Hand2 Loss-of-Function in Hand1-Expressing Cells Reveals Distinct Roles in Epicardial and Coronary Vessel Development

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Rationale: The basic helix–loop–helix (bHLH) transcription factors Hand1 and Hand2 are essential for embryonic development. Given their requirement for cardiogenesis, it is imperative to determine their impact on cardiovascular function.

Objective: To deduce the role of Hand2 within the epicardium.

Method and Results: We engineered a Hand1 allele expressing Cre recombinase. Cardiac Hand1 expression is largely limited to cells of the primary heart field, overlapping little with Hand2 expression. Hand1 is expressed within the septum transversum, and the Hand1 lineage marks the proepicardial organ and epicardium. To examine Hand factor functional overlap, we conditionally deleted Hand2 from Hand1-expressing cells. Hand2 mutants display defective epicardialization and fail to form coronary arteries, coincident with altered extracellular matrix deposition and Pdgfr expression.

Conclusions: These data demonstrate a hierarchical relationship whereby transient Hand1 septum transversum expression defines epicardial precursors that are subsequently dependent on Hand2 function. (Circ Res. 2011; 108:00-00.)

Key Words: Hand1 ■ Hand2 ■ bHLH ■ epicardium

When cardiac gene programs are hobbled, there is rarely an ablation of the cardiac lineage, but more often defective cell differentiation and/or tissue morphogenesis, which manifests as congenital heart defects (CHDs). Multiple cell lineages collectively form the heart.1 Although myocardial derivatives ultimately provide the cardiac musculature that powers circulation, it is the extracardiac cell lineages (the cardiac neural crest cells [cNCC], coronary endothelium, and epicardium) that define the framework on which these cardiomyocytes develop.2,3–5 Although the heart functions as an integrated whole, our understanding of its individual components is not uniform. For example, the second heart field (SHF) and cNCC have been extensively studied,6 whereas the differentiation programs of the primary heart field (PHF) and coronary vasculature are less well defined.

Both cNCC and epicardial cell lineages undergo epithelial-to-mesenchymal transition (EMT) and directly interact with differentiating cardiomyocytes. cNCCs enable septation of the outflow tract (OFT) into the aorta and pulmonary trunk. Epicardial mesothelium migrates from the proepicardial organ (PE) to cover the surface of the heart.7 Subsequently, an epicardial subpopulation undergoes secondary EMT, invades the heart, and differentiates into coronary smooth muscle and cardiac fibroblasts.8–11 Cardiac morphogenesis requires communication between cardiac and extracardiac cell populations.2,3,12 The signaling cascades through which these cell lineages communicate to are just coming to light.

Expression of the Twist-family basic helix–loop–helix (bHLH) transcription factors Hand1 and Hand213,14 partially overlaps within the developing heart and cNCC.15–18 Hand2 expression marks the cardiac crescent and linear heart tube and is downregulated within the left ventricle at the onset of cardiac looping.18 At comparable developmental stages, the left ventricle and myocardial cuff express Hand1.15,16,19 Understanding where and when Hand1 and Hand2 function during embryonic development is a challenge because of their dynamic spatiotemporal expression profiles.13

Hand factors are critical for cardiac morphogenesis.13,14 Systemic ablation of Hand2 results in a single ventricle, increased apoptosis, and lethality by embryonic day (E)9.5,15,16 Hand1 knockout mice die by E9.5 from extraembryonic and vascular defects.16,19,20 Conditional NCC-specific Hand2 ablation

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Hand1 and associated VSDs. Significantly, E14.5 and present persistent truncus arteriosus (PTA), DORV expressing cells to cardiogenesis using a WT1Cre ablation within the fibroblasts, and a nonfunctional coronary vasculature. bryos display defective epicardial EMT, decreased cardiac the epicardial derivatives of organ (PE), and is thus deleted from the PE, epicardium and temporally downstream of Hand1 results establish that the Hand2 haploinsufficiency increases phenotypic severity suggesting genetic and functional overlap.24,25

Here, we investigate the contributions of Hand1-expressing cells to cardiogenesis using a Cre recombinase-expressing Hand1 allele.26 The Hand1 lineage robustly marks the left ventricular myocardium, a CNCC subpopulation, and, unexpectedly, the epicardium and its derivatives.

To deduce functional/genetic interactions between Hand factors during cardiogenesis, we conditionally deleted Hand2 within the Hand1 lineage (H2CKO). H2CKO embryos die by E14.5 and present persistent truncus arteriosus (PTA), DORV and associated VSDs. Significantly, Hand2 expression lies temporarily downstream of Hand1 within the septum transversum (ST)-derived cells that migrate into the proepicardial organ (PE), and is thus deleted from the PE, epicardium and the epicardial derivatives of H2CKO embryos. H2CKO embryos display defective epicardial EMT, decreased cardiac fibroblasts, and a nonfunctional coronary vasculature. Hand2 ablation within the WTICre lineage phenocopies Hand1Cre-mediated Hand2 ablation. Gene expression analyses reveal an altered ratio of PdgfcrePdgfβ mRNA, a decreased profile of fibroblast markers, and disorganized extracellular matrix (ECM). These changes are associated with increased epicardial cell apoptosis. Together, our data shows that Hand2 performs an essential role during epicardialization, which directly impacts epicardial cell differentiation, and formation of the coronary vasculature.

