Transcriptional Regulation of Heart Development in Zebrafish

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Abstract: Cardiac transcription factors orchestrate the complex cellular and molecular events required to produce a functioning heart. Misregulation of the cardiac transcription program leads to embryonic developmental defects and is associated with human congenital heart diseases. Recent studies have expanded our understanding of the regulation of cardiac gene expression at an additional layer, involving the coordination of epigenetic and transcriptional regulators. In this review, we highlight and discuss discoveries made possible by the genetic and embryological tools available in the zebrafish model organism, with a focus on the novel functions of cardiac transcription factors and epigenetic and transcriptional regulatory proteins during cardiogenesis.

Keywords: zebrafish; heart development; transcription factors; epigenetic regulators

1. Introduction

Cardiac morphogenesis and the maintenance of cardiac physiology require dynamic and carefully coordinated transcription programs. Studies in pluripotent stem cells and multiple model organisms have delineated critical signaling events involving Bmp, Fgf, Wnt, and Notch that induce the expression of a set of cardiogenic transcription factors to drive cardiac specification and differentiation (for review see [1–3]). Recent studies have revealed an additional layer of epigenetic regulation that contributes to the tight temporal and spatial control of cardiac gene expression during development (for review see [4–6]). Misregulation of cardiac transcription programs not only results in severe developmental defects, but is also associated with many congenital heart diseases (CHD), the leading inborn cause of mortality in infants. A deeper mechanistic understanding of the gene programs governing normal heart development will provide insights into pathogenic events leading to CHD and may result in the development of new therapeutic strategies.

Over the last two decades the zebrafish has emerged as a powerful model organism for studying cardiac development. The mature zebrafish heart has a simplified structure, consisting of only one atrial and one ventricular chamber. Despite its anatomical differences with the four-chambered mammalian heart, the developmental mechanisms of zebrafish cardiogenesis closely parallel those of other vertebrate animal models (Figure 1). Cardiac progenitors in zebrafish are descended from cells located at the ventrolateral marginal region of the early blastula [7]. At the onset of gastrulation, these mesodermal cells are among the first to involute and migrate toward the embryonic axis. The activation of an evolutionarily conserved cardiac transcription program including GATA factors, Hand2, T-box proteins, and Nkx2.5 specifies cardiac progenitors within the anterior lateral plate mesoderm (ALPM), which subsequently migrate toward the midline of the embryo, turn on expression of cardiac sarcomere proteins, and fuse to form a beating heart tube. As has been shown in mammals, the continued growth and maturation of the zebrafish heart depend on the recruitment of additional cardiac progenitor
cells from the second heart field (SHF), which make a large contribution to the distal ventricle and outflow tract [8].

Because of the transparency of zebrafish embryos and their amenability to embryonic and molecular manipulations, the simple zebrafish heart is a convenient vertebrate model for examining the specification of cardiac progenitors, heart tube formation, and the growth and maturation of the cardiac chambers. In this review, we will discuss recent discoveries made possible by the genetic and embryological tools available in the zebrafish model organism, with a focus on the novel functions of cardiac transcription factors and epigenetic and transcriptional regulatory proteins in the myocardium during cardiogenesis.

Recent studies have also identified crosstalk between GATA factors and other critical cardiogenic signaling pathways [15–17]. For example, Tmem88a, a suppressor of canonical Wnt signaling,

2. Cardiac Transcription Factors

2.1. GATA Factors Control Cardiomyocyte Progenitor Migration and Regulate Cardiac Fate Specification by Restricting Wnt Signaling

GATA factors function at the top of the hierarchy of cardiac genes, and regulate multiple aspects of cardiogenesis by both tissue autonomous and non-autonomous mechanisms. In zebrafish, gata4, 5, and 6 are expressed in the ALPM and endoderm and control expression of myocardial and endodermal genes [9,10]. Similar to what has been observed in Gata4 knockout mice [11,12], loss of Gata5 function in zebrafish faust mutants disrupts migration of the endoderm to the midline [9,10]. Consistent with the crucial role the endoderm plays in migration of cardiac cells to the midline [11,13], faust mutants exhibit cardia bifida [9]. In addition to this non-tissue autonomous phenotype, gata5-deficient zebrafish embryos have a reduced number of cardiac progenitors, pointing to a cell intrinsic role for GATA factors in the production of cardiomyocytes [9]. Consistent with this view, overexpression of Gata5 expands the expression domains of cardiac genes including nkx2.5 within the ALPM [9]. Additionally, zebrafish embryos lacking both Gata5 and Gata6 show a complete absence of nkx2.5-positive progenitor cells, highlighting a redundant requirement of GATA factors for cardiac fate specification [14].

