Hand Factors in Cardiac Development

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Abstract

Congenital heart defects account for 1% of infant mortality and 10% of in-utero deaths. As the vertebrate embryo develops, multiple tissue types develop in tandem to morphologically pattern the functional heart. Underlying cardiac development is a network of transcription factors known to tightly control these morphological events. Members of the Twist family of basic helix-loop-helix (bHLH) transcription factors, Hand1 and Hand2, are essential to this process. The expression patterns and functional role of Hand factors in neural crest cells (NCC), endocardium, myocardium, and epicardium is indicative of their importance during cardiogenesis; however, to date, an extensive understanding of the transcriptional targets of Hand proteins and their overall mechanism of action remain unclear. In this review, we summarize the recent findings that further outline the crucial functions of Hand factors during heart development and in post-natal heart function.

Keywords
Heart; Cardiac development; Molecular Biology; Hand1; Hand2

CARDIAC MORPHOGENESIS

Congenital heart defects (CHDs) are the leading complication in pediatric mortalities (Ottaviani & Buja 2017). CHDs result from defects, genetic or environmental, of the developing heart in the first trimester of pregnancy. Cardiogenesis initiates as early as E6.5 in mice, when cardiac progenitor cells (CPCs) are specified in the anterior lateral plate mesoderm (Tam et al. 1997). As gastrulation proceeds, two molecularly distinct CPCs arise, the primary (PHF) and secondary heart field (SHF; Kelly et al. 2014). The cells of the PHF coalesce at the midline to form a linear tube that begins beating due to pacemaker activity in the venous pole (Sylva et al. 2014). The heart tube is composed of inner endothelial/endocardial cell layer and an outer myocardial layer. Proliferating SHF cells add to the outflow and inflow tracts causing the elongation of the heart tube and its subsequent looping in an asymmetric fashion by E9.5 (van den Berg et al. 2009; Vanden Berg & Levin 2013; Francou et al. 2017).
Multiple cell lineages contribute to the developing heart. NCCs are multipotent, migratory cells that arise from the dorsal neural tube (Noisa & Raivio 2014). Cardiac NCC migrate into the heart through the caudal pharyngeal arches (PAs) to septate the outflow tract, to form a patent aorta and pulmonary artery, as well as contribute to pulmonary and aortic valves (Keyte & Hutson 2012).

The epicardium forms the outer cell layer of the heart and is required for cardiac morphogenesis (Carmona et al. 2010). In mice, as cardiac looping begins, proepicardial villi from the pericardial side of the septum transversum initiate contact with the heart (Rodgers et al. 2008). By E10.5, these epicardial cell precursors cells cover the cardiac surface excluding the outflow tract (OFT). Around E12.5, a population of epicardial cells undergo secondary epithelial to mesenchymal transition (EMT) contributing to the coronary smooth muscle, cardiac fibroblasts and connective tissues of the pulmonary and aortic valves (Krainock et al. 2016).

The endocardium cell layer, in addition to providing a patent surface layer for circulation acts in conjunction with the myocardium as the heart develops. Myocardial-endocardial signaling has been shown to be critical for cardiac morphogenesis including, trabeculation, septation, and the development of the cardiac conduction system (Haack & Abdelilah-Seyfried 2016). In addition, populations of endocardial cells undergo EMT, populating the outflow tract (OFT) and atrial-ventricular cushions which ultimately contribute to the tricuspid and mitral valves (de Lange et al. 2004; MacGrogan et al. 2014). Endocardial contributions to coronary endothelium has also been proposed with some controversy (Tian et al. 2015).

In concert, these diverse cell populations all integrate to form a functional heart and each cell lineage relies on a finely tuned gene regulatory network driven by transcription factors modulating this complex integration. The basic Helix-Loop-Helix (bHLH) transcription factors Hand1 and Hand2 play key roles within the gene regulatory networks of NCC, epicardium, myocardium, and endocardium and in this review, our focus will be on recent studies involving Hand factors in these cell lineages.

**HAND FACTORS**

Hand factors are bHLH proteins that form homo- or hetero- dimers with bHLH partners and regulate gene expression (Firulli et al. 2000; Firulli et al. 2003; Firulli et al. 2005). They function as hetero- or homo- dimers and bind to consensus E - (CANNTG) or D – (CGNNTG) box sequences within the regulatory regions of gene targets (Massari & Murre 2000). Expression and lineage-tracing experiments have determined distinct and overlapping expression domains for these proteins in the developing cNCC, epicardium, myocardium, and endocardium. (Barnes & Firulli 2009).