**Results**

**Hand1 Expression Within the ST Marks the Progenitors of the PE**
As Hand1 lineage cells, but not Hand1-expressing cells, are observed within the epicardium at E10.5 (see Online Figure 1), we sought the Hand1-expressing progenitors of these cells. The PE is derived from the anterior ST and gives rise to the epicardium.32 ISH was performed for both Hand1 and Tbx18, a marker of the PE, at E9.5 (Figure 1A through 1E). Tbx18 is expressed throughout the PE but is not detectable within the ST. In contrast, Hand1 expression is not detectable within the PE but is expressed robustly throughout the ST, thus identifying the source of the Hand1-marked epicardium. X-Gal staining of Hand1LacZ/ embryos confirms expression within the LV and the ST (Figure 1F). X-Gal staining of the Hand1 lineage shows Hand1-marked cells dispersed throughout the ST and within the more proximal Tbx18-expressing region, supporting established models that PE cells derive from migratory ST cells (Figure 1G through 1I).33 These results establish that the Hand1 lineage cells observed throughout the cardiac fibroblasts, coronary vasculature, epicardium and PE originate from a Hand1-expressing ST cell population.

**Distinct Hand Factor Expression During Heart Morphogenesis**
E8.5 Hand1 lineage analysis reveals that the linear heart tube is not completely derived from Hand1-expressing cells, indicating that Hand1 expression initiates after heart tube fusion. To account for a possible temporal delay of Cre expression, we performed whole-mount ISH for Hand1 at E7.5 (Figure 2). Hand1 is detected throughout the extra embryonic mesoderm, chorion, and allantoic rudiment (Figure 2A). As the chorion is directly adjacent to the cardiac crescent (Figure 2A and 2B, white arrow), we performed a
double label ISH for both Hand1 and the cardiac marker Mlc2a. Hand1 expression does not directly overlap with Mlc2a expression at E7.5, indicating that, consistent with the Hand1 lineage analysis, Hand1 cardiomyocyte expression initiates subsequent to linear heart tube fusion (Figure 2B).

Whole-mount ISH at E8.5 (Figure 2C through 2F) shows Hand1 expression within the posterior heart tube, whereas Hand2 expression is observed throughout the entire cardiac field. Sagittal sections of whole mount–stained embryos confirm that Hand1 expression is restricted to the early ventricular chamber and cuff myocardium. Hand2 expression is robust throughout the endocardium, SHF and OFT. Interestingly, Hand2 expression is not detected within the ventricular myocardium at E8.5 and, in whole-mount view, the Hand2 endocardial expression is visible through the thin myocardial wall (Figure 2F, red arrow). At E9.5, Hand1 and

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**Figure 2.** Hand factor expression is distinct. Single- and double-labeled Mlc2a and Hand1 whole-mount RNA ISH of E7.5 embryos show that Hand1 is not expressed within the cardiac crescent (white arrow) but is expressed within extra embryonic mesoderm, allantoic rudiment, and the chorion (A and B). E8.5 whole mounts of Hand1 and Hand2 reveal complimentary expression during cardiac development (C through F). Hand2 expression is confined to the SHF and endocardium (black arrow). cc indicates cardiac crescent; eem, extraembryonic mesoderm; ht, heart tube; Im, lateral mesoderm; oft, outflow tract; v, ventricle.
By E14.5, H2CKO embryos displayed pericardial hemorrhaging, anasarca, and liver hypoplasia (Figure 4C, 4D, 4G, and 4H). OFT septation defects, including PTA and DORV, were also observed, reflecting cNCC and/or SHF-derived myocardial defects (Figure 4A, 4B, 4E, 4F, 4I, and 4J). Hypertrabeculation/noncompaction and VSDs were also observed (Figure 4K through 4N), although, H2CKO sarcomeric ultrastructure was indistinguishable from controls (Online Figure III, Q and R).

Hand2ΔNCC die from a sympathetic nervous system (SNS) norepinephrine deficit.22,23 As Hand1 and Hand2 are coexpressed in some NCCs, H2CKO embryos possibly die from SNS defects. Hand2ΔNCC lethality can be rescued by the β-adrenergic agonist isoproterenol.22,23 Unlike Hand2ΔNCC embryos, isoproterenol-treated Hand1Cre-mediated H2CKO embryos fail to survive to birth (Figure 4), indicating that SNS defects do not account for embryonic lethality.

Hand2 Is Required for a Functional Epicardium

Given the temporal relationship observed between Hand1 expression in the ST and Hand2 within the PE/epicardium, we examined H2CKO epicardial defects (Figure 5A and 5B). E14.5 H2CKO display abnormal compaction, an absence of epicardium, and a lack of coronary lumens, indicating a possible epicardial phenotype (Figure 5A and 5B). ISH analyses of epicardial markers at E10.5 (data not shown) and at E12.5 for Tcf21 suggest that specification and formation of the early epicardium occurs normally (Figure 5C and 5D). To confirm direct disruption of H2CKO epicardial development, we generated a WT1ERT2Cre H2CKO, in which Hand2 is ablated in the ST, PE and epicardium, but not the myocardium and NCC lineages (Figure 5E through 5H; see Online Figure IV for a WT1ERT2Cre, Hand1Cre, and Hand2 cardiac lineage expression comparison). Potentially complicating these analyses, WT1ERT2Cre marks lateral mesoderm, where Hand2 is expressed, and early allelic WT1ERT2Cre induction (E8.5) can produce ectopic Cre activity.4 HoxB6-Cre-mediated Hand2 deletion in lateral mesoderm generates viable neonates.23 Tamoxifen-mediated WT1ERT2Cre allelic induction at E9.5 resulted in no ectopic myocardial R26R reporter activity (Online Figure V). At E13.5, Hand2ERT2Cre, WT1ERT2Cre mutant embryos phenocopied H2CKO embryos, displaying a poorly organized epicardium and reduced WT1-marked cell lineage (Figure 5E through 5H). Myocardial noncompaction was also evident, further suggesting that this phenotype is non–cell-autonomous. These mutants appear to phenocopy the epicardial defects and E14.5 embryonic lethality associated with H2CKO embryos (Figure 5A, 5B, 5G, and 5H). These 2 independent epicardial Hand2 ablations indicate that Hand2 may play multiple roles during epicardiogenesis, the initiation of secondary epicardial EMT, and the terminal differentiation of post EMT epicardial cell populations.