Recent studies have also identified crosstalk between GATA factors and other critical cardiogenic signaling pathways [15–17]. For example, Tmem88a, a suppressor of canonical Wnt signaling,
is downregulated in zebrafish gata5/6 double morphants. Both excessive and reduced tmem88a expression disrupt ntx2.5 expression in the ALPM, consistent with the biphasic function of Wnt signaling in cardiac development and highlighting a dosage-sensitive requirement for Tmem88a [16]. Similarly, knockdown of Tmem88a during directed cardiac differentiation of human ES cells increases Wnt/β-catenin signaling and results in a reduced number of cardiac progenitor cells [17], pointing to a critical role of Tmem88a in mediating crosstalk between GATA factors and the Wnt signaling pathway.

### 2.2. Hand2 Regulates Cardiac Differentiation and Morphogenesis

Hand2 is a transcription factor of the basic-helix-loop-helix family whose expression domain demarcates the heart forming region in the ALPM [18]. Forced expression of hand2 in early zebrafish embryos promotes cardiac progenitor formation at the expense of the neighboring hematopoietic and endothelial cell lineages, suggesting an early impact of hand2 on cardiac progenitor cell formation. Interestingly, the initial specification of cardiac progenitor cells does not require Hand2 activity as ntx2.5-positive cardiac progenitor cells do emerge from the ALPM in hand2-deficient embryos. However, hand2 deficient cardiac progenitor cells are much less efficient at generating differentiated cardiomyocytes. As a result, hand2-deficient embryos have fewer cardiomyocytes and these cardiomyocytes are not sufficient to support endocardial differentiation [18,19].

Myocardial precursor form polarized epithelia in zebrafish embryos following their specification from the ALPM [20,21]. In hand2 mutants, these polarized myocardial precursors fail to migrate and fuse at the midline, resulting in cardia bifida [22]. When transplanted into a wild-type host, hand2 mutant cardiomyocytes are able to migrate to the midline and integrate into the heart tube, suggesting that the migration of cardiomyocytes is dependent on Hand2 function in their environment [23]. Fibronectin is a key component of the ECM surrounding the cardiac epithelium, and cardiac progenitor migration is sensitive to its expression levels [20]. Interestingly, Fibronectin accumulation is increased in hand2 mutants and decreased upon overexpression of Hand2 [21,23]. Reduction of Fibronectin levels in hand2 mutants restored migration of cardiac progenitors to the midline [23], indicating that Hand2 promotes the migration and fusion of the cardiac epithelium by limiting Fibronectin deposition and highlighting a mechanism by which a cardiac transcription factor prepares the embryonic environment for normal cardiac morphogenesis.

### 2.3. Ntx2.5 Maintains Cardiac Chamber Identity

Ntx2.5 is a homeodomain transcription factor that is one of the earliest markers of the cardiac lineage. Genetic studies in Drosophila, zebrafish, and mice have revealed divergent functions of Ntx2.5 during early heart development. In flies, Ntx2.5 activity is essential for cardiac specification, and tinman/ntx2.5 mutants completely lack a dorsal vessel [24,25]. On the contrary, a heart tube forms normally in ntx2.5-deficient zebrafish and mouse embryos, but the subsequent differentiation and maturation of its chambers are impaired [26–28].

Loss-of-function of both Ntx2.5 and Ntx2.7, two zebrafish homologs of mammalian Ntx2.5 [29,30], results in a dysmorphic heart tube with an oversized, hyperplastic atrial chamber and a small ventricular chamber with a reduced number of cardiomyocytes [28,31]. In zebrafish, chamber-specific gene programs are established after two days of development such that distinct sets of genes are restricted to the atrium (i.e., amhc) and the ventricle (i.e., vmhc). Interestingly, amhc-positive cells are observed outside of the atrium near the arterial pole of the ntx-deficient hearts, suggesting that ntx genes are required for the maintenance of chamber identity [28,31]. Reintroduction of Ntx2.5 expression using a heat shock-inducible transgene during the time frame when cardiomyocytes are differentiating restores normal chamber-specific cardiomyocyte production in ntx2.5 mutant embryos, suggesting that early expression of Ntx2.5 may prime cardiomyocytes for their later chamber identity [32]. Furthermore, the temporary overexpression of Ntx2.5 in ntx2.5 mutants during embryonic stages permits normal cardiac function into adulthood, and demonstrates that in contrast to the established ongoing requirement of ntx2.5 in mature cardiomyocytes of adult mice,
Nkx2.5 is dispensable for adult zebrafish cardiac function [32]. Nevertheless, given the redundant roles of nklx2.5 and nklx2.7 during early cardiac development [28,31], more study is required to determine the full extent of nklx gene functions in the adult zebrafish heart.