*Hand1* cardiac expression is first detectable in the mouse embryo at E8.5 in the posterior ventricle as well as a small domain of the developing OFT termed the myocardial cuff (Fig. 1; Barnes et al. 2011). As heart looping proceeds, *Hand1* expression is robust within the left ventricle (LV) between E9.5 –E13.5, is detected in both cNCC and SHF-derived myocardial
cuff of the OFT, and pericardium (Barbosa et al. 2007; Barnes et al. 2010). Hand1 expression is not detectable within endocardial or epicardial cells; however, the epicardium, and all its derivatives, is Hand1-lineage derived (Barnes et al. 2010; Barnes et al. 2011).

Hand2 expression within cardiac and endocardial progenitors is detectable at E7.75 within the cardiac crescent (Fig. 1; Barnes et al. 2011). Hand2 is robustly expressed within the SHF pharyngeal mesoderm that underlies and contributes to the growing heart tube (Tsuchihashi et al. 2011; Barnes et al. 2011). Through cardiac looping, low levels of Hand2 myocardial expression is observed but endocardial expression is most robust (Fig. 1; VanDusen, Vincentz, et al. 2014). Hand2 expression is also observed within cardiac NCC and myocardial cuff within the outflow tract as well as in the proepicardial organ and forming epicardium (VanDusen, Casanovas, et al. 2014). Systemic knockout of Hand2 is embryonically lethal at E10.5 due to right ventricular (RV) hypoplasia and vascular malformations (Srivastava et al. 1997).

HAND FACTORS IN CARDIAC NEURAL CREST CELLS

cNCC migrate into the caudal PAs between E9.5 and E10.5 and play a key role in the patterning of the OFT (Yutzey & Kirby 2002). Hand gene expression is not detected in cNCC until post-migration (Vincentz et al. 2008) suggesting that their function lies in the patterning and differentiation of cNCC into OFT structures. Indeed, loss-of-function studies for Hand1 and Hand2 show an interesting functionally redundant role. When Hand1 is deleted with Wnt1-Cre, resulting Hand1 conditional knockouts are viable and fertile (Barbosa et al. 2007). However, reduction in Hand2 gene dosage leads to mice born in expected ratios but die shortly after birth due to a failure to suckle (Barbosa et al. 2007). Examination of gene expression shows dysregulation of Pax9, Msx2 and Prx2 in the developing mesenchyme. Loss of Hand1 in conjunction with presence of only one copy of Hand2 or deletion of Hand2 in the pharyngeal arches leads to defects in neural crest derived distal midline mesenchyme development (Barbosa et al. 2007).

More recently, conditional knockout of Hand2 using Wnt1-Cre display misalignment of the OFT, defective aortic arch arteries accompanied by ventricular septal defects (VSDs; Hendershot et al. 2008; Holler et al. 2010). Gene expression analysis of Wnt1-Cre; Hand2 conditional knockouts show changes in genes involved in neural crest cell cycle and migration (Holler et al. 2010).

Loss of Hand2 results in reduction of Hand1 expression in cranial neural crest cells (Barron et al. 2011). However, Hand1 expression within the cardiac neural crest cells is not dependent on Hand2 (Vincentz et al. 2016).

HAND FACTORS IN EPICARDIUM

Hand1 expression is observed in the septum transversum at E9.5 but Hand1 expression is not observed in the epicardium (Barnes et al. 2011). Lineage tracing obtained from a Cre knocked into the Hand1 allele showed that Hand1-lineage marks the proepicardium, epicardium, and epicardial derivatives – the cardiac fibroblasts and coronary smooth muscle (Barnes et al. 2010; Barnes et al. 2011). Thus, there is a temporal cascade of Hand1 and
Hand2 expression; epicardial precursor cells express Hand1 and as these cells migrate to the proepicardial organ, Hand2 expression is turned on. When Hand2 is conditionally deleted in the Hand1 lineage (H2CKO), epicardial expression of Hand2 is lost indicating that within the epicardium, Hand2 lies downstream of Hand1 (Barnes et al. 2011). These H2CKO display defective epicardial EMT, decreased cardiac fibroblasts and nonfunctional coronary vasculature that leads to embryonic death by E16.5. Using a tamoxifen-inducible WT1Cre to ablate Hand2 in epicardial cells leads to a similar phenotype (Barnes et al. 2011). During secondary epicardial EMT, Pdgfra is involved in cardiac fibroblast differentiation and Pdgfrβ is important for vascular smooth muscle differentiation (Krainock et al. 2016). In H2CKOs, Pdgfra is downregulated whereas Pdgfrβ expression is upregulated (Barnes et al. 2011). These data suggest an important role for Hand factors in the function of the epicardium.

