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Hand2 loss-of-function in *Hand1*-expressing Cells Reveals Distinct Roles In Epicardial And Coronary Vessel Development

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Abstract

Rationale—The 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—Deduce the role of *Hand2* within the epicardium.

Method & 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 (ST) 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 ECM deposition and *Pdgfr* expression.

Conclusion—These data demonstrate a hierarchal relationship whereby transient *Hand1* ST expression defines epicardial precursors that are subsequently dependent upon Hand2 function.

Keywords

Hand1; Hand2; bHLH; epicardium

Introduction

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]. While myocyte derivatives ultimately provide the cardiac musculature that powers circulation, it is the extra-cardiac cell lineages (the cardiac neural crest cells (cNCC), coronary endothelium, and epicardium) that define the framework upon which these

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cardiomyocytes develop [2–5]. 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], while 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 track (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 extra-cardiac 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 *Hand2* [13, 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 due to 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 E9.5 [18, 20]. *Hand1* knockout mice die by E9.5 from extraembryonic and vascular defects [16, 19, 21]. Conditional NCC-specific *Hand2* ablation (*Hand2*$^{\Delta NCC}$) causes OFT defects, associated ventricular septal defects (VSD), Double Outlet Right Ventricle (DORV) and decreased SHF myocardial proliferation [22, 23]. Myocardial-specific *Hand2* deletion causes right ventricular hypoplasia and death by E12.5 [23]. Reciprocally, myocardial-specific *Hand1* deletion causes left-ventricular hypoplasia and although embryos survive until birth, *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 sub-population, 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*). *H2CKOs* embryos die by E14.5 and present Persistent Truncus Arteriosis (PTA), DORV and associated VSDs. Significantly, *Hand2* expression lies temporally 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 *H2CKOs. H2CKOs* display defective epicardial EMT, decreased cardiac fibroblasts, and a nonfunctional coronary vasculature. *Hand2* ablation within the *WT1*^{Cre}-lineage phenocopies *Hand1*^{Cre}-mediated *Hand2* ablation. Gene expression analyses reveal an altered ratio of *Pdgfrα:Pdgfrβ* mRNA, a decreased profile of fibroblast markers, and disorganized 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.

Methods

See the online supplement for detailed methods.

Targeting *Hand1* and Generation of Mice

The construction of the targeting vector, generation of targeted ES cells, chimera and germline mice is described in detail in [26]. Tamoxifen was administered for the as described [4].

In Situ Hybridization

Section and Wholemount *in situ* hybridization (ISH) was performed essentially as previously described [26, 2829].

Primary Epicardial Culture

Primary epicardial cultures were isolated and cultured as previously described [31].

Immunohistochemistry

Embryos were fixed in 4% PFA overnight then embedded in paraffin or cryoprotected and sectioned at 7mm as previously reported [26].

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 supplement Fig. I), we sought the *Hand1*-expressing progenitors of these cells. The PE is derived from the anterior ST and gives rise to the epicardium [33]. *ISH* was performed for both *Hand1* and *Tbx18*, a marker of the PE, at E9.5 [3, 34] (Fig. 1A-E). *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 $Hand1^{LacZ/+}$ embryos confirms expression within the LV and the ST (Fig. 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 (Fig. 1G-I) [3, 34]. 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 wholemount ISH for *Hand1* at E7.5 (Fig. 2). *Hand1* is detected throughout the extra embryonic mesoderm, chorion, and allantoic rudiment (Fig. 2A). As the chorion is directly adjacent to the cardiac crescent (Fig. 2A,B 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 (Fig. 2B).

Wholemount *ISH* at E8.5 (Fig. 2C-F) shows *Hand1* expression within the posterior heart tube while *Hand2* expression is observed throughout the entire cardiac field. Sagittal sections of wholemount-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 wholemount view, the *Hand2* endocardial expression is visible through the thin myocardial wall (Fig. 2F, red arrow). At E9.5, *Hand1* and *Hand2* expression overlaps within the SHF-derived myocardial cuff and the left ventriclular myocardium (not shown). Collectively, these data reaffirm that Hand factor expression is dynamically regulated and that, during early heart morphogenesis, this expression overlaps solely within the SHF-derived myocardial cuff.

