Hand2 elevates cardiomyocyte production during zebrafish heart development and regeneration

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ABSTRACT

Embryonic heart formation requires the production of an appropriate number of cardiomyocytes; likewise, cardiac regeneration following injury relies upon the recovery of lost cardiomyocytes. The basic helix-loop-helix (bHLH) transcription factor Hand2 has been implicated in promoting cardiomyocyte formation. It is unclear, however, whether Hand2 plays an instructive or permissive role during this process. Here, we find that overexpression of hand2 in the early zebrafish embryo is able to enhance cardiomyocyte production, resulting in an enlarged heart with a striking increase in the size of the outflow tract. Our evidence indicates that these increases are dependent on the interactions of Hand2 in multimeric complexes and are independent of direct DNA binding by Hand2. Proliferation assays reveal that hand2 can impact cardiomyocyte production by promoting division of late-differentiating cardiac progenitors within the second heart field. Additionally, our data suggest that hand2 can influence cardiomyocyte production by altering the patterning of the anterior lateral plate mesoderm, potentially favoring formation of the first heart field at the expense of hematopoietic and vascular lineages. The potency of hand2 during embryonic cardiogenesis suggested that hand2 could also impact cardiac regeneration in adult zebrafish; indeed, we find that overexpression of hand2 can augment the regenerative proliferation of cardiomyocytes in response to injury. Together, our studies demonstrate that hand2 can drive cardiomyocyte production in multiple contexts and through multiple mechanisms. These results contribute to our understanding of the potential origins of congenital heart disease and inform future strategies in regenerative medicine.

KEY WORDS: Hand2, Zebrafish, First heart field, Second heart field, Cardiac regeneration

INTRODUCTION

The assembly of the embryonic heart is a complex procedure involving the differentiation, migration and organization of the proper number of cardiomyocytes in order to form a functional contractile organ. Regulation of the genesis of cardiomyocytes requires the specification of heart fields with appropriate boundaries, as well as the controlled proliferation of the progenitor cells that emerge from these fields. An understanding of the genetic pathways that influence cardiomyocyte production may illuminate mutations that underlie congenital heart disease (CHD) (Fahed et al., 2013). Moreover, cardiomyocyte production, in vitro or in vivo, is a primary goal of cardiovascular regenerative medicine, and insight into genes that drive cardiomyocyte formation can enhance strategies for repairing hearts damaged by myocardial infarction (Laflamme and Murry, 2011; Choi and Poss, 2012; Xin et al., 2013). However, our understanding of the precise functions of factors that facilitate cardiomyocyte production remains incomplete.

The bHLH transcription factor Hand2 has been implicated as an important regulator of cardiomyocyte production. In mice, loss of Hand2 function results in hypoplasia of the right ventricle and outflow tract (Srivastava et al., 1997), suggesting that Hand2 promotes the development of cardiomyocytes derived from the second heart field (SHF). Furthermore, removal of Hand2 function from the SHF via tissue-specific deletion of a conditional allele interferes with survival of this progenitor population (Tsuhishashi et al., 2011). The effects of loss of Hand2 function are exacerbated when combined with either a conditional knockout or a hypomorphic allele of the related gene Hand1, suggesting functional redundancy between Hand2 and Hand1 that could mask earlier or broader roles of these factors in regulating cardiomyocyte production in mice (McFadden et al., 2005; Firulli et al., 2010).

In zebrafish, an early requirement for hand2 during cardiomyocyte production has been clearly demonstrated. Zebrafish hand2 mutants display a striking cardiac phenotype that features a dramatic deficit of cardiomyocytes (Yelon et al., 2000). This defect is evident from an early stage, as indicated by a substantial reduction in the number of cells that initiate expression of myocardial differentiation markers. Fate mapping has shown that hand2 expression demarcates the heart-forming region within the anterior lateral plate mesoderm (ALPM), and, in hand2 mutants, the progenitors residing in this region are ineffective at generating differentiated cardiomyocytes (Schoenebeck et al., 2007). Thus, hand2 is important for facilitating cardiomyocyte production. However, it is not clear whether hand2 regulates cardiomyocyte production by influencing cell fate decisions or by affecting proliferative capacity, nor is it known whether hand2 plays an instructive or a permissive role in this setting.

The possibility that Hand2 instructively directs cardiomyocyte production has been highlighted by recent studies in which forced expression of Hand2 was shown to increase the efficiency of reprogramming mammalian fibroblasts toward a myocardial fate (Song et al., 2012; Nam et al., 2013). Introduction of Hand2, Gata4, Mef2c and Tbx5 into neonatal mouse fibroblasts increased the frequency of reprogramming approximately three- to fourfold over that observed with introduction of only Gata4, Mef2c and Tbx5 (Song et al., 2012). Additionally, in human fibroblasts, Hand2 has been shown to be essential for initiation of cardiac
contractile gene expression programs during reprogramming, whereas other factors, such as *Gata4*, *Mef2c*, *Thx5* and *Myocd*, play redundant roles in this regard (Nam et al., 2013). Thus, Hand2 can play a pivotal part in enhancing protocols for cardiac regeneration and repair; however, little is known about how forced expression of Hand2 mediates this role.

To gain new insight into the ability of Hand2 to drive cardiomyocyte production, we have examined the effects of *hand2* overexpression on the embryonic and adult zebrafish heart. We find that overexpression of *hand2* in the early embryo can increase cardiomyocyte numbers, with a particularly striking impact on the size of the cardiac outflow tract. Our data suggest that this cardiac enlargement results from increased progenitor proliferation within the SHF, as well as from increased cardiomyocyte specification within the first heart field (FHF). In addition, we find that overexpression of *hand2* in the adult heart enhances myocardial proliferation following injury. Together, our data suggest that *hand2* can play important and instructive roles in elevating cardiomyocyte production in multiple contexts: by promoting specification of FHF cardiomyocytes, by enhancing proliferation of SHF cardiac progenitors and by augmenting proliferation of regenerating cardiomyocytes. These results refine our understanding of the origins of CHD, particularly in the context of partial trisomy distal 4q, which involves duplication of *HAND2* (Tamura et al., 2013). Moreover, our findings highlight ways in which *hand2* overexpression could facilitate future approaches for cardiac regeneration and repair.