Loss of Hand2 Disrupts Epicardial Gene Expression and Coronary Vasculature Patency

As conditional Hand2 loss-of-function leads to epicardial defects, we sought to further identify the mechanistic role for
Hand2 within the epicardium. Platelet-derived growth factor receptors (Pdgfrs) play essential roles in cell fate and specification of the mature epicardium.14,35 E12.5 ISH reveals decreased Pdgfrα/H9251 expression within the H2CKO epicardium (Figure 6A and 6B). The number of cells expressing Periostin (Postn), a marker of the cardiac fibroblast cell fate,36 is visibly decreased in the myocardium of E12.5 H2CKO mutants (Figure 6C and 6D). Together, these data suggest that Hand2 functions in epicardial cell fate determination during or after secondary EMT.

We performed CD31 (Online Figure VI, A and B) and Flk1 immunohistochemistry (Figure 6E and 6F) at E12.5 to examine coronary vessel formation. Flk1-positive cells running through the epicardium are absent. Phalloidin counterstaining indicates that the epicardium is intact, though absent of potential Flk1-positive lumens (Figure 6F). The Hand1 lineage does not contribute to coronary endothelial cell populations (Online Figure VI, C through F),26 and therefore the absence of coronary vasculature within H2CKO embryos likely results from non–cell-autonomous mechanism(s). To take advantage of induction, we administered tamoxifen at E11.5 CD31-positive coronary lumens are visible within H2CKO embryos when WT1ERT2Cre is activated at E11.5, although they appear less prevalent than in embryos that lack the WT1ERT2Cre allele (Online Figure V, C and D), suggesting that critical Hand2 epicardial function occurs between E9.5 and 11.5.

To further address the impaired function of H2CKO epicardium, we generated epicardial primary cultures (EPCs) from wild-type and H2CKO embryos and isolated total RNA for microarray analyses (Online Figure II, A through D). Gene ontology from our microarray analysis indicates significant differences in developmental, morphological, and cardiovascular gene programs within EPCs (Online Figure VI, G). Quantitative RT-PCR on RNA isolated from wild type and H2CKO EPCs was performed to validate changes in gene expression observed in the microarray (Online Figure VI, H). Quantitative RT-PCR confirms expression of Hand2 within the epicardium and its ablation within the H2CKO EPCs. Importantly, the ratio of Pdgfrα to Pdgfrβ is greatly altered in H2CKO EPCs. Expression of Pdgfrα is significantly reduced, whereas, Pdgfrβ expression is significantly upregulated (Online Figure VI, H). Hand2 regulation of Pdgfrα is direct (Online Figure VI, I). In epicardial cells, Pdgfrβ-mediated signaling promotes a smooth muscle fate, whereas the role of Pdgfrα signaling is currently unclear.14 The decrease in Postn-positive myofibroblasts within the compact zone suggests that Pdgfrα impacts fibroblast differentiation. Together, these data suggest that Hand2 directly impacts epicardial cell fate through a Pdgfr-dependent mechanism.

Hand2 Alters Fn1 Fibril Assembly and Organization

As H2CKO epicardial defects appear direct, we looked at the impact of a loss of Hand2 on epicardial mesothelium integ-
Fibril assembly regulates the organization and stability of ECM proteins, is capable of promoting EMT and adhesion-dependent growth, and is associated with integrin-mediated cell signaling. Additionally, defects in Fn1 underlie integrin-mediated valve leaflet defects in the lymphatic system. Immunohistochemistry shows that Fn1 is expressed in the E12.5 epicardium and is neatly organized around the developing coronaries (Figure 7A). H2CKO embryos retain epicardial Fn1; however, it appears disorganized (Figure 7B). To look at Fn1 organization more closely, we compared EPCs from wild type and H2CKO embryos (Figure 7C through 7F). Immunohistochemistry reveals that Fn1 fibrils form an organized lattice in wild type EPCs. Fn1 deposition appears abnormally uniform and sheet-like throughout the H2CKO explant (Figure 7C through 7F). Fn1 dysfunction suggests a role for Hand2 in ECM assembly and epicardium homeostasis. Increased Alcian Blue staining indicates that ECM organization and/or deposition is altered in the H2CKO epicardium (Figure 7G and 7H).

Gene ontology from microarray analysis to identify enriched biological processes indicates enrichment in ECM based processes, such as cell–cell signaling, assembly, connective tissue development, and motility (Figure 7I). Quantitative RT-PCR on wild type and H2CKO EPCs detected no changes in Fn1 expression, as has been reported in mice (Figure 7J). In addition to its role as an ECM component, Fn1 promotes intracellular signaling via interactions with cell surface integrins. To see whether the observed H2CKO Fn1 disorganization may alter cell signaling, we assessed integrin expression by quantitative RT-PCR (Figure 7J). Indeed, expression of the Fn1 receptor, Itga4, is significantly upregulated within H2CKO EPCs. Itga4 and Itgb1 together form an Fn1 receptor pair. Itga4 influences epicardial Fn1 polymerization. Itga4 overexpression impairs incorporation of new Fn1 into preexisting polymer structures, whereas Itga4 deletion causes embryonically lethal epicardial defects in mice. As Itga4 and Fn organization are essential for epicardiogenesis, the observed dysregulation of these ECM components suggests that Hand2 plays an important role in maintaining a normal epicardial ECM environment.
Gene ontology data indicate enrichment for cell cycle regulation genes. Loss-of-function of Hand2 has been implicated in apoptosis.20 Moreover, ECM disorganization and deposition are thought to be proapoptotic.47 Activated-Caspase3 and Phospho-Histone H3 immunohistochemistry at E12.5 to assess H2CKO epicardial apoptosis and proliferation (Figure 7K and 7L; Online Figure VII, A through D) reveals no change in proliferation, but a significant increase in epicardial apoptosis, These data suggest that the process of epicardial EMT is not affected in H2CKO as EMT is linked with proliferation,48 but the impaired function of the ECM in H2CKO embryos maybe tied both to the function, integrity, and survival of the epicardium.

