Published in final edited form as:
Birth Defects Res A Clin Mol Teratol. 2011 June ; 91(6): 485–494. doi:10.1002/bdra.20796.

Hand factors as regulators of cardiac morphogenesis and implications for congenital heart defects

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Abstract

Almost 15 years of careful study have established the related bHLH transcription factors *Hand1* and *Hand2* as critical for heart development across evolution. Hand factors make broad contributions, revealed through animal models, to the development of multiple cellular lineages that ultimately contribute to the heart. They perform critical roles in ventricular cardiomyocyte growth, differentiation, morphogenesis, and conduction. They are also important for the proper development of the cardiac outflow tract, epicardium, and endocardium. Molecularly, they function both through DNA-binding and through protein-protein interactions, which are regulated transcriptionally, post-transcriptionally by microRNAs, and post-translationally through phospho-regulation, Although direct Hand factor transcriptional targets are progressively being identified, confirmed direct targets of Hand factor transcriptional activity in the heart are limited. Identification of these targets will be critical to model the mechanisms by which Hand factor bHLH interactions affect developmental pathways. Improved understanding of Hand factor-mediated transcriptional cascades will be necessary to determine how Hand factor disregulation translates to human disease phenotypes. The following review summarizes the insight animal models have provided into the regulation and function of these factors during heart development, and the recent findings that suggest roles for *HAND1* and *HAND2* in human congenital heart disease.

Keywords

Basic helix-loop-helix; HAND1; HAND2; dimerization; Transcription; DNA-binding; Cardiogenesis; Heart development; Congenital heart defect

Introduction

Cardiac development involves the coordination of multiple cell types and differentiation programs

The heart is composed of disparate cell types, which progressively assume divergent cell fates as cardiogenesis advances. For example, cells derived from the epicardium ultimately differentiate into both cardiac fibroblasts and the smooth muscle of the coronary vasculature. However, in certain instances, these distinct cell types must assume similar characteristics to function as a cohesive unit. For example, the left and right ventricles are derived from distinct cell populations, termed the primary and secondary heart fields (PHF and SHF), respectively, and exhibit unique morphological characteristics, such as chamber shape and wall thickness. However, these two chambers also express common metabolic, cytoskeletal

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and cell-cell junction proteins that enable myocytes to synchronize contractile function. These conserved muscle gene programs may reflect the influence of pan-cardiac transcription factors, such as Nkx2.5 and Gata4, which are expressed in both forming ventricles. Interestingly, closely related factors such as Hand1 and Hand2, which perform both unique and redundant transcriptional functions, may both draw distinctions between the developing ventricular chambers and regulate a common coterie of cardiac myogenic and morphogenic promoters within the defined primary and secondary cardiac fields. Further, these factors may operate in similar unique and redundant manners within other developing heart tissues, such as the cardiac outflow tract (OFT) and the proepicardium (PE).

Regulation of Hand factor function

Hand1 and Hand2, members of the Twist-family of basic Helix-Loop-Helix (bHLH) proteins, function as either homo- or heterodimers, binding consensus elements termed E-boxes (CANNTG), or related, degenerate sequences termed D-boxes (CGNNTG), and regulating downstream effector genes (Barnes and Firulli, 2009; Conway et al. 2010; Firulli, 2003). They possess two characterized mechanisms of regulation. Twist-family member function can be regulated through adjustment of relative gene expression levels (Fig. 1). For example, *Hand2* and *Twist1*, a related Twist-family bHLH transcription factor, perform antagonistic functions in the developing limb, and proper anterior-posterior limb patterning depends upon maintenance of relative expression levels between these two factors (Firulli et al. 2005). It is likely that Twist-family members function in evolutionarily established equilibrium not only in the limb, but also in other tissues in which they display overlapping expression domains, such as those of the developing heart.

Hand1 and Hand2 function is also dictated by dimer partner choice, and this choice can be influenced via phosphorylation of evolutionarily conserved threonine and serine residues within the bHLH domain (Fig. 1; (Firulli et al. 2003). Twist-family members are characterized by conserved serine and threonine residues located in helix I. Phosphorylation of these residues alters both the dimerization affinity of these factors and, consequently, their DNA binding specificity (Firulli et al. 2007). Additionally, this phosphoregulation may direct Hand factor subcellular localization, consequently affecting dimerization (Martindill et al. 2007).

Both Hand1 and Hand2 play critical roles during cardiogenesis, as has been demonstrated through systemic and tissue-specific gene ablation studies. Hand factors contribute to the maturation of several substructures within the developing heart, namely the cardiomyocytes of the ventricles, myocardial and cNCC contributions to the OFT, and the mesothelial cell population forming the epicardium (Barnes et al. 2010). Hand2, potentially in conjunction with Twist1, also likely functions within the endothelial populations of the endocardium and cardiac vales. In the primitive heart tube, *Hand* genes are upregulated as the early heart tube undergoes looping, with *Hand2* expression marking myocardium in the outflow tract (OFT), and right ventricle (RV), and *Hand1* expression making in the outer curvature of the left ventricle (LV) and, to a more restricted degree than *Hand2*, the OFT myocardial cuff (Fig. 2). *Hand1* and *Hand2* are also dynamically expressed in the cNCCs in partially overlapping patterns. They are both strongly expressed within the NCCs that populate the pharyngeal arches, with *Hand2* more broadly expressed and *Hand1* more restricted to the medio-ventral cNCCs proximal to the OFT, which will ultimately contribute to the OFT cushions (Fig. 2). They are further expressed in some, but not all, of the cNCCs invading the OFT cushions. Recent studies also indicate that *Hand1* and *Hand2* are expressed complementarily in the progenitors of the epicardium (Barnes et al. 2010). *Hand1* is expressed in mesothelial cell populations in the E8.5 mouse embryo, including the septum transversum (Barnes et al. 2010). Genetic lineage trace analyses indicate that as *Hand1* is downregulated in these cells, they contribute to the proepicardial organ, where cells express *Hand2*. The cells of the

proepicardial organ progressively migrate to envelop the heart, ultimately undergoing EMT and contributing to the cardiac fibroblasts and the smooth muscle cells of the coronary vasculature. Thus *Hand1* and *Hand2* are expressed in spatiotemporally distinct manners as these cells mature and differentiate and likely play a role in defining these extra-cardiac cell populations.