Hand1^{Cre}-mediated ablation of *Hand2* within the, PE, and epicardium

To explore potential Hand factor functional overlap, we intercrossed *Hand2*^{fx} [23] and *Hand1*^{Cre} mice to ablate *Hand2* within the *Hand1*-lineage (*H2CKO*). *Hand2* expression is robust throughout the PE and detectable in both E10.5 epicardial cells and isolated primary epicardial cell cultures (arrow, Fig. 3A,C; Online Fig. II). *Hand2* expression was not detected within the epicardium or the PE of *H2CKOs*, confirming that *Hand1*-positive ST cells ultimately populate the PE and epicardium (Fig. 3B,D). *Hand1* and *Hand2* are not coexpressed within the ST or PE; rather, *Hand1* expression within the ST precedes PE/epicardial upregulation of *Hand2*. Thus, *Hand1*^{Cre} conditionally ablates *Hand2* function within the PE and epicardium.

Hand1-lineage *H2CKO* reveals novel phenotypes and embryonic lethality

H2CKO embryos were recovered at slightly below expected Mendelian ratios through E12.5 (Fig. 4). At E14.5, roughly 50% of *H2CKOs* collected were dead. E12.5 *H2CKOs* appeared phenotypically normal (data not shown). Cardiac patterning, and both *Hand1* and cardiomyocyte marker expression were normal (Online Fig. IIIA-P).

By E14.5, *H2CKOs* displayed pericardial hemorrhaging, anasarca, and liver hypoplasia (Fig. 4C, D, G, H). OFT septation defects, including PTA and DORV, were also observed, reflecting cNCC and/or SHF-derived myocardial defects (Fig. 4A, B, E, F, I-J). Hypertrabeculation/noncompaction and VSDs were also observed (Fig. 4K-N), although, *H2CKO* sarcomeric ultra-structure was indistinguishable from controls (Online Fig. IIIQ, R).

Hand2$^{\Delta NCC}$ die from a sympathetic nervous system (SNS) norepinepherine deficit [22, 23]. As *Hand1* and *Hand2* are coexpressed in some NCCs, *H2CKOs* possibly die from SNS defects. *Hand2*$^{\Delta NCC}$ lethality can be rescued by the β-adrenergic agonist isoproterenol [22, 23]. Unlike *Hand2*$^{\Delta NCC}$ embryos, isoproterenol treated *Hand1*^{Cre}-generated *H2CKOs* fail to survive to birth (Fig. 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 (Fig. 5A, B). E14.5 *H2CKO* display abnormal compaction, an absence of epicardium, and a lack of coronary lumens, indicating a possible epicardial phenotype (Fig. 5A,B). *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 (Fig. 5C,D). To confirm direct disruption of *H2CKO* epicardial development, we generated a *WT1*^{ERT2Cre} *H2CKO* [4], in which Hand2 is ablated in the ST, PE and epicardium, but not the myocardium and NCC lineages (Fig. 5E-H). (See Online Fig. IV for a *WT1*^{ERT2Cre}, *Hand1*^{Cre}, and *Hand2* cardiac lineage/expression comparison). Potentially complicating these analyses, *WT1*^{ERT2Cre} marks lateral mesoderm, where *Hand2* is expressed, and early allelic *WT1*^{ERT2Cre} induction (E8.5) can produce ectopic Cre activity [4]. *HoxB6-Cre*-mediated *Hand2* deletion in lateral mesoderm

generates viable neonates [23]. Tamoxifen-mediated *WT1*^{ERT2Cre} allelic induction at E9.5 resulted in no ectopic myocardial *R26R* reporter activity (Online Fig. V). At E13.5, *Hand2*$^{fx/-}$, *WT1*^{ERT2Cre} mutant embryos phenocopied *H2CKO* embryos, displaying a poorly organized epicardium and reduced *WT1*-marked cell lineage (Fig. 5E-H). Myocardial non-compaction 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 *H2CKOs* (Fig. 5A, B, G, H). These two 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 (Pdgfr) play essential roles in cell fate and specification of the mature epicardium [35, 36]. E12.5 ISH reveals decreased *Pdgfrα e*xpression within the *H2CKO* epicardium (Fig. 6A,B). The number of cells expressing *Periostin* (*Postn),* a marker of the cardiac fibroblast cell fate [32], is visibly decreased in the myocardium of E12.5 *H2CKO* mutants (Fig. 6C-D). Together these data suggest that Hand2 functions in epicardial cell fate determination during or after secondary EMT.