Discussion

Collectively, these data reveal a novel and essential function for Hand factors in epicardiogenesis (Online Figure VIII, A and B). Transient ST expression of Hand1 marks cells that successively populate the PE, epicardium and their derivatives. Hand1 ST expression temporally precedes Hand2 PE expression. Hand2 ablation causes significant epicardial defects including directly impaired Pdgfra regulation, abnormal differentiation and Fn1 organization, and Itga4 upregulation. Also evident in H2CKO embryos is increased epicardial apoptosis, potentially reflecting altered Fn-mediated signaling and/or cell migration. Fn1 and Itga4, have been associated with similar mesodermal, epicardial, and cardiovascular decline.45,46,49

Epicardiogenesis initiates normally in Itga4−/− mice; however, the epicardium subsequently detaches from the myocardium and degrades.45,46 Itga4 overexpression alters Fn/integrins interactions and disrupts Fn deposition, demonstrating that integrin levels significantly impact Fn organization and function.52 Our observations suggest that Hand2 is necessary to maintain the balance between this receptor/ligand pair. Ultimately, Hand2 ablation leads to compromised epicardial function and a failure to form a patent coronary vasculature, both phenotypes observed in other mouse models presenting coronary malformations.50

Fn1 is a multifunctional ECM protein that establishes cytoskeletal organization, motility, and cell signaling pathways required for proliferation and growth. As epicardial cells must migrate, alter morphology, and differentiate into functional cell types, the epicardial phenotypes observed in H2CKO embryos mechanistically fit a model of altered Fn1 function. Defects in Fn1 deposition are associated with increased fibrosis and apoptosis,51 all characteristic of the H2CKO epicardium. Fn1 mRNA expression is not altered within H2CKO epicardium, demonstrating that Hand2 regulation of Fn1 is indirect. Zebrafish hand regulates fn1 indirectly through ECM organization, rather than by directly regulating gene expression.52 These observations suggest a critical, evolutionarily conserved role for Hand2 during maturation of epicardial-derivatives.
Previous studies suggest that Hand factors have partially overlapping expression domains and are functionally redundant during cardiac patterning.\(^{25}\) Detailed Hand1 and Hand2 expression profiles show less spatiotemporal myocardial overlap than previously indicated. Hand2 deletion within the Hand1 expression domain does not cause significant myocardial patterning or differentiation defects (Online Figure III), suggesting that the H2CKO myocardial phenotypes are non–cell-autonomous. Hand2 deletion using cardiac-specific Cre-drivers causes SHF defects and early embryonic lethality.\(^{23}\) To completely rule out cell autonomous Hand2 function within PHF-derived myocytes, PHF-specific Hand2 deletion would be required.

WT1\(^{ERT2Cre}\)– and Hand1\(^{Cre}\)–mediated Hand2 deletions produce epicardial phenocopies, indicating a novel Hand2 function during epicardial development. Whereas the defects in the epicardium appear direct, WT1 is expressed in lateral mesoderm derivatives in addition to the epicardium.\(^{50}\) Lineage analysis following E9.5 WT1\(^{ERT2Cre}\) induction detects no cardiomyocyte expression (Online Figure V). Hand2 deletion using a lateral mesoderm and extraembryonic tissue-specific HoxB6\(^{Cre}\)\(^{23}\) which, because Hand2 is not detected within extraembryonic tissues,\(^{18}\) effectively generates a lateral mesoderm-specific Hand2 deletion, results in viable embryos.\(^{1,23}\) Hand2 function in the lateral mesoderm is therefore not critical to embryonic survival, and we conclude that PE/epicardium-specific Hand2 ablation disrupts coronary vasculature maturation and thus contributes to H2CKO lethality.

The Hand1 lineage does not contribute to coronary vascular endothelium.\(^{26}\) The current understanding identifies the origin of the coronary vessel endothelium as the sinus venosus,\(^{9}\) and presumes that the functional epicardium interacts with the coronary endothelium to both establish and maintain coronary vessel patency. H2CKO embryos display epicardial lineage–specific defects that impact both fibroblast and smooth muscle cell fates. No Hand1 lineage–dependent cells are detectable in the epicardium (Online Figure I).\(^{26}\) Hand2 ISH similarly marks the entire epicardium, suggesting that Hand2 is not enriched within a subset of epicardial cells. Pdgfrs govern specific lineage subsets during epicardial development.\(^{54}\) Pdgfra is downregulated and, conversely, Pdgfrβ is upregulated within the H2CKO epicardium. Luciferase transactivation data suggests direct Pdgfra regulation (Online Figure VII, I). Although Pdgfra’s epicardial function is unknown, it is essential in other EMT-derived cell populations.\(^{35}\) This altered ratio could reflect a Pdgfrβ compensation for decreased Pdgfra expression; however, Pdgfrβ drives epicardially-derived cell differentiation to smooth muscle cell.\(^{34}\) As the epicardium gives rise to both fibroblast and coronary smooth muscle, and Postn is expressed in cardiac fibroblasts invading the myocardium, (Figure 6), Pdgfra may govern the fibroblast cell fate.\(^{55}\) Hand2 appears to modulate the Pdgfr ratios that govern these divergent cell programs. Recently, it has been shown that Hand2 overexpression causes a significant increase in fibroblast marker expression, further implicating Hand2 in the cardiac fibroblast cell fate program.\(^{54}\)