As relative Twist family member expression levels dictate their cellular function, unraveling the mechanisms by which *Hand1* and *Hand2* expression is regulated in these tissues is pivotal to understanding their roles in cardiogenesis. Thus far, studies identifying the direct transcriptional regulators of *Hand1* and *Hand2* in the heart are limited. A *Hand2 cis*-regulatory element that is bound and activated by Gata factors and that drives gene expression in the RV and OFT has been isolated (McFadden et al. 2000). The *cis*-regulatory elements governing *Hand1* expression in the heart remain elusive.

Cellular protein levels can additionally be controlled posttranscriptionally by microRNAs (miRNAs) (Ambros, 2004; Kloosterman and Plasterk, 2006; Zhao and Srivastava, 2007). As miRNAs can serve to “fine-tune” protein synthesis (Baek et al. 2008; Selbach et al. 2008), they are strong candidate regulators of the precise cellular Twist family member levels required to properly implement developmental programs. The closely related miRNAs, *mirR-1-1* and *mirR-1-2*, are both expressed in the developing heart where, as shown through *in vivo* overexpression studies, they negatively regulate ventricular cardiomyocyte proliferation (Zhao et al. 2005). *In silico* analyses identified *Hand2* as a putative target of these miRNAs, and indeed, Hand2 protein levels are reduced in *miR-1* overexpressing hearts, while RNA levels remain unchanged (Zhao et al. 2005). Conversely, targeted deletion of *mirR-1-2* leads to a 4-fold upregulation of Hand2 protein expression (Zhao et al. 2007). Currently, no microRNAs have been associated in regulating Hand1 protein levels, although *in silco* analysis indentifies several seed-sequences within its 3’ UTR. Thus, cardiac Hand factor expression levels are partially controlled by microRNAs.

Hand factors regulate specific aspects of cardiomyocyte growth, differentiation, morphogenesis, and conduction

Disruption of *Hand1* function in the mouse heart causes myocardial defects

Insight into the functional roles Hand1 and Hand2 play during cardiogenesis stems largely from animal models. Cardiovascular defects are observed when the *Drosophila* Hand factor orthologue, *Hand*, is disrupted, indicating an evolutionarily conserved requirement for these factors during cardiogenesis (Lo et al. 2007). Systemic ablation of *Hand1* in mice results in embryonic lethality at E9.5, most likely owing to defects in trophonoblast differentiation (Firulli et al. 1998; Riley et al. 1998) and extra-embryonic vascularization (Morikawa and Cserjesi, 2004). *Hand1*$^{-/-}$ KO embryos experience growth arrest at E7.5, and fail to undergo turning. Heart development in these mutants arrest and the primitive heart tube fails to undergo looping (Firulli et al. 1998; Riley et al. 1998). Rescue of extra-embryonic phenotypes through tetraploid aggregation extends embryonic viability to E10.5, and while these rescued embryos undergo turning normally, the heart tube nonetheless fails to loop (Riley et al. 1998). These mutant hearts display diminished cardiomyocyte cell numbers and an absence of ventricular trabeculation (Riley et al. 1998). Hypomorphic alleles of *Hand1* enable analyses of mutant heart development at later stages (Firulli et al. 2010). Embryos homozygous for each of these two alleles express *Hand1* mRNA at either 40% or 30%, and survive to E12.5 and E10.5, respectively (Firulli et al. 2010). Although the hearts in these mutants loop normally, they display a thin LV myocardium, hypotrabeculation, and diminished expression of the LV-specific markers, *Nppa*, *Chisel*, and *Cited1* (Firulli et al. 2010). *Hand1* has also been ablated tissue-specifically to analyze associated cardiac

phenotypes in further detail. Deletion of *Hand1* specifically in the myocardium, with the exception of a few escapers (<2%) in the case of α*MHC-Cre*, results in perinatal lethality (McFadden et al. 2005). These mice display a spectrum of congenital heart defects, including membranous ventricular septal defects (VSDs), overriding aorta, hyperplastic atrioventricular (AV) valves, and double outlet right ventricle (DORV). Although LV chamber size is reduced in these mutants at mid gestation (E11.5–E13.5), ventricular size recovers by birth. However, the muscular ventricular septum is noticeably thickened in these mutants at this stage, which may indicate that the "rescue" of ventricular size in these mutants occurs through a hypertrophic mechanism.

Misexpression of *Hand1* in the mouse heart causes myocardial defects

Overexpression studies have, in conjunction with loss-of-function analyses, provided insight into the role of *Hand1* during cardiogenesis. Overexpression of *Hand1* within its endogenous expression domain results in a range of ventricular phenotypes, including a variable reduction in ventricular expansion and aberrant cardiomyocyte differentiation (Risebro et al. 2006). Cardiomyocytes in these mutants display elevated myocyte density and hypertrophy. These gain-of-function phenotypes are strikingly similar to those associated with loss of *Hand1* function. As perturbation of the cellular bHLH factor balance results in inappropriate dimer formation, potentially derailing normal development, comparable structural abnormalities could result from either too much or too little Hand1.