**RESULTS**

**Overexpression of hand2 increases cardiomyocyte production**

To determine whether increased *hand2* function can enhance cardiomyocyte production, we injected zebrafish embryos with *hand2* mRNA. Although we have previously injected *hand2* mRNA into wild-type embryos (Yelon et al., 2000; Garavito-Aguilar et al., 2010), our prior studies did not closely examine its impact on heart size. Here, we found that injected embryos exhibited expanded expression of *cmlc2*, a marker of differentiated cardiomyocytes, within the ALPM at 18 somites (Fig. 1A,B). Measurements of the region of *cmlc2* expression revealed a significant increase in area within the *hand2*-overexpressing embryos (Fig. 1E). To examine whether increased *hand2* expression leads to enhanced heart size at later stages, we injected *hand2* mRNA into embryos carrying Tg(5.1myl7:nDsRed2) (Mably et al., 2003), which facilitates quantification of fluorescent cardiomyocyte nuclei, and counted the number of cardiomyocytes present in the heart at 36 h post-fertilization (hpf). We found a significant increase in cardiomyocyte number in embryos overexpressing *hand2* (Fig. 1F). Together, these data suggest that increased expression of *hand2* can expand the heart-forming region within the ALPM, leading to a larger heart. However, *hand2* overexpression did not induce *cmlc2* expression outside the ALPM, indicating that cardiomyocyte production depends on the interaction of Hand2 with other ALPM factors.

The influence of Hand2 on cardiomyocyte production is dependent on protein-protein interactions and not on direct DNA binding

Previous studies have shown that Hand2 can function independently of direct DNA binding in some contexts (McFadden et al., 2002; Liu et al., 2009). Mice in which the *Hand2* gene was replaced with a DNA binding-deficient form of Hand2 exhibited relatively normal hearts at E11.5 (Liu et al., 2009), in contrast to the severe ventricular hypoplasia seen in *Hand2* mutants by E10.5 (Srivastava et al., 1997). It is presumed that the DNA binding-deficient form of Hand2 can influence transcription through dimerization with other bHLH factors, as well as through interactions with larger protein complexes (Rychlik et al., 2003; Xu et al., 2003). To test whether the early role of Hand2 during cardiomyocyte production is dependent on DNA binding or dimerization, we evaluated whether previously characterized DNA binding-deficient and dimerization-deficient versions of Hand2 can expand *cmlc2* expression.

Replacement of three arginines (residues 109-111) in the basic domain of mouse Hand2 with acidic residues [glutamic acid, aspartic acid and glutamic acid (EDE)] has been shown to abolish DNA binding (McFadden et al., 2002; Liu et al., 2009). This region is highly conserved between mouse and zebrafish (supplementary material Fig. S1): 98% of the amino acids in the basic helix-loop-helix domain are identical, including these three arginine residues (100-102 in zebrafish). In addition, replacement of a phenylalanine (residue 119) with a proline in the first helix of mouse Hand2 has been shown to disrupt its dimerization (McFadden et al., 2002; Liu et al., 2009). Mice in which the *Hand2* gene was replaced with a DNA binding-deficient form of Hand2 exhibited relatively normal hearts at E11.5 (Liu et al., 2009), in contrast to the severe ventricular hypoplasia seen in *Hand2* mutants by E10.5 (Srivastava et al., 1997). It is presumed that the DNA binding-deficient form of Hand2 can influence transcription through dimerization with other bHLH factors, as well as through interactions with larger protein complexes (Rychlik et al., 2003; Xu et al., 2003). To test whether the early role of Hand2 during cardiomyocyte production is dependent on DNA binding or dimerization, we evaluated whether previously characterized DNA binding-deficient and dimerization-deficient versions of Hand2 can expand *cmlc2* expression.

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**Fig. 1. hand2 overexpression increases cardiomyocyte production.** (A-D) *In situ* hybridization depicts *cmlc2* expression at 18 somites in (A) wild-type embryos, (B) *hand2*-overexpressing embryos (*hand2* OE), (C) embryos expressing a DNA binding-deficient form of *hand2* (*hand2* EDE) and (D) embryos expressing a dimerization-deficient form of *hand2* (*hand2* P); dorsal views, anterior upwards. Broader expression of *cmlc2* is found within the ALPM of embryos overexpressing *hand2* or *hand2* EDE. (E) Average area of *cmlc2* expression, in arbitrary units, in wild-type embryos and in embryos overexpressing *hand2*, *hand2* EDE or *hand2* P. Error bars indicate s.d.; asterisks indicate significant differences from wild type. Overexpression of *hand2* or *hand2* EDE increases area of *cmlc2* expression (*n*=13-25; *P*<0.001), whereas overexpression of *hand2* P does not (*n*=11; *P*=0.87). (F) Bar graph compares average number of cardiomyocytes at 36 hpf in wild-type embryos and in embryos overexpressing *hand2*. Error bars indicate s.d.; asterisk indicates a significant increase compared with wild type (*n*=17-19; *P*<0.001).
null allele of hand2 substantially rescued cmlc2 expression at 21 somites in (A) wild-type embryos, (B) hanP mutant embryos, (C) hanP mutant embryos injected with hand2 EDE mRNA and (E) hanP mutant embryos injected with hand2 P mRNA; dorsal views, anterior upwards. The hanP mutation is a null allele of hand2 resulting from a deletion that removes the entire hand2 gene (Yelon et al., 2000). At this stage, wild-type cardiomyocytes have formed a ring (A), whereas few cardiomyocytes have formed in the hanP mutants (B). Cardiomyocyte formation is substantially rescued with the injection of hand2 (C) or hand2 EDE (D) mRNA, but not of hand2 P mRNA (E). (F) Average area of cmlc2 expression, as in Fig. 1E. Asterisks indicate significant differences between the phenotype of hanP mutant embryos and the phenotypes of hanP mutant embryos overexpressing versions of hand2. Overexpression of hand2 or hand2 EDE partially rescues the area of cmlc2 expression in hanP mutants (n=14; *P<0.001), whereas hand2 P does not (n=14; P=0.085). mouse, we constructed corresponding variants of zebrafish hand2 and injected mRNA encoding each variant into wild-type embryos. Overexpression of the DNA binding-deficient form of hand2 (hand2 EDE) expanded the area of cmlc2 expression in a manner similar to the overexpression of wild-type hand2 (Fig. 1A-C,E). However, overexpression of the dimerization-deficient version of hand2 (hand2 P) did not expand the cmlc2 expression domain (Fig. 1A,D,E). Furthermore, injection of hand2 EDE mRNA substantially rescued cmlc2 expression in embryos homozygous for a null allele of hand2 (handP) (Yelon et al., 2000), reminiscent of the effects of injecting wild-type hand2 into hanP mutants (Fig. 2A-D,F). By contrast, injection of hand2 P mRNA did not alter cmlc2 expression in hanP mutants (Fig. 2B,E,F). These results suggest that Hand2 dimerization, but not its direct binding to DNA, is necessary for its influence on cardiomyocyte production.