We performed CD31 (Online supplement; Fig. VIA, B) and Flk1 immunohistochemistry (Fig. 6E, F) 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 (Fig. 6F). The *Hand1*-lineage does not contribute to coronary endothelial cell populations (Online Fig. VIC-F) [26], and therefore the absence of coronary vasculature within *H2CKOs* 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 *H2CKOs* when *WT1*^{ERT2Cre} is activated at E11.5, although they appear less prevalent than in embryos that lack the *WT1*^{ERT2Cre} allele (Online Fig. VC-D), suggesting that critical Hand2 epricardial 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 *H2CKOs* and isolated total RNA for microarray analyses (Online Fig. IIA-D). Gene ontology from our microarray analysis indicates significant differences in developmental, morphological, and cardiovascular gene programs within EPCs (Online Fig. VIG). 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 Fig. VIH). 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 Fig. VIH). Hand2 regulation of *Pdgfrα* is direct (Online supplement; Fig. VI,I). In epicardial cells, Pdgfrβ-mediated signaling promotes a smooth muscle fate, while the role of Pdgfrα signaling is currently unclear [35]. 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* upon epicardial mesothelium integrity. *Fn1* is downregulated in zebrafish *hand2* mutants [37].

Fn1 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 [38–40]. Additionally, defects in *Fn1* underlie integrin-mediated valve leaflet defects in the lymphatic system [41]. Immunohistochemistry shows that Fn1 is expressed in the E12.5 epicardium and is neatly organized around the developing coronaries (Fig. 7A). *H2CKOs* retain epicardial Fn1; however, it appears disorganized (Fig. 7B). To look at Fn1 organization more closely, we compared EPCs from wild type and *H2CKOs* (Fig. 7C-F). 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 (Fig. 7C-F). 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 (Fig. 7G, H).

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 (Fig. 7I). qRT-PCR on wild type and *H2CKO* EPCs detected no changes in *Fn1* expression, as has been reported in mice (Fig. 7J) [42].

In addition to its role as an ECM component, Fn1 promotes intracellular signaling via interactions with cell surface integrins [43]. To see if the observed *H2CKO* Fn1 disorganization may alter cell signaling, we assessed *integrin* expression by qRT-PCR (Fig. 7J). Indeed, expression of the Fn1 receptor, *Itgα4*, is significantly upregulated within *H2CKO* EPCs. Itgα4 and Itgβ1 together form an Fn1 receptor pair. Itgα4 influences epicardial Fn1 polymerization. *Itgα4* overexpression impairs incorporation of new Fn1 into preexisting polymer structures [44], while *Itgα4* deletion causes embryonically lethal epicardial defects in mice [45, 46]. As Itg*α4* 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 indicates 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 pro-apoptotic [47]. Activated-Caspase3 and Phospho-Histone H3 immunohistochemistry at E12.5 to assess *H2CKO* epicardial apoptosis and proliferation (Fig. 7K, L; Online Fig. VIIA-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 *H2CKOs* 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 Fig. VIIIA-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 *Pdgfrα* regulation, abnormal differentiation and Fn1 organization, and *Itgα4* upregulation. Also evident in *H2CKOs* is increased epicardial apoptosis, potentially reflecting altered Fn-mediated signaling and/or cell migration. Fn1 and *Itgα4*, 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]. *Itgα4* overexpression alters Fn/integrins interactions and disrupts Fn deposition, demonstrating that integrin levels significantly impact Fn organization and function [44]. 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 *H2CKOs* 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 deposition and influences ECM deposition during lateral mesoderm remodeling [37]. Consistent with our data, *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 Fig. 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 cardiomyocytes, 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. While 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 Fig. V). *Hand2* deletion using a lateral mesoderm and extraembryonic tissue-specific $HoxB6^{Cre}$ [23], which, as *Hand2* is not detected within extraembryonic tissues [18], effectively generates a lateral mesoderm-specific *Hand2* deletion, results in viable embryos [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]. 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. *H2CKOs* display epicardial lineage-specific defects that impact both fibroblast and smooth muscle cell fates. No *Hand1*-lineage independent cells are detectable in the epicardium (Online Fig. 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 [35]. *Pdgfrα* is downregulated and, conversely, *Pdgfrβ* is upregulated within the *H2CKO* epicardium. Luciferase transactivation data suggests direct *Pdgfrα* regulation (Online Fig. VII,I). Although Pdgfrα's epicardial function is unknown, it is essential in other EMT-derived cell populations [36]. This altered ratio could reflect a *Pdgfrβ* compensation for decreased *Pdgfrα* expression; however, *Pdgfrβ* drives epicardially-derived cell differentiation to smooth muscle cell [35]. As the epicardium gives rise to both fibroblast and coronary smooth muscle, and *Postn* is expressed in cardiac fibroblasts invading the