Hand1 misexpression studies have also uncovered a role for Hand1 during interventricular septum formation. *Hand1* misexpression throughout both ventricles, achieved by targeting a *Hand1* expression cassette to the *Mlc2v* locus, leads to an increased expansion of the outer curvatures of the right and left ventricles, although the myocardium itself is thin (Togi et al. 2004). Interestingly, no interventricular groove or septum forms in these knock-in embryos, although *Tbx5* expression remains chamber-specific. This loss of septum phenotype is not recapitulated when *Hand1* misexpression is confined to the RV. *Hand1*-lineage analysis reveals that *Hand1*-marked cells are largely excluded from the septum, suggesting that forced ectopic *Hand1* expression causes this phenotype (Barnes et al. 2010). Thus, in addition to its role in ventricular cardiomyocyte expansion, *Hand1* may function to establish the boundary of the nascent ventricular septum.

In addition to ventricular proliferation and differentiation, *Hand1* influences cardiac conduction. Additional genetic studies have used a doxycycline inducible tet-on system to overexpress *Hand1* roughly six-fold in adult mouse myocardium (Breckenridge et al. 2009). One month after transgene induction, these *Hand1*-overexpressers display a mild ventricular hypertrophy (~10% increase in cell diameter). Despite displaying no overt change in their behavior or cardiac pressure/volume relationships, *Hand1*-overexpressing mice displayed an increased 12-month mortality compared to control animals. Surface ECGs revealed that, compared to controls, *Hand1*-overexpressers displayed significantly prolonged PR, QRS and RR intervals (but not QTc) both 28 days and 250 days after induction. Pacing experiments designed to assess the ventricular tachycardia (VT) threshold of *Hand1*-overexpressing hearts revealed a significantly prolonged sinus node recovery time (SNRT600). These hearts showed an increased susceptibility to VT, and VT lasted longer than in control mice. Thus, these mice likely die from ventricular arrhythmia. Immunohistohemistry reveals that Connexin43 (Gja1, Cx43), a major component of the ventricular cardiomyocyte gap junction, is downregulated in the ventricular cardiac intercalated discs of *Hand1* overexpressers. However, qRT-PCR reveals that *Gja1* mRNA is not similarly downregulated, indicating a post-transcriptional mechanism of regulation. Conversely, β-catenin shows both increased protein levels within the intercalated discs and increased mRNA levels in *Hand1* overexpressing ventricles. *Gja1* downregulation has been linked with ventricular arrhythmias and sudden death in both transgenic models (Gutstein et al.

2001; Litchenberg et al. 2000) and human patients (Dupont et al. 2001; Kostin et al. 2004; Sepp et al. 1996), whereas β-catenin protein upregulation at the intercalated disc has been associated with human hypertrophic cardiomyopathy (Masuelli et al. 2003). These data provide molecular evidence that cardiomyocyte electrical coupling is compromised in *Hand1* overexpressers. Importantly, these molecular and physiological defects could be ameliorated through the withdrawal of doxycycline.

Confirmed direct transcriptional targets of Hand1 are rare. The actin monomer-binding protein *Thymosin β4* was identified in a differential screen of wild-type and *Hand1*$^{-/-}$ embryoid bodies as a downstream target of Hand1 (Smart et al. 2002). Further studies have confirmed that *Thymosin β4* is a direct transcriptional target of Hand1 (Smart et al. 2010). Although the contribution of this regulatory relationship to cardiogenesis has not been defined, evidence suggests Thymosin β4 may stimulate vascular and myocardial growth while inhibiting myocardial cell death (Shrivastava et al. 2010).

Disruption of *Hand2* function in the mouse generates cardiac phenotypes

Ablation of *Hand2* in mice similarly generates cardiac defects and results in early embryonic lethality. *Hand2*$^{-/-}$ mutants die by E10.5, displaying hypoplasia of both the ventricles and the pharyngeal arches (Srivastava et al. 1997; Thomas et al. 1998), as well as vascular defects (Yamagishi et al. 2000). Like *Hand1*, it has been proposed that Hand2 also regulates cardiomyocyte differentiation, specifically in the right ventricle (Srivastava et al. 1997). Alternatively, it is thought that Hand2 inhibits apoptosis in the developing right ventricle (Srivastava, 1999; Yamagishi et al. 1999). Although expression of the ventricle-specific marker *myosin light chain 2v* (*Mlc2v*) is maintained in *Hand2*$^{-/-}$ mutants (Srivastava et al. 1997), the domain of a transgene driving *lacZ* under the control of the *Hand2* RV-specific enhancer is reduced (McFadden et al. 2000), suggesting either that the RV is reduced in these mutants, or that Hand2 regulates its own expression. The *Hand2* RV-specific enhancer does contain highly conserved E-box elements; however, as mentioned, it has been shown to be dependent on the presence of conserved Gata *cis*-elements (McFadden et al. 2000). Conditional alleles of *Hand2* are now available (Hendershot et al. 2007; Morikawa et al. 2007). Myocardial deletion of *Hand2* using the cardiac *troponin Cre* (*cTNTCre*; (Morikawa and Cserjesi, 2008) shows a near phenocopy of the *Hand2* systemic knockout. Further studies incorporating the various heart-specific *Cre* drivers available will be important to better understand the etiology of the *Hand2*$^{-/-}$ myocardial phenotype.