Hand1\(^{Cre/+}\), Hand2\(^{+/−}\) mutants also display cardiac OFT defects. Wnt1-Cre–mediated NCC-specific Hand2 deletion results in aortic arch defects, DORV, and associated VSDs.\(^{22,23}\) As Hand1\(^{Cre}\)–mediated cNCC Hand2 ablation is temporally later and spatially more restricted then Hand2\(^{NCC}\) but entails heterozygosity of Hand1, the high penetrance of a more severe NCC-dependent PTA phenotype suggests that genetic and, possibly, functional interactions between Hand1 and Hand2 are critical for OFT septation. Established genetic interactions between Hand1 and Hand2 support this model.\(^{2,4}\) It is also consistent with established dimer regulation mechanisms governing the biological output of Twist-family bHLH factors. Indeed, dysregulation of Twist1 dimerization causes the human disease Saethre–Chotzen syndrome and directly reflects molecular antagonism between Twist1 and Hand2.\(^{55}\) Thus, Hand1 and Hand2 dimer choice may prove crucial to OFT morphogenesis. Experiments to explore this possibility mechanistically are currently underway.

Our data defines unique Hand1 and Hand2 expression domains within the developing murine heart. Hand1 is largely restricted the left ventricle. SHF expression is restricted to the myocardial cuff\(^{50}\) and is the sole domain of coexpression with Hand2. At the onset of cardiac looping, Hand1 expression/lineage is detectable within the forming left ventricle. Although we observe a thin compact zone and hypertrabeculation within H2CKO embryos, the cell autonomy of these defects cannot be deduced. Cardiac-specific Hand2 ablation causes SHF defects and early embryonic death.\(^{23}\) Although Hand1\(^{−}\)\(^{−}\)/Hand2\(^{−}\)\(^{−}\) mice allow insight into a possible role for Hand2 in the PFH myocardium, a PHF-specific Cre will be required to address this directly. In summary, these studies demonstrate that, in addition to its established functions within the cNCC and myocardium, Hand2 modulates cell-signaling mechanisms that dictate epicardial cell fates and ECM organization, thus playing a novel and critical role in the function and differentiation of the epicardium and, consequently, proper cardiac function.

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**Disclosures**

None.

**References**


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**Novelty and Significance**

**What Is Known?**

- Hand1 and Hand2 are dynamically expressed during early heart development.
- Myocardial and neural crest deletion of Hand factors results in cardiac defects.

**What New Information Does This Article Contribute?**

- Hand1 is expressed in the septum transversum, which marks the proepicardial and epicardial lineages.
- Hand2 is expressed within both the proepicardium and epicardium, and the deletion of Hand2 in these tissues results in the failure to form a functional coronary vasculature.
- Defects in Hand2 mutants suggest that Hand2 plays a role in epicardial cell fate and modulation of extracellular matrix within the epicardial space.

Hand1 and Hand2 are basic helix-loop-helix transcription factors that are dynamically expressed in both cardiac and extracardiac cell types. Hand proteins function as dimers over E box elements located in the regulatory regions of many target genes. Thus understanding where these proteins are coexpressed is essential for defining the transcriptional programs and the tissue-specific functions that are regulated by Hand factor transcriptional activity. To begin to address these questions, we use our *Hand1Cre* allele to specifically delete *Hand2* in all cell lineages where *Hand1* is expressed. Interestingly, we observe the unexpected findings that Hand factors play a critical role in the development of the coronary vasculature. Conditional null *Hand2* embryos die at embryonic day 14.5 with noncompacted and hypertrabeculated myocardium that is secondary to defects in pericardial cell epithelial-to-mesenchymal transition, differentiation, and organization of extracellular matrix. Hand factors have been shown to play critical roles in myocardium and cardiac neural crest. The results of this study now define a functional role for Hand1 and Hand2 as in the formation and function of the epicardium, which is essential for coronary vessel formation.
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**Methods**

**Targeting Hand1 and Generation of Mice**

To construct the targeting vector, a 3.4Kb EGFPCre cassette (kindly provided by Dr. Simon J. Conway) was isolated with a XhoI/HindIII digest and ligated into pBluescript SK+. A 3.0kb BstEII fragment, upstream from the Hand1 translational initiation codon was isolated with a XhoI digest and ligated 5’ of the EGFPCre cassette. A 1.75kb Frt-flanked neomycin cassette was isolated from ploxFlpneo (provided by Thomas Saunders U. Michigan) with an EcoRI/NotI digest and ligated 3’ of the EGFPCre cassette. A 1.6kb Sall/HindIII fragment from within the first intron of the gene and extending into the second exon was isolated with a NotI digest and cloned 3’ of the neomycin cassette. The targeting vector was linearized with XhoI, prior to electroporation into CCE 916 wildtype ES cells, and plated onto G418-resistant STO feeder layers. Following positive selection with G418, individual ES colonies were isolated and analyzed by Southern blot for homologous recombination at the Hand1 locus, as previously described. Homologous recombination was observed at a frequency of 1:4 in the 150 ES cell clones analyzed. Four independent clones were injected into blastocysts obtained from C57/B6 mice, which were subsequently implanted into pseudopregnant Swiss foster females. Chimeras that were obtained transmitted the Hand1 mutation through the germline and the resulting offspring were intercrossed with homozygous R26\(^{Fki}\) (FLPeR) mice on a pure 129/SvJaeSor genetic background to permanently delete the neomycin cassette. Offspring were then intercrossed to remove the R26\(^{Fki}\) allele.

**Genotyping and Tamoxifen Administration**

ES cell DNA, as well as tail genomic DNA from Hand1\(^{EGFPCre\DeltaNeol^+}\) mice, were analyzed for the Hand1 mutation by Southern-blot analysis. Insertion of the EGFPCre and neo genes into the Hand1 locus introduced an EcoRI site that could be used to distinguish the wild-type
and targeted alleles, which yielded 10.0Kb and 4.0Kb fragments, respectively, following Southern analysis of EcoRI-digested DNA and hybridization with a labeled BssHII-Xhol probe from the region 3’ of the targeted mutation. Following removal of the neo gene, the 4.0Kb fragment shifted to a 2.25Kb fragment. Since the targeting construct did not contain a negative selection gene, an 861bp Ncol fragment from the EGFPCre cassette was labeled and hybridized to EcoRI-digested DNA, yielding a 10.0Kb band. Ectopic bands were not detected.