**HAND FACTORS IN CARDIOMYOCYTES**

The Hand factors are excellent markers of ventricular identity; changes in their expression correlate with altered cardiac morphogenesis. For example, knockdown of Tbx20 results in decreased expression of both Hand1 and Hand2 by E9.0 resulting in defective heart formation and hypoplasia of the RV and outflow tract (Takeuchi et al. 2005). Similarly, knockout of Bmpr1a in cardiac progenitors using MesP1-Cre leads to loss of Hand1 expressing cells (Klaus et al. 2007). The requirement of patterning by Hand factors early in the developing embryo highlights their importance in the development of the ventricles.

Hand1 systemic knockout mice die by E9.5 due to defects in extra embryonic tissue and cardiac morphogenesis (Firulli et al. 1998; Riley et al. 1998). Heart development in Hand1 homozygous null embryos is arrested due to failure of heart tube fusion at the caudal portion (Firulli et al. 1998). Conditional myocardial deletion of Hand1 using the cardiomyocyte specific αMHC-Cre and Nkx2.5-Cre leads to reduced ventricular growth and maturation resulting in neonatal lethality (McFadden et al. 2005). Mice expressing a Hand1 hypomorphic allele with 30% Hand1 mRNA expression, survive longer than the systemic knockout, E10.5 to E12.5, exhibiting thin left ventricular myocardium along with extra-embryonic tissue defects (Firulli et al. 2010). In order to examine the role of Hand1 in adult tissue post myocardial infarction, adult male mice haploinsufficient for Hand1 were subjected to ligation of left anterior descending coronary artery (S. Lu et al. 2016). Post myocardial infarction, Hand1+/− mice show better heart function and decreased Matrix Metalloproteinase-9, MMP-9 expression, which might be a possible mechanism for protection post injury due to decreased cardiomyocyte apoptosis (S. Lu et al. 2016). In adult rats, post injury, Hand1 expression is downregulated (Thattaliyath, Livi, et al. 2002).

Hand1 gain-of-function analysis using MLC2V promoter leads to enlarged RV and LV and loss of the intraventricular septum (Togi et al. 2004). Conditional Hand1 gain-of-function in adult cardiomyocytes using a doxycycline inducible system results in arrhythmias (Breckenridge et al. 2009).

Recently the cis-regulatory elements that control Hand1 expression in the myocardial cuff, cNCC, and, LV have been identified (Fig 2; Vincentz et al. 2017). Analysis of the conserved
regions upstream of the Hand1 locus suggests putative enhancer DNA-binding sites (Fig. 2). A 744bp conserved enhancer region was isolated and shown to drive Hand1 expression exclusively in the LV (Fig 2; Vincentz et al. 2017). This Hand1 enhancer was used to drive Cre recombinase expression selectively in the LV. Hand1^LV Cre. Loss of the Hand1 lineage LV cardiomyocytes using the Hand1^LV Cre with Diphtheria Toxin A (R26R^DTA) allele results in hypoplastic LV at E10.5. Surprisingly however, by E16.5, LV sizes are indistinguishable from controls, suggesting a compensatory growth by non-Hand1 lineage cardiomyocytes in the developing LV. Since there is overlapping expression of Hand2 in the myocardium by E11.5 and Hand2 has been shown to compensate for loss of Hand1, further studies were performed ablating both Hand1 and Hand2 using Hand1^LV Cre. The loss of both Hand factors leads to a LV cardiomyocyte hyperplasia resulting in a severely occluded LV lumen, disorganized IVS, hyperplastic mitral valves and double outlet RV. The Hand1^LV knockout mice survive at a rate of 50% to birth with adults that have a severely compromised systolic function as measured by fractional shortening and ejection fraction.

Cardiac marker analysis shows expansion of IVS (Irx2 and Dkk) and compact zone (Tbx20 and Hey2) marker genes. An increase in proliferation of cells in the LV trabeculae was shown to cause the LV hyperplasia phenotype. Ablation of Hand1, Hand2 and both Hand1/Hand2 with Hand1^LV Cre in the presence of R26R^DTA rescues this phenotype. This study demonstrates that the Hand1 negative cardiomyocytes populating the LV are sufficient to generate a functional chamber; however, both Hand1 and Hand2 are required for normal LV development. Loss of both genes in the LV leads to a mispatterning of the LV and IVS as determined by marker gene expression.