Functional interactions and redundancy between *Hand1* and *Hand2*

As Hand1 and Hand2 exhibit similar functional properties, both sharing dimer partners and binding to E- and D-boxes, it is thought that they may perform redundant functions. Treatment of chick hearts, which express *Hand1* and *Hand2* in uniform and completely overlapping patterns, with antisense oligonucleotides against both factors causes heart development to arrest during looping. When either *Hand1* or *Hand2* antisense oligonucleotides are used individually, heart development proceeds normally (Srivastava et al. 1995). These results indicate that, in the chick, Hand1 and Hand2 can functionally compensate for each other's absence.

Studies in the mouse have provided additional evidence that *Hand1* and *Hand2* share biological functions. As mentioned, conditional ablation of *Hand1* in the myocardium causes a reduction of the LV during mid-gestation (McFadden et al. 2005). Breeding these embryos onto a *Hand2*$^{+/-}$ haploinsufficient background exacerbates this phenotype. Unlike *Hand1* conditional knockouts, *Hand1*$^{fx/-}$;*Hand2*$^{+/-}$;*Nkx2.5-Cre(+)* embryos are not recovered after E10.5 (McFadden et al. 2005) and present a comparatively thin, poorly trabeculated myocardium. Thus, Hand1 and Hand2 can perform redundant functions, or at

least participate within the same molecular networks, and a cumulative critical gene dosage of both factors is necessary for proper heart development. Hand1 and Hand2 can form heterodimers with each other (Firulli et al. 2000; Firulli et al. 2003), and their respective contribution to such a heterodimer would by default be defined as functionally redundant. Rigorous assessment of functional redundancy between *Hand1* and *Hand2* necessitates gene replacement strategies, which have yet to be reported. Conversely, breeding a *Hand1* conditional allele onto a *Hand2* null background (*Hand1*$^{fx/+}$;*Hand2*$^{-/-}$;*Nkx2.5-Cre[+]*) led to an increased deposition of cardiac jelly, the dense extracellular matrix (ECM) that lies between the myocardium and endocardium (McFadden et al. 2005). As these embryos die at E9.5, however, the ultimate developmental impact of this increased matrix deposition cannot be assessed.

Hand factors function independently of DNA binding in developing cardiomyocytes

Given that *Hand1* and *Hand2* encode transcription factors, it would be convenient to suppose that the loss-of-function phenotypes observed for each mutant reflect disruption of Hand factor-mediated transcriptional programs. Studies using a targeted allele of *Hand2* in which basic domain function has been abolished have shown that Hand2 is able to partially function in vivo in the absence of DNA binding (Liu et al. 2009). Indeed, although the RV and OFT of systemic *Hand2* mutants are severely hypoplastic, causing lethality by E10.5, the heart is structurally normal, if somewhat smaller, in these DNA-binding mutants. Somewhat similarly to the phenotypes observed in *Hand1* misexpressing mutants, *Hand2* DNA-binding mutants display abnormal myocardial thickness and ventricular septum formation. At E11.5 these mutants have a thin left ventricular myocardium, and though the interventricular groove begins to form, it is developmentally delayed and its myocytes fail to thicken. Although histologically similar to wild-type counterparts at E9.5, expression of the ventricle-specific homeobox transcription factor *Irx4* is slightly decreased in *Hand2* DNA-binding mutants, suggesting that Hand2-mediated regulation of *Irx4* expression requires DNA binding. These findings indicate that, during cardiogenesis, Hand2 has both DNA-binding dependent and independent functions, both of which mechanistically rely on dimerization. Furthermore, it is important to consider that when Hand factors are expressed in ectopic locations that their presence will have profound effects on the bHLH dimer pool within those cells. To fully understand relevant gain-of-function future efforts will require faithful upregulation only within cardiomyocytes that express either *Hand1* or *Hand2*.

Given that, together, Hand factors regulate multiple aspects of ventricular development, the possibility arises that different gene sets, and thereby different developmental processes, are regulated mechanistically through distinct modes of Hand factor function, It has been shown that, in the developing sympathetic nervous system, Hand2 synergistically, with the homeoprotein Phox2a, upregulates transcription from the *dopamine β-hydroxylase* promoter independent of DNA binding (Rychlik et al. 2003; Xu et al. 2003). In the heart, Hand2 may similarly interact with other DNA-bound *trans*-activators to regulate transcription as a component of a larger protein complex. Alternatively, Hand2 may heterodimerize with negative-regulatory bHLH factors, for example, the HES-related transcription factors (HRT; also referred to as Hey, Hesr, HERP, or CHF; (Firulli et al. 2000), providing relief of repression for certain genes. Indeed Hand2 has been shown to interact with Nkx2.5 synergistically to activate transcription of the *Nppa* promoter through a DNA-binding independent mechanism (Thattaliyath et al. 2002). These possibilities add an additional layer of complexity to Hand factor function. In the context of human birth defects, it is important to bear in mind that, while partial or full loss of Hand factor function may be detrimental, mutations which cause these two factors to interact with protein binding partners in aberrant ways may also lead to congenital disease.

Insights into *hand* function during cardiomyocyte growth and morphogenesis from zebrafish

In addition to cardiomyocyte maturation, studies in zebrafish have demonstrated that Hand factors regulate cardiac morphogeneis. In contrast to the mammalian heart, which develops from the cardiac crescent, he zebrafish heart originates as two bilateral cardiogenic fields that migrate medially, fusing to form the primitive heart tube.

Unlike medaka, tetradon and stickleback, which have both *hand1* and *hand2* genes (ENSEMBL), the zebrafish genome only contains *hand2*. Similar to what is seen in mice, Twist proteins are expressed in the endocardial cushions, but not the myocardium (Yeo et al. 2009). Thus, functional studies in zebrafish are not clouded by potential functional redundancy between factors. The sole zebrafish *hand* factor is disrupted in *Hands off* (*han*) mutants.