2.4. Tbx20 Is Required for Cardiac Progenitor Formation

Tbx20 is an ancient T-box protein whose cardiac expression initiates at the time when cardiac progenitor cells emerge from mesoderm and persists to adulthood [33–37]. In mice, Tbx20 overexpression promotes cardiomyocyte proliferation whereas Tbx20 deficiency results in impaired cardiomyocyte differentiation and cardiac developmental arrest after formation of the linear heart tube [38–42]. Ablation of Tbx20 activity in adult cardiomyocytes disrupts the structure of myofibrils and causes arrhythmia in both Drosophila and mouse models [36,43], and mutations in tbx20 are associated with diverse cardiac pathologies including cardiomyopathy in humans [43–49].

While Tbx20’s function in the regulation of cardiac chamber morphogenesis and the maintenance of cardiac physiology and cellular structure appears to be conserved in all species examined, its role in cardiac progenitor cell formation is still unclear. In flies, the cardiac progenitor population is severely compromised when both tbx20 homologues are downregulated [50], but Tbx20 ablation in mice does not impact the formation of the cardiac crescent and the primitive heart tube forms in Xenopus and zebrafish tbx20 morphants [39–42,51,52]. This has led to the conclusion that Tbx20 activity is dispensable during early cardiogenesis in vertebrates. However, our recent discovery that cardiac progenitor cells are diminished in zebrafish tbx20 null mutant embryos argues against this hypothesis (Lu, Langenbacher and Chen, unpublished data). Further investigation is needed to determine whether compensatory mechanisms exist in the mouse to allow cardiac crescent formation in the absence of Tbx20 or if the zebrafish represents a transition state in the evolution of tbx20 gene function in vertebrates.

3. Epigenetic and Transcriptional Regulators

The accessibility of regulatory DNA sequences to transcription factors and RNA polymerase II is dynamically regulated by covalent histone modifications, DNA methylation, and ATP-dependent chromatin remodeling. As a result, epigenetic regulation plays a critical role in driving cardiac gene expression and cardiac fate specification. Furthermore, epigenetic regulators control the morphogenesis and maturation of the heart. For example, loss of function of the Histone 3 Lysine 4 methyltransferases Smyd3 and Setd7 in zebrafish embryos produces severe defects in cardiac chamber morphogenesis, and Histone deacetyrase activity controls valvulogenesis in zebrafish by promoting myocardial expression of Bmp4 at the atrioventricular boundary [53,54]. Here we will focus on two complexes, BAF and PAF1, which play a central role in transcriptional regulation of zebrafish cardiac development.

3.1. BAF Chromatin Remodeling Complex Induces a Cardiac Mesoderm Fate

The SWI/SNF (switching defective/sucrose non-fermenting) complex is an ATP-dependent chromatin remodeling complex that was initially purified from yeast. Its vertebrate homolog, the BAF (Brg1/Brm-associated factor) complex, contains 12 protein subunits including Baf60c/Smarcd3 and Brg1/Smarca4. Many components of the BAF complex are significantly upregulated in patients with hypertrophic cardiomyopathy [55–57] and are indirectly implicated in the pathogenesis of CHD [58,59]. The BAF complex regulates cardiogenesis by interacting with cardiac transcription factors like Tbx1, Tbx5, Nkx2.5, and Gata4 [60–62]. Loss of function of Brg1/Smarca4 and Baf60c/Smarcd3 in mouse embryos results in a wide range of congenital heart defects and the cardiac defects caused by Brg1/Smarca4 haploinsufficiency are exacerbated in an Nkx2.5, Tbx20, or Tbx5 heterozygous background, highlighting the genetic interaction between the BAF complex and the central members of the cardiac transcription program [55,60,63].