myocardium, (Fig. 6), Pdgfrα may govern the fibroblast cell fate [53]. 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*$^{fx/-}$ 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*$^{\Delta NCC}$ 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 [24]. 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 [26], and is the sole domain of co-expression 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 *H2CKOs*, the cell autonomy of these defects cannot be deduced. Cardiac-specific *Hand2* ablation causes SHF defects and early embryonic death [23]. Although *Hand1*^{Cre} 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations & Acronyms

CHDs	congenital heart defects
cNCCs	cardiac neural crest cells
SHF	second heart field
PHF	primary heart field
EMT	epithelial-mesenchymal-transition

OFT	outflow tract
PE	proepicardium
bHLH	basic helix-loop-helix
CKO	conditional knock-out
VSD	ventricular septal defect
DORV	double outlet right ventricle
PTA	persistent truncus arteriosis
ST	septum transversum
PE	proepicardium
EPCs	epicardial primary cultures

References

1. Buckingham M, Meilhac S, Zaffran S. Building the mammalian heart from two sources of myocardial cells. Nat Rev Genet. 2005; 6:826–835. [PubMed: 16304598]
2. Jiang X, Rowitch DH, Soriano P, McMahon AP, Sucov HM. Fate of the mammalian cardiac neural crest. Development. 2000; 127:1607–1616. [PubMed: 10725237]
3. Cai CL, Martin JC, Sun Y, Cui L, Wang L, Ouyang K, Yang L, Bu L, Liang X, Zhang X, Stallcup WB, Denton CP, McCulloch A, Chen J, Evans SM. A myocardial lineage derives from Tbx18 epicardial cells. Nature. 2008; 454:104–108. [PubMed: 18480752]
4. Zhou B, Ma Q, Rajagopal S, Wu SM, Domian I, Rivera-Feliciano J, Jiang D, von Gise A, Ikeda S, Chien KR, Pu WT. Epicardial progenitors contribute to the cardiomyocyte lineage in the developing heart. Nature. 2008; 454:109–113. [PubMed: 18568026]
5. Wada AM, Willet SG, Bader D. Coronary vessel development: a unique form of vasculogenesis. Arterioscler Thromb Vasc Biol. 2003; 23:2138–2145. [PubMed: 14525796]
6. Black BL. Transcriptional pathways in second heart field development. Semin Cell Dev Biol. 2007; 18:67–76. [PubMed: 17276708]
7. Snarr BS, Kern CB, Wessels A. Origin and fate of cardiac mesenchyme. Dev Dyn. 2008; 237:2804–2819. [PubMed: 18816864]
8. Olivey HE, Svensson EC. Epicardial-myocardial signaling directing coronary vasculogenesis. Circ Res. 2010; 106:818–832. [PubMed: 20299672]
9. Red-Horse K, Ueno H, Weissman IL, Krasnow MA. Coronary arteries form by developmental reprogramming of venous cells. Nature. 2010; 464:549–553. [PubMed: 20336138]
10. Snider P, Standley KN, Wang J, Azhar M, Doetschman T, Conway SJ. Origin of cardiac fibroblasts and the role of periostin. Circ Res. 2009; 105:934–947. [PubMed: 19893021]
11. Souders CA, Bowers SL, Baudino TA. Cardiac fibroblast: the renaissance cell. Circ Res. 2009; 105:1164–1176. [PubMed: 19959782]
12. Zhou B, Pu WT. More than a cover: epicardium as a novel source of cardiac progenitor cells. Regen Med. 2008; 3:633–635. [PubMed: 18729786]
13. Barnes RM, Firulli AB. A twist of insight - the role of Twist-family bHLH factors in development. Int J Dev Biol. 2009; 53:909–24. [PubMed: 19378251]
14. Firulli AB. A HANDful of questions: the molecular biology of the heart and neural crest derivatives (HAND)-subclass of basic helix-loop-helix transcription factors. Gene. 2003; 312:27–40. [PubMed: 12909338]
15. Cserjesi P, Brown D, Lyons GE, Olson EN. Expression of the Novel Basic Helix-Loop-Helix Gene eHAND in Neural Crest Derivatives and Extraembryonic Membranes during Mouse Development. Developmental biology. 1995; 170:664–678. [PubMed: 7649392]