Hand2 systemic knockout in mouse results in severe hypoplasia of the RV and dilated aortic sac by E9.5 and embryos die by E10.5 (Srivastava et al. 1997). These apoptotic RVs misexpress markers such as Irx4 (Srivastava et al. 1997; Yamagishi et al. 2001). Overexpression of Hand2 in ventricles leads to absence of IVS which is a further indication that Hand2 expression is critical for the patterning of ventricles (Togi et al. 2006). Interestingly, Hand2 along with Gata4, Mef2c and Tbx5 are able to reprogram adult mouse cardiac fibroblasts into functional cardiomyocytes both in vitro and in vivo (Song et al. 2012).

When Hand2 is ablated using Nkx2.5-Cre, cardiac looping takes places with the development of RV and LV, but mice die by E12.5 due to the lack of cardiomyocyte expansion (Tsuchihashi et al. 2011). This study also knocked out Hand2 with Isl1-Cre, which is expressed in all early SHF cells, leading to embryonic death by E10.5 and severe hypoplasia of the right ventricle, similar to the systemic knockout. The investigators next used Mef2c AHF-Cre, which marks cells that give rise to the outflow track and right ventricle including the IVS, to ablate Hand2. These embryos die by E13.5, have thin RV myocardium and VSDs. Hand2 is required for the early survival of SHF progenitors and loss of Hand2 in these populations results in defective myocardium and IVS (Tsuchihashi et al. 2011).

Hand2 has been shown to regulate Nppa independent of its DNA binding ability (Thattaliyath, Firulli, et al. 2002). Hand2 ChIP-seq analysis was performed using a mouse
line targeted with 3xFLAG epitope tag to the N-terminus of Hand2 by Laurent et al to study transcriptional targets of Hand2 on a genome wide scale (Laurent et al. 2017).

Gene ontology analysis of the Hand2 cistrome and whole mount in-situ hybridization showed that genes involved in EMT, specifically Has2 and Snai1 to be direct targets of Hand2 at E9.5 in the heart. The Snai1 locus was shown to contain two Hand2 responsive cis-regulatory elements that drive Snai1 expression in the heart (Laurent et al. 2017).

Spatial and temporal regulation of Hand2 expression was shown to involve the microRNA, miR-1 (Zhao et al. 2005). In these experiments, perturbation of Hand2 expression by knocking out miR-1 led to changes in proliferation of ventricular myocytes. Hand2 mediated RV maturation depends on activation of enhancer regions in the Hand2 promoter that bind GATA4 (McFadden et al. 2000). This is further confirmed by conditional deletion of Gata4 in the heart with Nkx2.5Cre which leads to the loss of expression of Hand2 (Zeisberg et al. 2005). More recently, upperhand been shown to directly regulate Hand2 expression in cis during development (Anderson et al. 2016). Systemic knockout of upperhand leads to loss of Hand2 expression in the ventricles and outflow tract.

In the adult, cardiac dysfunction is most commonly attributed to cardiomyopathies that impair LV function such as hypoplastic left heart syndrome (HLHS) and LV non-compaction (LVNC). The Hand factors have been implicated in the etiology of these diseases (Jiang et al. 2014). Formalin fixed heart tissue samples from human patients with HLHS were reported to contain protein mutations in Hand1 (Reamon-Buettner et al. 2008; Wang et al. 2011). However, further analysis from fresh frozen tissue from HLHS patients did not recapitulate this finding (Esposito et al. 2011; Durbin et al. 2017). Analysis of the molecular consequences of this mutation in mice was conducted by generating a transgenic animal with mutant Hand1 allele that has a nucleotide deletion at codon 126, causing a frameshift mutation leading to termination after 13 amino acids with a stop flox cassette that allows conditional activation (Firulli, Toolan, et al. 2017). When crossed with Nkx2.5Cre, embryos die by E14.5 accompanied by cardiac outflow tract and IVS abnormalities. Using αMHC-Cre or Mef2c AHF-Cre to express mutant protein in cardiomyocytes results in reduced phenotype and limited viability. As was determined by examination of fresh frozen human tissue samples (Esposito et al. 2011; Durbin et al. 2017), LVs of Hand1A126FS mutant mice are not hypoplastic (Firulli, Toolan, et al. 2017).