Induction of hand2 overexpression after gastrulation expands cardiomyocyte production

In some embryos injected with hand2 mRNA, we observed morphological defects that could result from abnormal gastrulation, such as shortened body axis or abnormal body curvature. To bypass effects on gastrulation, we established an inducible system for hand2 overexpression. We constructed a series of transgenes in which hand2 gene (Yelon et al., 2000). At this stage, wild-type cardiomyocytes have formed a ring (Fig. 2A,D,E). Furthermore, injection of hand2 EDE mRNA expanded the area of cmlc2 expression in embryos homozygous for a null allele of hand2 (handP) (Yelon et al., 2000), reminiscent of the effects of injecting wild-type hand2 into hanP mutants (Fig. 2A-D,F). By contrast, injection of hand2 P mRNA did not alter cmlc2 expression in hanP mutants (Fig. 2B,E,F). These results suggest that Hand2 dimerization, but not its direct binding to DNA, is necessary for its influence on cardiomyocyte production.

The cardiac expansion induced by hand2 overexpression requires phosphorylation-independent dimerization of Hand2

The consequences of injection of the hand2 EDE and hand2 P mRNAs suggest that the role of Hand2 during cardiomyocyte production requires its dimerization but not its direct binding to DNA (Fig. 2). Phosphorylation of conserved threonine and serine residues within the first helix of Hand factors has previously been shown to be important for their choice of dimerization partners (Firulli et al., 2003, 2005). For example, changes in phosphorylation of T107 and S109 of mouse Hand1 influence the affinity for formation of Hand1-Hand1 homodimers relative to the formation of Hand1 heterodimers with E proteins (Firulli et al., 2003, 2005). To determine whether the dimerization interactions that influence cardiomyocyte production require Hand2 phosphorylation, we constructed the transgene Tg(hsp70:FLAG-hand2P-2A-mCherry) [hereafter referred to as Tg(hsp70:hand2P)] (referred to as Tg(hsp70:hand2P)). The zebrafish Hand2 variant...
expressed by this transgene features substitution of the residues T103 and S105 with alanines (supplementary material Fig. S1), thereby preventing their phosphorylation; extrapolating from prior studies of Hand1 and Twist1, these changes may promote affinity for homodimerization rather than heterodimerization with E proteins (Firulli et al., 2003, 2005).

We compared the effects of inducing expression of hand2, hand2AA, and hand2P, using inducible transgenes to overexpress each variant (supplementary material Fig. S3). Induction of expression of the phosphorylation-deficient version of hand2 at 10 hpf resulted in expansion of cmlc2 expression at 18 somites, similar to the effects of inducing wild-type hand2 expression (Fig. 3A,B,D,E). Likewise, overexpression of the phosphorylation-deficient version of hand2 caused formation of an enlarged heart and an elongated outflow tract by 36 hpf, as is the case for overexpression of wild-type hand2 (Fig. 3G,I,K). By contrast, induction of expression of the dimerization-deficient version of hand2 did not expand the area of cmlc2 expression (Fig. 3C,E) and did not yield a larger heart or outflow tract (Fig. 3J). These data suggest that an unphosphorylated form of Hand2 mediates its dimerization and role in the context of cardiac expansion.

**Increased proliferation in late-differentiating cells contributes to cardiac expansion in embryos overexpressing hand2**

The enlarged hearts in embryos overexpressing hand2 led us to investigate the cellular mechanism through which hand2 influences cardiomyocyte expansion. As overexpression of Hand1 has been shown to enhance proliferation within the outflow tract in mice (Risebro et al., 2006), we were especially interested in determining the origins of the elongated outflow tract in hand2-overexpressing embryos. First, we examined the expression of mef2cb at 36 hpf; at this stage, mef2cb marks a population of SHF-derived cells that contributes to the outflow tract (Lazic and Scott, 2011; Hinits et al., 2012). We found excess mef2cb expression at the arterial pole of the heart in hand2-overexpressing embryos (Fig. 4A-D), suggesting that the observed outflow tract expansion is the product of excess SHF-derived cells, rather than simply a morphological anomaly. In addition, mef2cb expression in embryos overexpressing the phosphorylation-deficient and dimerization-deficient versions of hand2 correlated with the observed outflow tract morphologies: embryos overexpressing hand2 AA exhibited an excess of mef2cb-expressing cells (Fig. 4G,H), whereas embryos overexpressing hand2 P did not (Fig. 4E,F). In contrast to their expanded mef2cb expression at the arterial pole, hand2-overexpressing embryos exhibited a normal pattern of islet1 expression at the venous pole (supplementary material Fig. S4), suggesting that hand2 has a more potent influence on outflow tract formation than on inflow tract formation.