16. Firulli AB, McFadden DG, Lin Q, Srivastava D, Olson EN. Heart and extra-embryonic mesodermal defects in mouse embryos lacking the bHLH transcription factor Hand1. Nat Genet. 1998; 18:266–270. [PubMed: 9500550]

17. Srivastava D, Cserjesi P, Olson EN. A subclass of bHLH proteins required for cardiac morphogenesis. Science. 1995; 270:1995–1999. [PubMed: 8533092]

18. Srivastava D, Thomas T, Lin Q, Kirby ML, Brown D, Olson EN. Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. Nat Genet. 1997; 16:154–160. [PubMed: 9171826]

19. Riley P, Anson-Cartwright L, Cross JC. The Hand1 bHLH transcription factor is essential for placentation and cardiac morphogenesis. Nat Genet. 1998; 18:271–275. [PubMed: 9500551]

20. Thomas T, Yamagishi H, Overbeek P, Olson E, Srivastava D. The bHLH factors, dHAND and eHAND, specifiy pulmonary and systemic cardiac ventricles independent of left-right sidedness. Developmental Biology. 1998; 196:228–236. [PubMed: 9576835]

21. Morikawa Y, Cserjesi P. Extra-embryonic vasculature development is regulated by the transcription factor HAND1. Development. 2004; 131:2195–2204. [PubMed: 15073150]

22. Holler KL, Hendershot TJ, Troy SE, Vincentz JW, Firulli AB, Howard MJ. Targeted deletion of Hand2 in cardiac neural crest-derived cells influences cardiac gene expression and outflow tract development. Dev Biol. 2010; 341:291–304. [PubMed: 20144608]

23. Morikawa Y, Cserjesi P. Cardiac neural crest expression of Hand2 regulates outflow and second heart field development. Circ Res. 2008; 103:1422–1429. [PubMed: 19008477]

24. Barbosa AC, Funato N, Chapman S, McKee MD, Richardson JA, Olson EN, Yanagisawa H. Hand transcription factors cooperatively regulate development of the distal midline mesenchyme. Dev Biol. 2007; 310:154–168. [PubMed: 17764670]

25. McFadden DG, Barbosa AC, Richardson JA, Schneider MD, Srivastava D, Olson EN. The Hand1 and Hand2 transcription factors regulate expansion of the embryonic cardiac ventricles in a gene dosage-dependent manner. Development. 2005; 132:189–201. [PubMed: 15576406]

26. Barnes RM, Firulli B, Conway SJ, Vincentz JW, Firulli AB. Analysis of the Hand1 Cell Lineage Reveals Novel Contributions to Cardiovascular,Neural Crest, Extra-Embryonic, and Lateral Mesoderm Derivatives. Dev Dyn. 2010; 239:3086–3097. [PubMed: 20882677]