Single nucleotide polymorphisms and haploinsufficiency at the Hand1 locus are associated with CHDs (Starkovich et al. 2016). Human CHDs have been linked with defects in Hand2 expression as well (Tamura et al. 2013; C.-X. Lu et al. 2016). Hand2 has also been shown to be induced in post-natal cardiomyopathies and required to drive pressure overload induced cardiac remodeling (Thattaliyath, Livi, et al. 2002; Dirkx et al. 2013). More extensive examination is required to determine the exact molecular interactions leading to these CHD phenotypes. The critical role that Hand1 and Hand2 play in early development would seem to preclude mutations in the protein itself. It is more likely that changes in the enhancer regions controlling the expression of these genes are responsible for human CHDs.
HAND FACTORS IN ENDOCARDIUM

Conditional knockout of Hand1 using αMHC-Cre displayed defects in endocardial cushions (McFadden et al. 2005). However, since lineage trace analysis do not identify Hand1 expressing cells in the endocardium (Barnes et al. 2010), these phenotypes are most likely due to defects in myocardial signaling.

Hand2 is strongly expressed in the endocardium (Fig. 1; Barnes et al. 2011). In mice where the Hand2 DNA binding domain is perturbed, at E11.5 show disorganized endocardial cushions with reduced trabeculation and are lethal by E12.5 (Liu et al. 2009). Hand2 ablation using the Met2c AHF-Cre, which marks SHF progenitors that contribute to the endocardium, shows defects in the tricuspid valve, tricuspid atresia (Tsuchihashi et al. 2011). However, cardiomyocyte specific loss of Hand2 does not show this phenotype (Tsuchihashi et al. 2011). Ablating Hand2 specifically in the endocardium using either the Tie2-Cre or Nfatc1Cre (H2CKO) leads to tricuspid atresia and double inlet LV (VanDusen, Casanovas, et al. 2014). This study also shows that loss of Hand2 in endocardial populations resulted in reduced trabeculae in the developing ventricles as assessed by expression of Bmp10. Hand2 function within the endocardium lies within the Notch signaling pathway as Hand2 is down-regulated in the endocardium of RBPJk knockout mice, the target transcription factor for canonical Notch signaling. An endocardial specific conditional knockout generated using Tie2-Cre deletion of EphrinB2 (EfnB2), a direct Notch target also leads to a loss of Hand2 expression. However, conditionally ablating Hand2 using the same Cre does not lead to a change in EfnB2 expression. Although, the trabeculation growth factor, Neuregulin1 (Nrg1), also an EfnB2 target, is downregulated in H2CKO as assessed by ISH and qRT-PCR and is directly regulated by Hand2 as shown by Nrg1 promoter analysis. Conditional gain-of-function of Hand2 in Nfatc1-Cre conditional knockouts of EfnB2 leads to a rescue of Nrg1 and Bmp10 positive trabeculae. Thus, these data demonstrated that Hand2 acts downstream of EfnB2 but upstream of Nrg1 to regulate endocardial development. H2CKO also exhibits a hypervascularization phenotype (VanDusen, Casanovas, et al. 2014). Genes involved in vascular differentiation, specifically components of Vegf signaling, were mesregulated with increased expression of VegfR3, Vegfa, and Lyve-1. Direct regulation by Hand2 was shown by promoter analysis of a Vegf signaling cofactor, Nrp1 using ChIP-seq and luciferase assays. These results show the importance of Hand2 in the development of the endothelial component of coronary vasculature.

The role that Hand factors play in development is well studied and some of the molecular pathways that these transcription factors are involved in have recently been elucidated. Although not discussed in this review, Hand factors also play an important role in other tissue types, most notably the limb (Osterwalder et al. 2014; Firulli, Milliar, et al. 2017) and craniofacial tissues (Firulli et al. 2014). The gene regulatory networks that involve Hand factors also remain an area of focus. Further work looking at the mechanism of action of Hand factors is necessary to fully elucidate their biological function.
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FIGURE 1:
Schematic representation of stages of mammalian heart development with expression of Hand1 (in green) and Hand2 (in yellow) as marked at the respective stages. CC: Cardiac Crescent; OFT: Outflow tract; SV: Sinus Venosus; endo: Endocardium; RA: Right Atrium, RV: Right Ventricle; LA: Left Atrium; LV: Left Ventricle.
FIGURE 2:
The mouse Hand1 locus as visualized in http://genome.ucsc.edu (Kent et al. 2002). The 5’ region has mammalian conserved regions (red boxes) that have been interrogated for their spatial and temporal specific activity. SG: sympathetic ganglia; LV: left ventricle; OFT: outflow tract; ST: septum transversum.