Next, we employed an EdU incorporation assay to investigate whether the expansion of the outflow tract or the general increase in cardiomyocyte number could be the result of increased proliferation in hand2-overexpressing embryos. Following heat shock at 10 hpf, we pulsed embryos with EdU for 30 min when they reached 17 hpf, thereby labeling proliferating cells during that interval (Fig. 5G). We then compared the numbers of EdU-positive and EdU-negative cardiomyocytes at 36 hpf in Tg(hsp70:hand2) embryos and their nontransgenic siblings (Fig. 5A-F). Strikingly, we found a significantly higher proliferation index in the hand2-overexpressing cardiomyocytes (Fig. 5H), indicating that increased proliferation
contributes to the enhanced cardiomyocyte production in these embryos. By contrast, in a parallel set of experiments in which we pulsed embryos with EdU at 23 hpf (supplementary material Fig. S5A-G), we did not detect an increased proliferation index in hand2-overexpressing cardiomyocytes (supplementary material Fig. S5H), suggesting that hand2 overexpression influences proliferation prior to 23 hpf.

Based on this series of results, we hypothesized that increased proliferation in hand2-overexpressing embryos occurs between 17 hpf and the time of initial heart tube assembly. To test this, we pulsed embryos with EdU at 17 hpf and analyzed the distribution of EdU-positive cells at 26 hpf in hand2-overexpressing and nontransgenic embryos (Fig. 6A-E). In addition to scoring EdU-positive cardiomyocytes, we used Tg(nkx2.5;ZsYellow) to facilitate scoring of EdU labeling in SHF-derived progenitor cells that reside near the arterial pole of the early heart tube and ultimately contribute to the outflow tract (Zhou et al., 2011); these progenitor cells express Tg(nkx2.5;ZsYellow) but do not yet express high levels of other myocardial differentiation markers. In these experiments, we found that hand2 overexpression caused a significant increase in the proliferation index of the SHF-derived cardiac progenitor cells near the arterial pole (Fig. 6F). However, hand2 overexpression did not enhance the proliferation index of the first heart field-derived (FHF-derived) cardiomyocytes that form the initial heart tube (Fig. 6F); we obtained similar results when assessing the cardiomyocyte proliferation index in embryos pulsed with EdU at 17 hpf and fixed at 23 hpf, and in embryos pulsed with EdU at 14 hpf and fixed at 26 hpf (supplementary material Fig. S6). Altogether, our data demonstrate that overexpression of hand2 has a potent and early impact on the proliferation of the SHF-derived, late-differentiating progenitor cells that will create the outflow tract.

**Overexpression of hand2 alters ALPM patterning**

Although our results suggest that enhanced proliferation of SHF-derived progenitors contributes to the enlarged outflow tract seen when hand2 is overexpressed, increased proliferation within the SHF may not account for all of the enhanced cardiomyocyte production in hand2-overexpressing embryos. Notably, the increased area of cmlc2 expression observed at 18 somites (Fig. 3A,B,E) seems to reflect increased production of cardiomyocytes by the FHF, as only early-differentiating, FHF-derived cells are thought to express cmlc2 at this stage (de Pater et al., 2009). Additionally, we have found increased numbers of differentiated cardiomyocytes in hand2-overexpressing embryos at 23 hpf (nontransgenic embryos, 127±25 cardiomyocytes; hand2-overexpressing embryos, 164±39 cardiomyocytes; n=9 or 10, P<0.05) and at 26 hpf (nontransgenic embryos, 154±25 cardiomyocytes; hand2-overexpressing embryos, 224±47 cardiomyocytes; n=10 or 11, P<0.001). As is the case at 18 somites, it is thought that cardiomyocytes have not yet been added from the SHF at 23 or 26 hpf (de Pater et al., 2009; Lazic and Scott, 2011). Furthermore, we did not detect an increased proliferation index in the cardiomyocyte population at 23 or 26 hpf (Fig. 6F; supplementary material Fig. S6F). Together, these data suggest that the early expansion of cardiomyocytes in hand2-overexpressing embryos reflects an influence of hand2 on the early-differentiating FHF, and that this effect is more likely to be the result of altered specification than of altered proliferation.

To evaluate whether overexpression of hand2 alters the patterning of the ALPM, we examined markers of lineages that are neighbors of the heart fields at early stages. scl and etsrp are expressed in progenitors of the blood and vessel lineages that are rostral to the heart-forming region that expresses hand2 (Schoenebeck et al., 2007). When blood and vessel lineage specification are inhibited by loss of scl and etsrp function, hand2 expression expands into the rostral ALPM and an increased number of cardiomyocytes form (Schoenebeck et al., 2007; Palencia-Desai et al., 2011). These findings suggest that scl and etsrp act to limit the extent of myocardial specification in the ALPM. Conversely, we found that overexpression of hand2 limited the expression of anterior hematopoietic and endothelial markers at 12 somites, including scl, etsrp and pu.1 (Fig. 7A-F); even so, these gene expression defects did not seem to cause major anomalies in vascular patterning or in endocardial development (supplementary material Fig. S7). At the same time, hand2-overexpressing embryos exhibited expanded expression of mef2cb (Fig. 7G,H), which is found in myocardial progenitor cells at this stage (Hinits et al., 2012). However, not all myocardial progenitor markers are similarly expanded, as we found that hand2-overexpressing embryos exhibit normal expression of nkx2.5 at 12 somites (Fig. 7I,J). This result suggests that hand2 functions downstream of nkx2.5, which is consistent with the normal nkx2.5 expression pattern observed in hand2 mutant embryos (Yelon et al., 2000; Schoenebeck et al., 2007). Finally, gata4 expression also appeared

![Fig. 4. Overexpression of hand2 causes outflow tract expansion.](image-url)
similar in nontransgenic and hand2-overexpressing embryos (Fig. 7K,L), indicating that increased hand2 expression does not alter the overall dimensions of the ALPM. Thus, in addition to a later capacity to promote proliferation within the SHF, hand2 overexpression can influence early patterning processes within the ALPM, possibly by promoting myocardial specification while limiting hematopoietic and endothelial specification.