Although their precardiac mesoderm is specified normally, *han* mutants have reduced myocardium (Yelon et al. 2000). The myocardium that does form, rather than fusing to form the medial heart tube, remains laterally displaced. Differentiation of ventricular cardiomyocytes, as assessed through *ventricular myosin heavy chain* (*vmhc*) expression, is strongly affected, with *han* mutants showing either no *vmhc*-expressing cells, or few, irregularly distributed *vmhc*-expressing cells. Although the majority of cardiac transcription factors examined, including *mef2c*, *gata4*, *gata5*, *gata6*, and the *Tbx20* ortholog, *hrt*, were all expressed normally in *han* mutant cardiomyocytes, expression of the T-box transcription factor *tbx5* is not maintained (Yelon et al. 2000).

han mutants display deficient cardiogenic lateral plate mesoderm (LPM) expansion mediolaterally within the embryo, as visualized through *gata4* expression, potentially by regulating cell movement and proliferation. These observations indicate that in zebrafish, hand2 regulates not only cardiac chamber-specific gene expression, but also a more general role in the early morphogenesis and differentiation of the LPM (Schoenebeck et al. 2007).

In zebrafish, hand2 is thought to play a permissive, rather than instructive, role in potentiating cardiomyocyte differentiation. Ectopic *hand2* expression does not generate ectopic cardiomyocytes (Yelon et al. 2000). Dimensions of the heart-forming region, as gauged by the relative domains of *nkx2.5*, which marks cardiogenic progenitors, and *scl*, which designates vascular and hematopoetic progenitors, are unchanged by a loss of *hand2*. However, fewer of these progenitors (~60%) ultimately contribute to the heart in *han* mutants. It is thought that a portion of cardiac progenitors initiates myocardial differentiation, but fail to complete the program.

han mutant cells behave indistinguishably from wild-type cells when transplanted into wild-type zebrafish embryos, migrating medially and integrating into the heart, while wild-type cells transplanted into *han* mutant embryos fail to move independently towards the midline (Garavito-Aguilar et al. 2010). Hand2 function during zebrafish cardiac field fusion is, therefore, non-cell autonomous. This interesting observation could suggest defects in extracellular matrix (ECM) that alter cell migration. Interestingly, only about 60% of these *han* mutant-derived donor cells differentiate into cardiomyocytes, consistent with the ~40% reduction of cardiomyocyte production typical of the *han* mutant heart field (Schoenebeck et al. 2007), while wild-type donor-derived cells differentiate into cardiomyocytes at comparable frequencies when implanted in either wild-type or *han* mutant hosts. Therefore, unlike its influence on cardiac cell migration, *hand2*-mediated potentiation of cardiomyocyte cell fate is likely cell-autonomous. Zebrafish hand2 thus performs independent functions to both potentiate myocardial differentiation cell-autonomously, and to promote cardiac fusion through an extracellular mediator.

Strong evidence that *hand2* modulates ECM has recently been reported. Fibronectin1 (fn1) is a large, adhesive glycoprotein that occupies the extracellular matrix and functions during certain forms of cell migration as an ECM substrate, chemotactic promoter, and an integrin signaling activator (Yamada, 2000). *Fn1* gene expression is upregulated and fn1 protein deposition is disorganized in *han* mutants (Garavito-Aguilar et al. 2010; Trinh et al. 2005). Conversely, embryos overexpressing *hand2* display reduced *fn1* expression and deposition. These *hand2* overexpressers phenocopy certain aspects of *fn1* mutant zebrafish (Trinh and Stainier, 2004), such as scattered cardiomyocytes, a disorganized myocardial monolayer, and delayed cardiac field fusion.

Further supporting antagonistic roles for *hand2* and *fn1* during early cardiogenesis, heterozygosity of *fn1* rescues cardiac fusion (but not cardiomyocyte production) in *han* mutants. As both excess and absence of fn1 leads to cardia bifida, these data strongly suggest that hand2 enables cardiac fusion through *fn1* modulation.

han mutant cardiomyocytes also exhibit polarity defects. Normal cardiomyocytes display distinct cellular polarity and defined subcellular localization of β-catenin (basolaterally), aPKC (apically), and ZO-1 (laterally). *han* mutants lack all of these polarized features, and their cardiomyocytes aggregate into clusters, rather than forming a monolayer. However, *fn1* heterozygous rescue partially restores cardiomyocytes to a monolayer conformation, although it fails to restore other features of cellular polarity.

Thus, hand2 plays an integral role in zebrafish cardiogenesis regulating gene expression during ventricular cardiomyocyte specification, ECM deposition during cardiac field migration and fusion, and apicobasal cell polarity necessary for heart tube extension.

Hand2 and the TALE (Three Amino acid Loop Extension)-class homeodomain proteins pbx2 and pbx4, as revealed by morpholino knockdown experiments, regulate a common set of downstream targets and share similar myocardial differentiation and morphogenesis reduction-of-function phenotypes (Maves et al. 2009). *Hand2* expression is upregulated in the cardiogenic fields of *pbx2*;*pbx4* morphants. Morphants in which *hand2*, *pbx2* and *pbx4* have all been knocked down phenocopy the cardia bifida associated with *hand2* null embryos, potentially revealing that the primary role of pbx proteins in the heart is to facilitate hand2 function.

