around standardised data sharing, file formats and computational pipelines are urgently required to facilitate technical feasibility [55].

Key Points

- Cardiogenesis in zebrafish is achieved by a complex series of parallel morphogenetic events that are tightly orchestrated within the first 100 hours of embryogenesis.
- Spatial transcriptomics ushers in a new era of gene expression profiling, revealing novel insights into cardiac development.
- Temporal expression data acquisition techniques for zebrafish heart are well-established, but analyses of these data remain challenging.

Author contributions

J.C.H., H.T.N., J.S., G.C., L.W., F.B. and M.R. wrote the manuscript with contributions from D.E. and E.P.

Conflict of Interest

None.

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