Overexpression of hand2 augments cardiomyocyte proliferation during cardiac regeneration

Mechanisms of embryonic heart development have served as necessary guides for understanding cardiac regeneration. The potent effects of hand2 in the early embryo raise the possibility that its expression could influence the efficacy of regeneration in the injured adult heart. In the zebrafish, cardiac injuries are repaired through a process in which spared mature cardiomyocytes reduce indicators of differentiation and proliferate to regenerate lost muscle, involving induction and function of gata4 in source cardiomyocytes (Jopling et al., 2010; Kikuchi et al., 2010; Gupta et al., 2013). In previous studies, we also observed increased expression of nks2.5, tbx5 and tbx20 in the injury site following resection of the adult zebrafish ventricle (Lepilina et al., 2006), although the induction of these genes was not detectable by others (Raya et al., 2003; Jopling et al., 2010). We additionally found activation of hand2 and gata5 regulatory sequences in endocardial cells after resection injury (Kikuchi et al., 2011a). To assess gene expression after a more severe injury, we employed transgene-driven cardiomyocyte ablation in Z-CAT transgenic fish, in which the combination of Tg(cmlc2:CreER) and Tg(β-actin2:loxsp-mCherry-STOP-loxsp-DTA) transgenes permits 4-HT-inducible expression of diphtheria toxin in cardiomyocytes (Wang et al., 2011). Enhanced expression of hand2, as well as nks2.5, tbx5 and tbx20, was evident in spared cardiomyocytes throughout the injured Z-CAT ventricle (Fig. 8A-H). Additionally, fluorescence in Tg(hand2:EGFP) myocardium and endocardium was boosted during regeneration (Fig. 8I-K), indicating that cardiac injury can activate hand2 regulatory sequences in multiple cell types.

To test whether induced hand2 expression in adult cardiomyocytes affects their regenerative capacity, we first examined cardiomyocyte proliferation in injured Z-CAT animals heterozygous for the han6 mutation (Yelon et al., 2000). Heterozygosity for han6 did not alter levels of ablation-induced cardiomyocyte proliferation, although we also did not detect reduced levels of hand2 mRNA in injured heterozygous hearts (data not shown). To artificially increase hand2 expression in cardiomyocytes after injury, we generated a new transgene that permits 4-HT-inducible hand2 expression, Tg(β-actin2:loxsp-mCherry-STOP-loxsp-hand2) [hereafter referred to as Tg(β-actin2:RSH)]. In fish carrying Tg(β-actin2:RSH) in combination with Tg(cmlc2:Cre:ER), a single 4-HT injection sharply increased myocardial hand2 expression but had no effect on cardiomyocyte proliferation in the absence of injury (data not shown). In Z-CAT animals carrying Tg(β-actin2:RSH), we experimentally elevated hand2 levels in spared cardiomyocytes in concert with inducing ablation injury (Fig. 9A). Under these conditions, we observed a 34% increase in cardiomyocyte proliferation in Z-CAT; Tg(β-actin2:RSH) animals at 7 days post-injection (dpi), compared with that seen in Z-CAT animals at the same timepoint (Fig. 9B). To confirm effects of hand2 on regenerative proliferation, we induced hand2 overexpression in Tg(cmlc2:Cre:ER); Tg(β-actin2:RSH) animals by injecting 4-HT 5 days before performing ventricular resection. In these experiments, we observed a 102% increase in cardiomyocyte proliferation at the resection plane, compared with the proliferation observed in vehicle-injected Tg(cmlc2:Cre:ER); Tg(β-actin2:RSH) animals (Fig. 9C-E). In total, our results indicate that elevation of hand2 expression helps boost cardiomyocyte production during regeneration, mirroring its effect in the embryo.

DISCUSSION

Taken together, our studies demonstrate three distinct mechanisms through which hand2 overexpression can promote enhanced cardiomyocyte production. First, overexpression of hand2 results in expansion of cardiomyocyte production within the early differentiating FH, potentially by promoting myocardial specification at the expense of neighboring blood and vessel lineages. Second, hand2 overexpression causes excess proliferation of cardiac progenitor cells within the late-differentiating SHF, leading to the formation of an abnormally elongated outflow tract. Third, induced myocardial expression of hand2 enhances regenerative proliferation of cardiomyocytes in response to injury.
Our previous studies have shown that loss of hand2 function dramatically reduces the cardiomyocyte population, emphasizing the necessity of hand2 for efficient cardiomyocyte production (Yelon et al., 2000; Schoenebeck et al., 2007). Integrating our current and past work, we conclude that hand2 plays important and instructive roles in promoting cardiomyocyte production via influences on both specification and proliferation.

Our data indicate that overexpression of hand2 is sufficient to induce ectopic cardiomyocyte formation in a region proximal to the typical FHF, but we do not find ectopic cardiomyocytes elsewhere within hand2-overexpressing embryos. Additionally, while hand2 overexpression is capable of hindering the expression of blood and vessel markers, hand2-null embryos do not exhibit expanded expression of blood and vessel genes (Schoenebeck et al., 2007). These findings suggest that other factors partner with Hand2 to promote myocardial specification in the FHF and that expression of these factors is restricted to a particular region of the ALPM. The significance of the partnerships between Hand2 and other factors is reinforced by our data demonstrating that the effects of hand2 overexpression on cardiomyocyte production appear to be independent of direct binding of DNA by Hand2 and dependent on Hand2 dimerization. It will be valuable for future studies to investigate which Hand2 dimerization partners are relevant to its influences on ALPM patterning.