27. Soriano P. Generalized lacZ expression with the ROSA26 Cre reporter strain. Nat Genet. 1999; 21:70–71. [PubMed: 9916792]

28. Vincentz JW, Barnes RM, Rodgers R, Firulli BA, Conway SJ, Firulli AB. An absence of Twist1 results in aberrant cardiac neural crest morphogenesis. Dev Biol. 2008; 320:131–139. [PubMed: 18539270]

29. Nagy, A. Manipulating the mouse embryo : a laboratory manual. 3. Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press; 2003.

30. Ishii Y, Langberg JD, Hurtado R, Lee S, Mikawa T. Induction of proepicardial marker gene expression by the liver bud. Development. 2007; 134:3627–3637. [PubMed: 17855432]

31. Rhee DY, Zhao XQ, Francis RJ, Huang GY, Mably JD, Lo CW. Connexin 43 regulates epicardial cell polarity and migration in coronary vascular development. Development. 2009; 136:3185–3193. [PubMed: 19700622]

32. Snider P, Hinton RB, Moreno-Rodriguez RA, Wang J, Rogers R, Lindsley A, Li F, Ingram DA, Menick D, Field L, Firulli AB, Molkentin JD, Markwald R, Conway SJ. Periostin is required for maturation and extracellular matrix stabilization of noncardiomyocyte lineages of the heart. Circ Res. 2008; 102:752–760. [PubMed: 18296617]

33. Watt AJ, Battle MA, Li J, Duncan SA. GATA4 is essential for formation of the proepicardium and regulates cardiogenesis. Proc Natl Acad Sci U S A. 2004; 101:12573–12578. [PubMed: 15310850]

34. Christoffels VM, Grieskamp T, Norden J, Mommersteeg MT, Rudat C, Kispert A. Tbx18 and the fate of epicardial progenitors. Nature. 2009; 458:E8–9. E9–10. [PubMed: 19369973]

35. Mellgren AM, Smith CL, Olsen GS, Eskiocak B, Zhou B, Kazi MN, Ruiz FR, Pu WT, Tallquist MD. Platelet-derived growth factor receptor beta signaling is required for efficient epicardial cell migration and development of two distinct coronary vascular smooth muscle cell populations. Circ Res. 2008; 103:1393–1401. [PubMed: 18948621]