Zebrafish are a powerful model organism, in that their hearts have the capacity to regenerate following mechanical damage, and the growth of adult fish can be modulated experimentally depending upon age and aquarium density. Unlike mammalian cardiomyocytes, which typically generate postnatal cardiac growth through the hypertrophy of existing cells, zebrafish cardiomyocytes are proliferative. During zebrafish heart regeneration, *hand2*-expressing cells appear along the apical ridge of the wound where they remain until regeneration is complete (Wills et al. 2008). Interestingly, myocardial *hand2* expression is greater in adult zebrafish undergoing slow growth than those maintaining a static size. This expression increases to levels comparable to those seen during cardiomyocyte regeneration in fish undergoing rapid growth. These results suggest that adult zebrafish cardiomyocytes reactivate factors that are active during early development, such as *hand2*, to generate new cardiomyocytes during periods of rapid growth and regeneration.

Animal models of *Hand1* and *Hand2* function in the OFT (cNCC and SHF)

In addition to their contributions to ventricular cardiomyocyte expansion and heart chamber morphogenesis, Hand factors play integral roles in the development of extracardiac tissues important for formation of the functional heart, among them the cardiac neural crest cells (cNCCs). The cNCCs originate from the dorsal lip of the neural tube caudal to the otic

placode, at the axial level of rhombomeres 5–7. These cells delaminate from the neural tube, migrating ventrally and invading the caudal pharyngeal arches (arches III, IV and VI), where they surround the pharyngeal arch arteries (Scholl and Kirby, 2009). They subsequently migrate into the cardiac jelly-filled lumen intervening the OFT myocardium and endocardium. Through extensive remodeling, the outflow tract is septated into aortic and pulmonary components, and the pharyngeal arch arteries become the great vessels of the aortic arch. The cNCCs themselves contribute to the aorticopulmonary septum and differentiate into the smooth muscle cells of the great arteries. Neural crest cell dysfunction disrupts various aspects of this remodeling process.

Hand1 and *Hand2* are both expressed dynamically in cNCCs that have migrated into the caudal pharyngeal arches (Cserjesi et al. 1995; Srivastava et al. 1997; Vincentz et al. 2008). *Hand1* continues to be expressed, although not uniformly, in cNCCs occupying the OFT cushions (Vincentz et al. 2008). Additionally, both *Hand1* and *Hand2* are expressed strongly in the developing myocardial cuff (Cserjesi et al. 1995; Srivastava et al. 1997; Vincentz et al. 2008). Thus, the OFT is one of the only regions of the developing heart in which *Hand1* and *Hand2* are strongly expressed in overlapping domains.

A broad spectrum of CHDs, including VSDs, aortic arch artery patterning defects, and aorticopulmonary valve defects, which represent a substantial proportion of all birth defects, can be attributed to cNCC dysfunction (reviewed in (Lie-Venema et al. 2007; Mitchell et al. 2007; Obler et al. 2008; Snarr et al. 2008; Srivastava and Olson, 2000; Stoller and Epstein, 2005; Waldo et al. 1998). Although *Hand2*$^{-/-}$ systemic mutants die too early to assess formation of the OFT, the general hypoplasia of the OFT myocardium (Srivastava et al. 1997) and pharyngeal arches (Thomas et al. 1998; Yamagishi et al. 2000) characteristic of these mutants strongly indicates that disruption of Hand2 would recapitulate a subset of these disease phenotypes. Loss of the catecholamine norepinepherine in the neural crest cell-derived sympathetic nervous system causes embryonic lethality at mid-gestation (Lim et al. 2000). Hand2 is required in the developing sympathetic nervous system to transcriptionally regulate biosynthetic enzymes, such as tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DBH), necessary to generate norepinephrine (Morikawa et al. 2007). Conditional ablation of *Hand2* in NCCs, consequently, causes embryonic lethality at E12.5 (Morikawa et al. 2007). However, these *Hand2* cNCC-CKOs embryos can be rescued to birth by supplementing the water of pregnant dams with the adrenoceptor agonist, isoproterenol (Morikawa and Cserjesi, 2008). *Hand2* cNCC-conditional knockouts (CKOs) then succumb, perinatally, to various OFT patterning defects, including pulmonary stenosis, B-type interrupted aortic arch (IAA-B), aberrant origin of the right subclavian artery (AORSA), often including retroesophageal right subclavian artery, and membranous VSDs coupled with double outlet right ventricle (DORV; (Holler et al. 2010; Morikawa and Cserjesi, 2008). Conflicting lineage trace analyses make it difficult to assess whether cNCCs contribute to the *Hand2* cNCC-CKO OFT qualitatively at levels below that of (Holler et al. 2010) or comparable to (Morikawa and Cserjesi, 2008) control embryos. Although vascular smooth muscle cell differentiation is dysfunctional in *Hand2*$^{-/-}$ systemic mutants (Yamagishi et al. 2000), differentiation of *Hand2* conditionally null cNCCs into smooth muscle cells is unimpeded (Morikawa and Cserjesi, 2008). *Hand2* conditionally null cNCCs do display proliferative defects (Holler et al. 2010). Expression of the semaphorin3C (Sema3C) receptor, plexinA2 (PlxnA2), is downregulated in *Hand2* cNCC-CKO OFTs, raising the possibility that *Hand2* null cNCCs are incapable of responding to Sema3C signaling, resulting in a partial phenocopy of the *Sema3C*$^{-/-}$ mutant phenotype (Morikawa and Cserjesi, 2008). Extensive microarray analyses of *Hand2* conditionally null cNCCs have identified a number of factors regulated by Hand2 and potentially important for cNCC migration (Holler et al. 2010), including decreased *Pdgfα* (Morrison-Graham et al. 1992; Orr-Urtreger and Lonai, 1992) and *Gja5* (*Cx40*), and increased integrin α9 (Itgα9; (Huang et

al. 2005; Young et al. 2001) and integrin α4 (Itgα4; (Pinco et al. 2001; Sheppard et al. 1994).