In addition to an instructive role for hand2 during specification of the FHF, our data suggest a previously unappreciated role for hand2 in promoting progenitor proliferation within the SHF. Hand factors have been shown to have roles in both promoting and inhibiting proliferation
(Risebro et al., 2006; Li et al., 2011), but this particular role of hand2 in the SHF has not been demonstrated by prior studies. The impact of hand2 on SHF progenitor proliferation contrasts with previous work that found a role for Hand2 in promoting survival of SHF progenitor cells (Tsuchihashi et al., 2011); although proliferation defects were not documented in these studies of conditional Hand2 knockout mice, it is possible that deficient SHF proliferation preceded the observed death of SHF cells in these embryos. Additionally, it is interesting to compare the effects of hand2 overexpression on the zebrafish SHF with the phenotype of mouse mutants in which Hand1-expressing cells were engineered to overexpress Hand1 (Risebro et al., 2006). In these mice, increased proliferation was seen in the distal outflow tract, but no expansion of SHF progenitor markers was observed, suggesting that Hand1 overexpression did not increase proliferation within the SHF itself but instead within the cardiomyocytes present or arriving at the outflow tract (Risebro et al., 2006). These effects of Hand1 overexpression on outflow tract proliferation seem distinct from our observed effects of hand2 overexpression on the SHF progenitor.

Fig. 8. Cardiac injury induces hand2 expression.
(A-H) In situ hybridization depicts expression of (A,E) hand2, (B,F) tbx5, (C,G) tbx20 and (D,H) nkx2.5 in ventricles of adult Z-CAT fish 7 days post-injection (dpi) of vehicle (A-D) or 4-HT (E-H). Expression of each gene is enhanced in spared myocardium following injury (E-H, arrowheads). (I-K) Expression of Tg(hand2:EGFP) (green) highlights the activation of hand2 regulatory sequences following injury. Images are single confocal slices of ventricular tissue at 7 dpi (I,J) and 14 dpi (K); insets demonstrate myosin heavy chain (MHC; red) localization in hand2-expressing cells.

Fig. 9. Overexpression of hand2 boosts cardiomyocyte proliferation in response to injury.
(A) Schematic representation of transgenes used for cardiomyocyte ablation and myocardium-specific hand2 overexpression in Z-CAT fish. (B) Proliferation indices for ventricular cardiomyocytes; error bars indicate s.e.m. Proliferation was assessed 7 days after 4-HT injection in Z-CAT fish with or without induced cardiomyocyte-specific hand2 overexpression. hand2-overexpressing fish exhibited significantly increased proliferation following ablation injury (n=9 or 10; *P<0.05). (C) Proliferation indices in injured cmlc2:CreER; β-act2:RSH; Tg(hand2:EGFP) fish. hand2-overexpressing fish exhibited significantly increased proliferation following resection injury (n=4 or 5; *P<0.005). (D,E) Representative examples of cardiomyocyte proliferation at 7 dpa at the resection plane (brackets) in injured cmlc2:CreER; β-act2:RSH fish that were treated with vehicle (D) or 4-HT (E). Mef2 (green) marks cardiomyocytes and PCNA (red) marks proliferating cells; arrows in insets indicate double-positive cells.
population outside the heart tube; this difference may relate to the conditional regulation of Hand1 expression in these mice, which do not overexpress Hand1 within the SHF.

Finally, our data indicate that hand2 overexpression can impact regenerative cardiomyocyte proliferation in the adult heart. These findings implicate the induction of hand2 expression during regeneration as a key component of the regenerative mechanism. We speculate that the ability of Hand2 to increase reprogramming efficiency of fibroblasts toward a cardiac fate (Song et al., 2012; Nam et al., 2013) could be related to our observation that Hand2 can enhance cardiomyocyte proliferation; alternatively, the function of Hand2 during reprogramming could be a reflection of our observed influences of Hand2 on myocardial progenitor specification or proliferation. To distinguish between these possibilities, it will be important to elucidate and compare the pathways regulated by Hand2 in each of these contexts, as well as in other settings, such as the Hand2-driven induction of hypertrophy in the postnatal mouse myocardium (Dirkx et al., 2013). In the long term, these investigations may allow further improvements in strategies for cardiac reprogramming, regeneration and repair.

In addition to their implications regarding future applications for Hand2 in regenerative medicine, our results provide intriguing insight into possible causes of CHD. Duplications and deletions of the 4q33 chromosomal region, which contains HAN2D, have been associated with CHD (Borochowitz et al., 1997; Byatt et al., 1997). Moreover, individuals with partial trisomy distal 4q (4q+ syndrome), a translocation that involves duplication of HAND2 and neighboring genes, exhibit striking cardiac and limb defects (Tamura et al., 2013). In a mouse model for 4q+ syndrome, rebalancing levels of Hand2 by breeding to Hand2-deficient animals ameliorated the heart and limb phenotypes, implicating overexpression of Hand2 as a cause of these defects (Tamura et al., 2013). Our data suggest possible ways in which HAND2 overexpression could cause CHD through alteration of cardiomyocyte production; future studies that elucidate the specific Hand2 partners and targets involved in FHF specification, SHF cardiomyocyte production; and also prevents read-through translation of Hand2 protein.

In situ hybridization
Whole-mount in situ hybridization was performed as previously described (Thomas et al., 2008) using probes for cmlc2 (myl7; ZDB-GENE-991019-3), mef2c (ZDB-GENE-040901-7), islet1 (id1; ZDB-GENE-980526-112), scl (sall1; ZDB-GENE-980526-501), etv2 (en2; ZDB-GENE-050622-14), pu.1 (pu.1; ZDB-GENE-980526-164), nkd2.5 (ZDB-GENE-980526-321) and gata4 (ZDB-GENE-980526-476). Embryonic stages were determined by counting somites prior to fixation. Area measurements were made by using the selection tool in ImageJ64 to score stained pixel area. Statistical analysis of data sets was performed using Microsoft Excel to conduct unpaired t-tests. We have found that cmlc2 area measurements at 18-20 somites correlate well with counts of cell outlines in the same images (data not shown), making area measurement a reasonable strategy for comparing estimated sizes of cardiomyocyte populations.