In fish, loss of function of Smarca4 or Smarcd3 causes progressive morphological and functional deterioration of the heart [61,63,64]. In addition, simultaneous overexpression of Gata5 and Smarcd3 in
zebrafish embryos leads to expanded expression of cardiac genes and an enlarged heart [61]. Similarly, Gata4/Smarcd3/Tbx5 expression promotes cardiomyocyte differentiation from non-cardiogenic mesoderm in mice [65], suggesting that GATA factors and the BAF complex have a conserved and joint role in promoting the cardiac gene program. Interestingly, when cells overexpressing both Gata5 and Smarcd3 were transplanted to regions outside of the cardiac field of wild-type host zebrafish embryos, they often contributed to the cardiac region of the ALPM and differentiated into cardiomyocytes, endocardium, and smooth muscle [61]. These findings from the zebrafish model suggest that Gata5 and Smarcd3 can induce a cardiac fate in a cell regardless of its position within the embryo as well as promote the differentiation of multiple cardiac lineages.

3.2. The PAF1 Complex Is Involved in Cardiac Progenitor Formation and Cardiomyocyte Differentiation

The Polymerase Associated Factor 1 complex (PAF1C) is an evolutionarily conserved protein complex that accompanies RNA PolII through the entire open reading frame of transcribed genes and regulates multiple transcriptional processes, including initiation, elongation, termination, 3' end formation, and transcription-coupled histone modification (for review see [66]). The PAF1C consists of five components: Leo1, Ctr9, Cdc73, Paf1, and Rtf1. Genetic studies have suggested a requirement of PAF1C components in multiple biological processes including cell cycle regulation, somite and neural crest development, and maintenance of stem cell pluripotency [67–70]. Loss of any one of the PAF1C components results in abnormal heart development in zebrafish, but, interestingly, the individual proteins of the PAF1C play distinct roles during cardiogenesis. Leo1 is required for chamber morphogenesis and the differentiation of cardiomyocytes at the atrioventricular boundary of the heart, while Ctr9, Cdc73, and Paf1 are required for generating an appropriate number of cardiomyocytes and elongation of the heart tube [69,71]. Strikingly, loss of Rtf1 causes the most severe cardiac phenotype; ablation of Rtf1 activity eliminates the cardiac progenitor population and abolishes heart formation [71]. Upon transplantation, rtf1-deficient donor cells do not contribute to the heart in wild type embryos, while wild-type donor cells are able to form cardiomyocytes in rtf1-deficient embryos, indicating that Rtf1 regulates cardiac development in a cell autonomous manner [71]. It is currently not known how individual components of the PAF1C assume different roles in cardiogenesis or why Rtf1 deficiency causes such a dramatic effect on heart formation. Given the multifunctional role that the PAF1C plays in transcription regulation, one possible explanation is that PAF1C members differentially regulate a transcriptional event critical for cardiogenesis. Alternatively, it is intriguing to note that Rtf1 is loosely associated with the PAF1C in metazoans and that PAF1C-independent regulation of transcriptional elongation by Rtf1 has been observed in fission yeast and HeLa cells [71–77]. Continued study is essential to clarify the biological significance of individual PAF1C components and provide insights into their regulation of the cardiac transcription program.

4. Summary

A multitude of novel findings in zebrafish have helped to identify the critical mechanisms by which cardiac transcription factors, chromatin remodeling proteins, and general transcriptional regulators shape the developing heart and demonstrate the power of using zebrafish as a model to increase our knowledge about cardiogenesis. A deeper understanding of the steps of cardiogenesis is critical for deciphering the molecular mechanisms underlying congenital heart defects and developing novel regenerative therapies for cardiovascular disease.

Unlike mammalian hearts, the zebrafish heart shows robust regenerative capabilities and can restore a fully functional heart even after severe injury. Following ventricular resection, new cardiomyocytes regenerate via the de-differentiation and proliferation of existing cardiomyocytes [78], and recent studies highlight the reactivation and function of embryonic cardiac gene programs during regeneration. Cardiomyocytes throughout the subepicardial ventricular layer trigger expression of gata4 before beginning to proliferate and contribute to heart regeneration [79,80]. Increased expression of hand2, nkx2.5, tbx5, and tbx20 has been observed in the myocardium surrounding sites of injury, and
higher levels of gata5 and hand2 expression were also detected within the injury site endocardium [81–83]. Strikingly, overexpression of Hand2 in adult zebrafish cardiomyocytes augments their proliferative and regenerative capacity [81]. Future studies exploring the functions of cardiac transcription factors, chromatin remodeling proteins, and general transcriptional regulators after injury may also lead to new therapies for cardiac regeneration and repair.

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