ROSA26R homozygous mice were genotyped using a probe located 5’ of the STOP Flox (provided by Dr. Phillipe Soriano). Southern analysis of EcoRV-digested DNA was hybridized with labeled probe and yielded a 3.8Kb band.

The Wnt1-Cre transgene was detected in genomic DNA isolated from tail samples using the primers 5’–TCGATGCAACGAGTGATGAG–3’ and 5’–TTCGGCTATACGTAACAGGG–3’, which recognize the sequence encoding Cre, and generate a ~470bp amplicon.

The Hand2^floxflox^ mice and Wt1^Ert2Cre^ mice (provided by William Pu Harvard) were genotyped as previously described^2,3^.

Tamoxifen was administered for the Wt1^Ert2Cre^ mice at E9.5 or E11.5 as previously described^2^.

**Mating Schemes and Histological Preparations**

Hand1^EgfPcreΔNeo^, Wt1^Ert2Cre^ and Wnt-Cre males were crossed to ROSA26R reporter mice^4^. Hand1^EgfPcreΔNeo^, Hand2^+/−^ and Wt1^Ert2Cre^, Hand2^+/−^ mice were crossed to Hand2^floxflox^, ROSA26R reporter mice (LacZ or eYFP) to generate conditional null Hand2 embryos.
X-gal staining, and histological preparations were done as previously described for paraffin embedded sections and wholemounts \textsuperscript{5}. For E15.5 and adult heart, embryos/tissues were dissected in PBS and fixed in a 1:1 mixture of 4\% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) and 1X PBS for 5 minutes at 4\textdegree{}C. Samples were then moved to 30\% sucrose in 1X PBS overnight at 4\textdegree{}C. Samples were then cryoprotected and stored at -80\textdegree{}C. Samples were sectioned at 10-20mm and washed for 5 minutes in 1X PBS. Sections were then post-fixed in 0.5\% PFA for 2 minutes, rinsed in 1X PBS then washed in 1X PBS for 10 minutes. Slides were then incubated with X-gal solution in the dark at 37\textdegree{}C overnight. Slides were rinsed in 1X PBS and then post-fixed in 4\% PFA for 20 minutes and then were washed three times in 1X PBS at room temperature for 5 minutes each. Slides were counterstained with Nuclear Fast Red.

**Section RNA In Situ Hybridization**

Section \textit{in situ} hybridization was performed essentially as previously described \textsuperscript{5}. Antisense digoxigenin-labeled riboprobes were transcribed with T7, T3, or SP6 (Roche) following linearization of template DNA.

**Wholemount RNA In Situ Hybridization**

Wholemount \textit{in situ} hybridization was performed essentially as previously described \textsuperscript{6}. Antisense digoxigenin-labeled riboprobes were transcribed for \textit{Hand1} and \textit{Tbx5}. For double in situ hybridization modifications were made as previously described \textsuperscript{7}. Briefly, a riboprobe for \textit{Hand1} was detected using anti-digoxigen antibody and NBT/BCIP substrate. After the removal of the antibody by incubating twice with glycine-HCl (pH 2.2), the \textit{Mlc2A} probe was detected using anti-fluorescein antibody and INT/BCIP substrate. After color development was completed, embryos were fixed with 4\% paraformaldehyde in phosphate-buffered saline (PBS).

**Primary Epicardial Culture**
Primary epicardial cultures were isolated and cultured as previously described for both WT and H2CKO 8. E11.5 hearts were explanted for 48 hours before removal. Isolated epicardial cells were cultured for an additional 48 hours before RNA isolation or immunocytochemistry.

**Immunocytochemistry**

Primary epicardial cultures were fixed in 4% PFA for 10 minutes, washed, and incubated in 0.1% Sodium Borohydride for 30 minutes. Cells were permeabilized with 0.15% Triton, blocked with 2% normal serum, and incubated with primary antibodies for Fn1 (Abcam). Secondary antibodies were conjugated with Alexa 488 (Molecular Probes) and counter stained with DAPI.

**Immunohistochemistry**

Embryos were fixed in 4% PFA overnight then embedded in paraffin or cryoprotected and sectioned at 7mm. Antibody staining for α-SMA (Sigma), Tyrosine Hydroxylase, and Tubb3 (Abcam) was done as previously described on paraffin embedded samples 9. Frozen sections were washed in PBS, blocked in 1.5% normal serum for 30 minutes followed by a serum-free protein block (Dako) for 10 minutes. Primary antibody for Flk1 (Abcam), Fn1 (Abcam), pHistone H3 (Abcam), aactivated-Caspase 3 (Promega) and GFP (Invitrogen) were incubated at 4°C overnight. Secondary antibodies were conjugated with Alexa 488 or 594 (Molecular Probes). Alexa 594-conjugated Phalloidin was used to dye F-Actin filaments (Molecular Probes). Images were collected at the same standardized conditions on a Leica CTR 5000 microscope using Leica Application Suite software. Wholemount immunohistochemistry was done as previously reported 10.

**Quantitative RT-PCR**
Total RNA was isolated from flash-frozen E12.5 hearts or primary epicardial cultures using the High Pure RNA Tissue or Isolation Kit (Roche). Total RNA served as a template to generate cDNA using the Transcriptor First Strand cDNA Synthesis Kit (Roche) or WGA1 Whole Genome Amplification Kit (Sigma). For quantitative real-time PCR, cDNA was amplified using Lightcycler 480 Probes Master (Roche). Target gene specific assays were designed in accordance with the UPL probe library (Roche). Relative gene expression was determined by using standard curves and normalized to GAPDH. 3-6 samples were collected per assay. Differences between groups were examined for statistical significance using the Student’s t-test. P values of <0.05 were regarded as significant.