Immunofluorescence
Whole-mount immunofluorescence was performed as previously described (Thomas et al., 2008), using the monoclonal antibodies MF20 and S46 (Developmental Studies Hybridoma Bank), a polyclonal antibody against Islet1 (GeneTex; GTX128201L) and the secondary antibodies goat anti-mouse IgG2b FITC (Southern Biotech), goat anti-mouse IgG1 TRITC (Southern Biotech) and goat anti-rabbit Alexa Fluor 488 (Molecular Probes). The numbers of Islet1-positive nuclei within MF20-positive cells were counted both in three-dimensional reconstructions and in optical sections.

Fluorescent in situ hybridization with immunofluorescence
Embryos were fixed in 2% formaldehyde for 20 min and then gently agitated in 0.5% Triton and 0.2% saponin to dissolve the yolk. Embryos were then fixed in 4% paraformaldehyde overnight at 4°C. An antisense fluorescein probe was used for detection of TSA Plus fluorescent solution (PerkinElmer), using an established protocol (Brend and Holley, 2009), followed by MF20 antibody staining.

Cell counting and EdU incorporation
Cardiomyocyte counting in Tg(hsp70:FLAG-hand2-2A-mCherry) embryos was performed using an established protocol (Schoenebeck et al., 2007). Cell counting in Tg(hsp70:FLAG-hand2-2A-mCherry) embryos was performed subsequent to EdU incorporation, using a modification of a described protocol (Zeng and Yelon, 2014). Dechorionated embryos were exposed to 10 mM EdU...
0.3× Danieau buffer with 15% DMSO for 30 min on ice. After a series of washes, embryos were incubated at 28.5°C until fixation. Embryos were fixed in 2% formaldehyde for 20 min and then gently agitated in 0.5% Triton and 0.2% saponin to dissolve the yolk, followed by fixation in 2% formaldehyde overnight at 4°C. Embryos were then stained with the primary antibody MF20 followed by an anti-mouse IgG secondary antibody conjugated with either Alexa Fluor 488 or 647 (Invitrogen). EdU incorporation was visualized using a Click-it imaging kit (Invitrogen) with either Alexa Fluor 594 or 647. Samples were then placed in SlowFade Gold anti-fade reagent with DAPI (Molecular Probes). Cardiomyocyte number was determined by examining DAPI-stained nuclei within MF20-positive cells both in three-dimensional reconstructions and in optical sections, and proliferation index was calculated as the percentage of these cells that exhibited EdU localization. A similar method was used to determine the number and proliferation index of Tg(tbx2.5:ZsYellow)-expressing MF20-negative cells clustered near the arterial pole of the heart tube. Statistical analysis of data sets was performed using Microsoft Excel to conduct unpaired t-tests.

Imaging
Bright-field and fluorescent images were captured with a Zeiss Axiozoom or a Zeiss Axioscope microscope, and processed using Zeiss AxiosVision and Adobe Creative Suite. Confocal z-stacks were collected by a Leica SP5 confocal microscope and analyzed using Imaris software (Bitplane).

Cardiac injury and histology
For cardiomyocyte ablation injury, we induced myocyte expression of diphtheria toxin A in zebrafish carrying both the Tg(cmlc2:CreER)t248 (Kikuchi et al., 2010) and Tg(β-actin2:loxp-mCherry-STOP-loxp-DTA)p346 (Wang et al., 2011) transgenes; this combination is referred to as Z-CAT (zebrafish cardiomyocyte ablation transgenes) (Wang et al., 2011). Anesthetized Z-CAT fish were injected with 0.5 mg/ml 4-hydroxytamoxifen (zebrafish cardiomyocyte ablation transgenes) (Wang et al., 2011). For ventricular resection injury, we performed surgeries as described previously (Poss et al., 2002), using probes for hand2 (ZDB-GENE-000511-1), tbx5 (ZDB-GENE-991124-7), nks2.5 (ZDB-GENE-980526-321) and tbx20 (ZDB-GENE-000427-7). Immunofluorescence with primary antibodies that recognize myosin heavy chain (F59; Developmental Studies Hybridoma Bank) and nkx2.5 (ZDB-GENE-000511-1), and McCallion, A. S. (2006). Evaluating the biological relevance of putative enhancers using Tol2 transposon-mediated transgenesis in zebrafish. Nat. Protoc. 1, 1297-1305.

References


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Supplemental Figure S1. Mouse and zebrafish Hand2 protein sequences are highly conserved. Alignment of amino acid sequences of mouse Hand2 (NP_034532.3; top row) and zebrafish Hand2 (AAI65015.1; bottom row), generated through pairwise BLASTP (Altschul et al., 1997). Identical and similar amino acids are indicated in the middle row. The three arginines replaced in the DNA binding-deficient form of Hand2 (Hand2 EDE) are shown in red. The phenylalanine replaced in the dimerization-deficient form of Hand2 (Hand2 P) is shown in green. The threonine and serine replaced in the phosphorylation-deficient form of Hand2 (Hand2 AA) are shown in blue.