Hand2 cNCC-CKOs, surprisingly, display an enlarged RV (Morikawa and Cserjesi, 2008). This phenotype is likely due to increased proliferation in the trabecular zone of the RV, reflective of a failure of trabecular cardiomyocytes to exit the cell cycle. The trabecular zone marker, atrial natriuretic peptide (*Nppa*), is downregulated in the RV, but not LV of *Hand2* cNCC-CKOs. Together, these data suggest that Hand2 functions non-cell autonomously in the cNCCs to, prior to the onset of *Nppa* expression at E10.5, to regulate SHF-derived cardiomyocyte proliferation vs. differentiation. This finding is intriguing, as it provides a model for the congenital disorder hyper-trabeculation/ventricular noncompaction (VHT), whose prevalence has only recently begun to be appreciated through advancements in imaging technology (Weiford et al. 2004). Although the genetic causes of this disorder are unknown, it is often associated with facial dysmorphism (Chin et al. 1990; Ichida et al. 1999) and other cardiac defects attributable to cNCC dysfunction (Kenny et al. 2007). Thus, NCC defects may ultimately prove to comprise the etiology of VHT.

No OFT phenotypes have yet been reported for *Hand1* conditional ablation in cNCCs. Genetic interactions have been reported between Hand1 and Hand2 in cranial NCCs (Barbosa et al. 2007). As mentioned previously, *Hand1* and *Hand2* expression overlaps in the cNCCs. Genetic interaction studies would be warranted and necessary to determine whether *Hand1* and *Hand2* perform redundant functions during OFT morphogenesis.

Observations of NCCs in *Twist1*$^{-/-}$ OFTs have suggested that *Hand1* and *Hand2* may perform unique functions within specific subpopulations of these cells. Among the normally compacted cNCC-derived ectomesenchyme of the *Twist1*$^{-/-}$ OFT cushions are amorphic cellular aggregates (Vincentz et al. 2008). These aggregates are of NCC origin, but differ from surrounding, phenotypically normal NCC ectomesenchyme in that they express high levels of *Hand1* and *Hand2* (Vincentz et al. 2008). Thus, the subpopulation of *Hand1*/*Hand2* co-expressing NCCs in the OFT cushions are uniquely affected by a loss of *Twist1* function. Whether Hand1 and Hand2 actively function to contribute to the phenotype of these mutant cells remains to be seen.

Hand2$^{-/-}$ mutants have diminished SHF-derived myocardium, most likely due to cardiomyocyte apoptosis (Thomas et al. 1998). Conditional ablation of *Hand2* in differentiated cardiomyocytes, via the *cTnt-Cre* mouse line, causes embryonic lethality between E9.5 and E13.5. These mice display RV and OFT hypoplasia with variable expressivity, with severely affected mutants phenocopying *Hand2*$^{-/-}$ systemic mutants and later-surviving mutants, despite also suffering from hypoplasia, displaying two distinct ventricles. These data confirm that Hand2 regulates the development of differentiated, SHF-derived cardiomyocytes.

Conversely, overexpression of *Hand1* in the myocardial cuff generates an OFT 1.5 – 2 fold longer than that of a wild-type embryo (Risebro et al. 2006). Markers of OFT cardiomyocyte differentiation, *Nppa* and *Wnt11*, are downregulated in the OFTs of these mutants. Immunohistochemistry for phospho-histone H3 reveals that the OFT cardiomyocytes in these mutants are over proliferative. As a whole, these data suggest that Hand factor function governs cell number in the SHF-derived OFT myocardium.

Hand factor function in the epicardium and endocardium

Derivatives of the epicardium and endocardium fulfill important roles in the developing heart. Although Hand factors have been shown to contribute to the development of several heart structures, their respective contributions to the development of the epicardial and

endocardial lineages has not been thoroughly studied. Cells of the endocardium undergo EMT, invading the AV cushions and ultimately contributing to the AV valves. In E11.5 *Hand1* cardiac-specific conditional knockouts, the AV cushions are hyperplastic in the absence of any detectable cell proliferation or cell death abnormalities (McFadden et al. 2005). The neonatal AV valves of these mutants are comparatively thicker than those of wild-type littermates. This phenotype is intriguing, as *Hand1* expression is never observed in the endocardium or AV cushions (Fig. 2). As such, Hand1 function may be required in the myocardium to regulate endocardial cell contribution to the AV cushions in a non-cell autonomous manner. It should be noted, however, that the direct Hand1 transcriptional target *Thymosin β4* is expressed within these tissues at E10.0, a time point consistent with an E11.5 phenotype (Smart et al. 2002). Thus, *Hand1* may be transiently or weakly expressed in the endocardium and/or AV cushions and has yet to be detected and reported, although lineage trace analyses using the *Hand1*^{EGFPCre} knock-in allele fail to identify a *Hand1*-expressing lineage in the endocardium (Barnes et al. 2010) and provide evidence against this hypothesis. Similarly, E11.5 Hand2 DNA binding-deficient mutants have enlarged and disorganized endocardial cushions (Liu et al. 2009). Unlike *Hand1*, *Hand2* is strongly expressed in the endocardium, and may be directly required, through a DNA binding-independent mechanism, to regulate AV cushion development. More so than the limited amount that is known about Hand factor function in the endocardium, the function of Hand1 and Hand2 in the epicardium is completely unexplored, although recent studies confirm that *Hand1*-expressing cells ultimately form the epicardium and its derivatives (Barnes et al. 2010). Additionally, development of the proepicardium is disrupted in zebrafish han mutants (Liu and Stainier). Extensive tissue-specific gene ablation studies will be required to unravel the cell autonomous and non-cell autonomous functions of *Hand1* and *Hand2* in the developing endocardium and, potentially, epicardium.