Online supplement Results

The Hand1-lineage marks the PHF and epicardium

The $\text{Hand1}^{\text{eGFP}^{\text{Cre}, \text{Neo}}}$ ($\text{Hand1}^{\text{Cre}}$) was generated using a strategy similar to that used to generate the $\text{Hand1}^{\text{LacZ}}$ allele. To establish the contribution of the Hand1-lineage to the developing heart, we intercrossed $\text{Hand1}^{\text{Cre}}$ mice with $\text{R26R}$ reporter mice. Embryos were analyzed for $\beta$-galactosidase expression. Comparison of X-gal staining from the $\text{Hand1}^{\text{LacZ}}$ and $\text{Hand1 ISH}$ indicate similar expression domains (Online Fig. IA-F). At E8.5 the $\text{Hand1}$-lineage observed is indistinguishable from that of $\text{Hand1}$ expression, marking cells in the lateral mesoderm, left ventricle, and OFT of the heart (Online Fig. IG). At E9.5, robust X-gal staining of the $\text{Hand1}$ lineage is observed throughout the outer curvature of the left ventricle, with no significant staining within the right ventricle corresponding directly with $\text{Hand1}$ expression (Online Fig. IB). This indicates that $\text{Hand1}$ is not expressed within the progenitors of the right ventricular myocardium prior to E9.5 (Online Fig. IH).
At E10.5, the Hand1-lineage analysis reveals divergence from the Hand1 expression pattern. At E10.5, Hand1 is expressed within the midline of the pharyngeal arches, the OFT, and the outer curvature of the left ventricle (Online Fig. IC, F). Lineage analysis in wholemount shows that the Hand1-lineage is restricted to the midline of the pharyngeal arches, but now stains the entire heart (Online Fig. I, l). Comparisons of E9.5 lineage-marked and Hand1LacZ embryos in section reveal an indistinguishable expression pattern to the Hand1LacZ within right and left ventricular myocardium (Online Fig. IJ, K). Surprisingly, the pan-cardiac X-gal staining observed in wholemount at E10.5 reflects Hand1Cre-mediated recombination within the nascent epicardium (Online Fig. IM). Hand1LacZ expression in conjunction with Hand1 ISH confirms that Hand1 is not actively expressed within epicardial cells (Online Fig. IC and L). X-gal staining of the Hand1-lineage in adult mice confirms the Hand1-lineage contributes to smooth muscle vasculature and to cardiac fibroblasts, of which both cell types are epicardial derivatives (Online Fig. IN and O).

Loss of Hand2 disrupts epicardial gene expression and patency of coronary vasculature

CD31 wholemount immunoreactivity is observed within a well-developed vascular plexus that covers the entire surface of the heart. In contrast, the H2CKO hearts display a greatly reduced vascular plexus that is restricted to the back of the heart just posterior to the sinus venosus, which is thought to be the source of coronary endothelium (Online Fig VIA, B red arrows define progression of the extending vessels) 11.

To confirm that H2CKO coronary defects are secondary to epicardial defects when Hand2 is conditionally ablated using Hand1Cre and not resulting from Hand2 ablation from myocardium or NCC, we generated H2CKOs with Hand1Cre and WT1ERT2Cre, and employed immunohistochemistry for Cre-lineage and Flk1 (Online Fig. VIC-F, see Online Fig. IV and V). WT1-lineage results confirm that the epicardium does not contribute to coronary endothelium in
wild type mice (Fig. VID). However, in WT1-generated H2CKOs, Flk1 positive coronary lumens could not be detected (Fig. VIF). As E9.5 day-induced WT1 ERT2Cre does not mark NCC or myocardium (Online Fig V). ², this supports the conclusion that Hand2 deletion within the PE/epicardium is causative to coronary development failure via a non-cell autonomous mechanism.

Hand2 transcriptionally regulates Pdgfra

Given that ISH and gene array analyses place Pdgfra downstream of Hand2, we tested whether Pdgfra regulation is directly controlled by Hand2 through luciferase reporter assays on an isolated 1.1kb upstream region of the Pdgfra gene (Online Fig. VI, I). This proximal Pdgfra 5’ region contains two highly conserved E-box cis-elements and the endogenous transcriptional start site (Online Fig. VI, I). Hand2 modestly transactivates this reporter clone 2.5 fold (P<0.05); however, co-transfection with both Hand2 and E12 results in a 40% reduction in promoter activity, suggesting that Hand2 functions either as a homodimer or a non-E-protein heterodimer. Collectively, these results suggest that the down regulation of Pdgfra message in H2CKO’s is a direct effect of Hand2 transcriptional regulation.
Online references