Supplemental Figure S2. Induction of hand2 overexpression at 10 hpf, but not at 24 hpf, produces a morphologically evident cardiac phenotype. (A-F) Lateral views of live embryos at 36 hpf depict (A-C) embryonic morphology and (D-F) mCherry fluorescence in (A,D) a nontransgenic embryo and (B,C,E,F) embryos carrying the transgene Tg(hsp70:FLAG-hand2-2A-mCherry) (abbreviated as Tg(hsp70:hand2)), after heat shock at (A,B,D,E) 10 hpf (tail bud) or (C,F) 24 hpf. Heat shock of transgenic embryos at 10 hpf leads to pericardial edema (B), and residual mCherry fluorescence is still evident in these embryos at 36 hpf (E). In contrast, edema is not observed in transgenic embryos after heat shock at 24 hpf (C); mCherry fluorescence remains pervasive in these embryos at 36 hpf (F).
Supplemental Figure S3. Levels of protein production in different transgenic lines. (A-H) Lateral views of live embryos at 30 hpf depict bright field images of representative embryos from different transgenic lines (A-D), along with corresponding mCherry fluorescence (E-H). (E) No mCherry fluorescence is induced in heat-shocked nontransgenic embryos. (F-H) mCherry fluorescence is readily detectable in representative embryos carrying the transgenes (F) Tg(hsp70:FLAG-hand2-2A-mCherry) (Tg(hsp70:hand2)), (G) Tg(hsp70:FLAG-hand2P-2A-mCherry) (Tg(hsp70:hand2P)), and (H) Tg(hsp70:FLAG-hand2AA-2A-mCherry) (Tg(hsp70:hand2AA)). (I) Western blot analysis compares levels of FLAG-tagged Hand2 protein in different transgenic lines. Embryos were deyolked and lysates were prepared as previously described (Link et al., 2006), and blots were probed with either a monoclonal anti-FLAG M2 antibody (F1804, Sigma, 1:2000) or a monoclonal anti-α-Tubulin antibody (T6728, Sigma, 1:10,000), followed by a rabbit anti-mouse IgG HRP-conjugated secondary antibody (ab97046, Abcam, 1:10,000). Proteins were visualized using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). Each lane contains lysate from 15 embryos at 36 hpf (2 hours following heat shock), and the lanes compare protein levels in nontransgenic embryos (WT), Tg(hsp70:hand2) embryos (hand2), Tg(hsp70:hand2P) embryos (hand2P), and Tg(hsp70:hand2AA) embryos (hand2AA). All three transgenic lines contain a ~23 kD FLAG-Hand2 protein, with comparable levels in Tg(hsp70:hand2) and Tg(hsp70:hand2AA) embryos and lower levels in Tg(hsp70:hand2P) embryos.
Supplemental Figure S4. Normal islet1 expression at the venous pole in embryos overexpressing hand2. (A-B) Immunofluorescence at 36 hpf for MF20 (red, visible throughout the heart) and Islet1 (green, visible in the nuclei of a subset of atrial cells). Frontal views, dorsal to the top; arrows point to the Islet1-positive population at the venous pole of the atrium. Following heat shock at 10 hpf, the population of Islet1-positive cells at the venous pole appears similar in hearts from (A) nontransgenic embryos and (B) Tg(hsp70:hand2) embryos. (C) Bar graph compares average number of Islet1-positive cardiomyocytes at 36 hpf in nontransgenic embryos and Tg(hsp70:hand2) embryos, following heat shock at 10 hpf. Error bars indicate standard deviation; no significant difference is observed between these two data sets (n=12, p=0.09). (D-E) In situ hybridization depicts islet1 expression at 36 hpf, following heat shock at 10 hpf. Frontal views, dorsal to the top; arrows point to the ring of islet1-expressing cells at the venous pole of the atrium. Expression patterns are similar in (D) nontransgenic embryos and (E) Tg(hsp70:hand2) embryos.
Supplemental Figure S5. No evident influence of hand2 overexpression on cardiomyocyte proliferation after initial heart tube assembly. (A-F) EdU incorporation in hearts of (A-C) nontransgenic and (D-F) Tg(hsp70:hand2) embryos at 36 hpf, following heat shock at 10 hpf and EdU pulse at 23 hpf; (A,C,D,F) partial reconstructions of confocal z-stacks with ventricle up and (B,E) representative single slices. Dots, arrows, and color schemes are as described for Fig. 5A-F. Note that the nontransgenic heart shown (A) contains a number of EdU-positive blood cells that were trapped during fixation; EdU-positive blood cells are less commonly observed within the hearts of hand2-overexpressing embryos (D), due to their impaired circulation. (G) Timeline of experimental design. (H) Bar graph compares proliferation indexes in nontransgenic (mCherry-negative) and Tg(hsp70:hand2) (mCherry-positive) embryos, as in Fig. 5H. No change in proliferation index is seen in hand2-overexpressing embryos (n=8-11; p=0.196).
Supplemental Figure S6. No evident influence of hand2 overexpression on cardiomyocyte proliferation within the heart tube at 23 hpf. (A-D) EdU incorporation in hearts of (A,B) nontransgenic and (C,D) Tg(hsp70:hand2) embryos at 23 hpf, following heat shock at 10 hpf and EdU pulse at 17 hpf; partial reconstructions of confocal z-stacks. Images depict the elongating cardiac cone, positioned with its arterial end toward the top. (A,C) White dots indicate EdU-positive (red) cells that are also MF20-positive (green) differentiated cardiomyocytes. (B,D) White dots indicate all nuclei (DAPI, blue) of myocardial cells, including both EdU-positive and EdU-negative cardiomyocytes. (E) Timeline of experimental design. (F) Bar graph compares proliferation indexes in nontransgenic (mCherry-negative) and Tg(hsp70:hand2) (mCherry-positive) embryos, as in Fig. 5H. No change in proliferation index is seen in hand2-overexpressing cardiomyocytes (n=10-11; p=0.252). Similarly, when we assessed EdU incorporation in hand2-overexpressing embryos at 26 hpf, following heat shock at 10 hpf and EdU pulse at 14 hpf, we did not see an increased proliferation index in hand2-overexpressing cardiomyocytes (proliferation index of 28 ± 4% in nontransgenic embryos compared to proliferation index of 27 ± 4% in hand2-overexpressing embryos; n=7-10, p=0.58).
Supplemental Figure S7. Endocardium is present in embryos overexpressing hand2. (A-H) Lateral views of live embryos at 30 hpf depict (A-B) embryonic morphology and (C-H) expression of the transgene Tg(kdrl:GRCFP) in the vasculature of (A,C,E,F) nontransgenic embryos and (B,D,G,H) Tg(hsp70:hand2) embryos, following heat shock at 10 hpf. (C-D) General vascular patterning and sprouting of intersomitic vessels (inset) is intact in embryos overexpressing hand2 (D). (E-H) Partial reconstructions of confocal z-stacks (E,G) and representative single slices (F,H) highlight the endocardium (arrows): just as in nontransgenic embryos (E,F), a thin layer of endocardial tissue lines the entire heart tube in embryos overexpressing hand2 (G,H).

SUPPLEMENTAL REFERENCES