HAND factors in human CHD

The respective reciprocal expression domains in the left and right ventricle are well-characterized features of *Hand1* and *Hand2* in the heart, marking them both as candidate contributing factors to human CHDs that affect the developing heart asymmetrically. For example, as the name suggests, Hypoplastic Left Heart Syndrome (HLHS), a severe form of CHD affecting 0.16–0.36 of every 1000 live births (Talner, 1998), specifically affects components of the left side of the heart, including the aorta, LV and both the aortic and mitral valves. The genetic causes of HLHS are an almost complete black box; however, one of the two human genes implicated in HLHS is *HAND1*.

Reamon-Buettner, et. al, sequenced the *HAND1* gene in 31 unrelated hypoplastic heart syndrome patients. 24 of these 31 patients exhibited a common frameshift mutation (A126fs) in the loop region of the bHLH domain, resulting in a truncated protein featuring has only a single complete α-helix. This truncated HAND1 is incapable of modulating transcription from D- or E-boxes either alone or in conjunction with bHLH binding partners. Thus, disruption of *HAND1* function is associated with human cardiac hypoplasia.

Gja1 is also implicated in HLHS. Gja1 protein expression is downregulated by overexpressing *Hand1* in mice (Breckenridge et al. 2009); however, no change in mRNA levels is reported, indicating that, while a regulatory relationship exists between *Gja1* and Hand1 in cardiomyocytes, current evidence suggests that it is not directly transcriptional. Hand1 and Hand2 may both transcriptionally regulate the related protein *Gja5* (*Cx40*; (McFadden et al. 2005), although cardiomyocyte expression of this factor is limited to the LV embryonically. Expression of *Gja5* is slightly decreased in *Hand1* cardiac-CKO embryos and is not detected in the ventricular myocardium of *Hand1*$^{fx/-}$;*Hand2*$^{+/-}$;*Nkx2.5-Cre(+)* embryos. Further expression studies in *Hand2* cardiac-CKO embryos, coupled with

in vitro DNA binding and *trans*-activation assays indicate that *Gja5* is a direct transcriptional target of Hand2 in these cells (Holler et al. 2010). Thus, regulation of Gja proteins may be an evolutionarily conserved mechanism by which Hand factors contribute to cardiogenesis and congenital disease.

The association of *HAND2* with human CHDs is not as well characterized; however, recent studies have provided tantalizing evidence that *HAND2* may contribute to human CHDs. *HAND2* maps to chromosome 4q33. A high incidence of CHDs, including VSDs, septal defects, pulmonary atresia, coarctation of the aorta, and tetralogy of Fallot, is associated with genomic duplications or deletions of 4q33 (Borochowitz et al. 1997; Byatt et al. 1997). Studies of 131 ethnic Han Chinese children with CHDs, such as tetralogy of Fallot, pulmonary stenosis, atrioventricular septal defects, and VSDs with DORV, identified three *HAND2* missense mutations, one isonymous mutation, and three mutations within the *HAND2* 5' and 3' untranslated regions in 12 of these patients (Shen et al. 2010). It should be noted that one of the missense mutations, S36N, is not in an evolutionarily conserved residue, is not predicted *in silico* to affect HAND2 protein function, and was also detected in one of the 250 healthy controls. Although this specific mutation is not a strong candidate causative factor in human CHDs, the relatively high frequency of *HAND2* mutations revealed by this study suggests that *HAND2* dysfunction may contribute to CHDs affecting the RV and OFT. It will be interesting to determine whether these mutations, whether through altered protein-protein interactions or DNA-binding, or through altered mRNA stability or translation, result in HAND2 hypo- or hyper-activity. Indeed, increased *HAND2* mRNA expression has been associated with tetralogy of Fallot, while increased *HAND1* mRNA expression has been associated with hypertrophic obstructive cardiomyopathy (Ritter et al. 1999), echoing the idea that either too much or too little HAND factor function may be detrimental to cardiac growth and morphogenesis. Functional validation of these mutant *HAND2* variants will be critical to elucidating their contribution to congenital heart disease.

Acknowledgments

We would like thank all the laboratories who contributed publications that fill this review. Infrastructural support at the Herman B Wells Center for Pediatric Research is in part supported by the generosity of the Riley Children's Foundation and Division of Pediatric Cardiology. This work is supported by the NIH RO1HL061677-11 1P01HL085098-04 (ABF), and AHA 0815426G (RMB).

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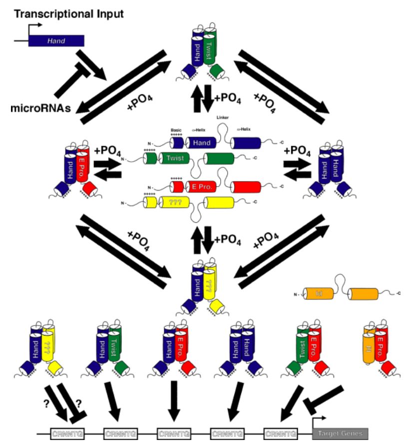

Figure 1.

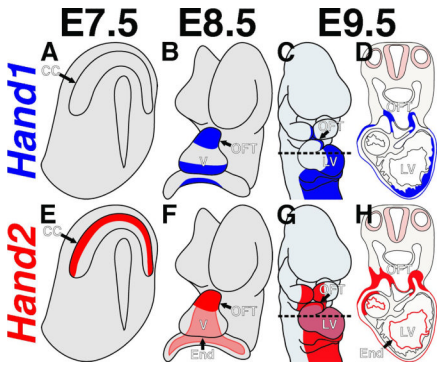

Figure 2.