Online Figure I: Validation of the *Hand1* lineage. (A-C) Wholemount RNA *in situ* hybridization of *Hand1*. (D-F, J, L) X-gal staining of the *Hand1*<sup>LacZ</sup> mouse line. (G-I, K, M-O) X-gal staining of the lineage map of *Hand1* using the *Hand1*<sup>Cre</sup>. In panel G, the absence of LacZ-positive cells throughout the heart tube (red arrow) indicates that *Hand1* is not expressed in the entire cardiac crescent as previously suggested<sup>1,12</sup>. Panel H shows that *Hand1* is not expressed in the SHF-derived cardiomyocytes at E9.5 but does lineage map to the left ventricle. Wholemount analysis in panel I shows that the *Hand1*-lineage appears to be widely expressed throughout the heart; however, transverse section analysis in panel M indicates that this surface expression is confined to the epicardium (black arrow). X-Gal stained *Hand1*-lineage in the right ventricle of adult mice confirms restriction to epicardial smooth muscle derivatives and cardiac fibroblasts (N, O). Ht, heart tube; lv, left ventricle; pa, pharyngeal arch; rv, right ventricle.
Online Figure II: Hand2 is expressed in epicardial cultures. RT-PCR on 4-day epicardial explants indicates Hand2 is expressed in epicardial cultures (A). WT1 immunocytochemistry indicates a high > 90% enrichment for epicardial cells within the explant cultures (B-D).
**Online Figure III:** Hand2 myocardial deletion does not result in cell autonomous myocardial defects. E10.5 *Hand2* ISH shows Hand2 expression at E10.5 throughout the left ventricular myocardium in WT and H2CKOs (A-B). Normal distribution of LacZ staining at E14.5 in the left ventricle of H2CKs indicates normal establishment and maintenance of the *Hand1*-lineage (C-D). *ANF* expression at E12.5 indicates preservation of normal heart function (E-F). Normal expression of *Cx40* at E12.5 and *Cited1* at E14.5 indicate *Hand1* is functioning properly (G-J). The endocardial gene program necessary for trabeculation remains intact in H2CKOs as evident by *Nrg1* staining at E12.5 (M-N). *Tbx5* expression at E10.5 shows normal patterning of the left ventricle (Q-R). α-SMA staining and electron microscopy illustrate normal cardiomyocyte periodicity indicates both normal function and differentiation of H2CKO cardiomyocytes (S-V).
**Online Figure IV:** Model of Cre Lineage: The model illustrates the regions impacted by the two cardiovascular Cre mouse lines utilized in this study in relation to Hand2 expression. Hand1\textsuperscript{Cre} will delete Hand2 from the cardiac NCC (~E9.0), cuff and left ventricle myocardium (~E8.5), and finally from the ST precursors that populate the PE and form the epicardium (ep) (~E8.5). Wt1\textsuperscript{Cre} will mark cells within the ST and PE (~E8.5), as well as lateral mesoderm and ventral mesenchyme (not shown) but no myocardium, endocardium or cNCC. Ncc, neural crest cells; ep, my, myocardium; en, endocardium.
Online Figure V: WT1 Lineage Analysis. (A) E12.5 β-galactosidase staining and eYFP Immunohistochemistry (B) for the WT1 lineage. Low power transverse sections show WT1-lineage cells composing the epicardium but not the cardiomyocytes of the right (rv) and left ventricles (lv), or NCC-derived OFT cushions. WT1-positive mesenchyme is observed within the medial regions of the embryo ventral to the jugular vein (jv) and aorta (ao). To further confirm WT1-lineage, Immunohistochemistry showing detection of eYFP (green) and nuclei counterstained with DAPI (blue) confirms lacZ analysis that WT1Cre does not mark cardiomyocytes. Tamoxifen was administered at E9.5 to initiate Cre Recombinase activation. Staining is restricted to the epicardial lineage and does not significantly contribute to myocardial recombination in the compact zone and trabeculae. Staining indicates there is not a direct myocardial recombination event. (C-D) Tamoxifen injections were initiated at E11.5 day to generate H2CKOs. CD31 marks the endothelium in red, WT1-lineage in green and nuclei in blue via DAPI. (D) Results show that later stage deletion of Hand2 allows for formation of surface coronaries although fewer vessels are observed suggesting an early role for Hand2 in coordinating vessel formation.
Online Figure VI: Wholemount immunohistochemistry for CD31 at E12.5 (A, B). A decrease in the extension of coronary vasculature around the heart is observed in H2CKOs (red arrows). E13.5 Hand1-lineage (C, E) and WT1 lineage (D, F) for wild type (C, D) and conditional knockouts of Hand2 (E, F). Immunohistochemistry for Flk1 (red) and Cre-Lineage (green) confirms coronary endothelium is not derived from the epicardium, but Hand2 conditional deletion in the epicardium leads to an absence of coronary vessel endothelium. Gene ontology microarray analysis showing affected pathways in H2CKO isolated epicardium (G). qRTPCR on WT (blue bar) and H2CKO (black bar) isolated epicardium; n=4 (H). Asterisks indicate statistical significance: *p < 0.05; **p <0.01. (I) Trans-activation of Pdgfrα luciferase by Hand2. 1.1kb of proximal Pdgfrα sequence was co-transfected with pcDNA1, Hand2, E12, or Hand2 and E12 in HEK293 cells. Graph shows fold activation +/- SEM and * denotes a significance P<0.002; n=9.
Online Figure VII: *Hand2* Epicardial Deletion Results In Cell Death. E12.5 immunohistochemistry for the *Hand1* lineage (green); and either pHistone H3 (red) (A,B) or activated-Caspase 3 (red) (C,D). White arrow marks representative staining. Nuclei counterstained with DAPI (blue). rv, right ventricle; at, atria. Every third right ventricular section from three animals was counted and results are graphed in figure 7.
Online Figure VIII: *Hand2* regulates multiple essential processes during epicardiogenesis. Developmental model showing the role of *Hand2* within the epicardium (A). Deletion of *Hand2* in the epicardium results in a disorganized ECM, which results in a decline in epicardial function and integrity and cell Fn1 cell signaling. Ultimately epicardial cell viability is compromised and coronary vessel formation and function is compromised. Putative secondary defect on myocardium noncompaction could also be cell autonomous; however, a PHF-specific Cre is required to address this directly.
Online Fig.I
Online Fig. III
Online Fig. V
Online Fig. VII
A

WT

H2CKO

- Myocardium
- Epicardium
- Endocardium/Coronary Vessels
- Extracellular Matrix

B

Hand2

↑ a-Caspase3

F11 Fibril

Disorganization

Cell Death Protection

PDGFRα
PDGFRβ

↓ Periostin

Extracellular Matrix

Cell Fate

Online Fig. VIII