36. Tallquist MD, Soriano P. Cell autonomous requirement for PDGFRalpha in populations of cranial and cardiac neural crest cells. Development. 2003; 130:507–518. [PubMed: 12490557]
37. Trinh LA, Yelon D, Stainier DY. Hand2 regulates epithelial formation during myocardial diferentiation. Curr Biol. 2005; 15:441–446. [PubMed: 15786591]
38. Sottile J, Hocking DC, Swiatek PJ. Fibronectin matrix assembly enhances adhesion-dependent cell growth. J Cell Sci. 1998; 111:2933–2943. [PubMed: 9730985]
39. Leiss M, Beckmann K, Giros A, Costell M, Fassler R. The role of integrin binding sites in fibronectin matrix assembly in vivo. Curr Opin Cell Biol. 2008; 20:502–507. [PubMed: 18586094]
40. Liao YF, Gotwals PJ, Koteliansky VE, Sheppard D, Van De Water L. The EIIIA segment of fibronectin is a ligand for integrins alpha 9beta 1 and alpha 4beta 1 providing a novel mechanism for regulating cell adhesion by alternative splicing. J Biol Chem. 2002; 277:14467–14474. [PubMed: 11839764]
41. Bazigou E, Xie S, Chen C, Weston A, Miura N, Sorokin L, Adams R, Muro AF, Sheppard D, Makinen T. Integrin-alpha9 is required for fibronectin matrix assembly during lymphatic valve morphogenesis. Dev Cell. 2009; 17:175–186. [PubMed: 19686679]
42. Yin C, Kikuchi K, Hochgreb T, Poss KD, Stainier DY. Hand2 regulates extracellular matrix remodeling essential for gut-looping morphogenesis in zebrafish. Dev Cell. 2010; 18:973–984. [PubMed: 20627079]
43. Ieda M, Tsuchihashi T, Ivey KN, Ross RS, Hong TT, Shaw RM, Srivastava D. Cardiac fibroblasts regulate myocardial proliferation through beta1 integrin signaling. Dev Cell. 2009; 16:233–244. [PubMed: 19217425]
44. Pae SH, Dokic D, Dettman RW. Communication between integrin receptors facilitates epicardial cell adhesion and matrix organization. Developmental Dynamics. 2008; 237:962–978. [PubMed: 18351655]
45. Yang JT, Rayburn H, Hynes RO. Cell adhesion events mediated by alpha 4 integrins are essential in placental and cardiac development. Development. 1995; 121:549–560. [PubMed: 7539359]
46. Sengbusch JK, He W, Pinco KA, Yang JT. Dual functions of [alpha]4[beta]1 integrin in epicardial development: initial migration and long-term attachment. Journal of Cell Biology. 2002; 157:873–882. [PubMed: 12021259]
47. Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol. 1994; 124:619–626. [PubMed: 8106557]
48. Wu M, Smith CL, Hall JA, Lee I, Luby-Phelps K, Tallquist MD. Epicardial spindle orientation controls cell entry into the myocardium. Dev Cell. 2010; 19:114–125. [PubMed: 20643355]
49. George EL, Baldwin HS, Hynes RO. Fibronectins are essential for heart and blood vessel morphogenesis but are dispensable for initial specification of precursor cells. Blood. 1997; 90:3073–3081. [PubMed: 9376588]
50. Moore AW, McInnes L, Kreidberg J, Hastie ND, Schedl A. YAC complementation shows a requirement for Wt1 in the development of epicardium, adrenal gland and throughout nephrogenesis. Development. 1999; 126:1845–1857. [PubMed: 10101119]
51. Tomita M, Okuyama T, Katsuyama H, Miura Y, Nishimura Y, Hidaka K, Otsuki T, Ishikawa T. Mouse model of paraquat-poisoned lungs and its gene expression profile. Toxicology. 2007; 231:200–209. [PubMed: 17215068]
52. Garavito-Aguilar ZV, Riley HE, Yelon D. Hand2 ensures an appropriate environment for cardiac fusion by limiting Fibronectin function. Development. 2010; 137:3215–3220. [PubMed: 20724450]
53. Richarte AM, Mead HB, Tallquist MD. Cooperation between the PDGF receptors in cardiac neural crest cell migration. Dev Biol. 2007; 306:785–796. [PubMed: 17499702]
54. Ieda M, ji-dong F, Delgado-Olguin P, Vedantham V, Hayashi Y, Bruneau BG, Srivastava D. Direct Reprogramming of Fibroblasts into Functional Cardiomyocytes by Defined Factors. Cell. 2010; 142:375–386. [PubMed: 20691899]
55. Firulli BA, Krawchuk D, Centonze VE, Virshup DE, Conway SJ, Cserjesi P, Laufer E, Firulli AB. Altered Twist1 and Hand2 dimerization is associated with Saethre-Chotzen syndrome and limb abnormalities. Nat Genet. 2005; 37:373–381. [PubMed: 15735646]

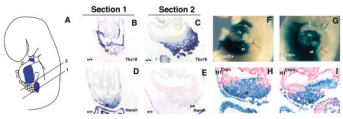

Figure 1.

The *Hand1* lineage gives rise to epicardial progenitors. (A) E9.5 illustration showing *Hand1* expression (blue) and depicting the planes of section in B-E (B). *Hand1* and *Tbx18* section *ISH* of E9.5 embryos on adjacent sections through the ST and the PE (B-E). *Hand1* expression is not detected within the PE but is expressed in the ST. X-gal staining of *Hand1*^{LacZ} (F) and *Hand1*^{eGFPCre} *R26R* activation *(G-I)* shows *Hand1*-lineage cells within the ST and in the PE.. lv, left ventricle; pe, proepicardium; st, septum transversum.

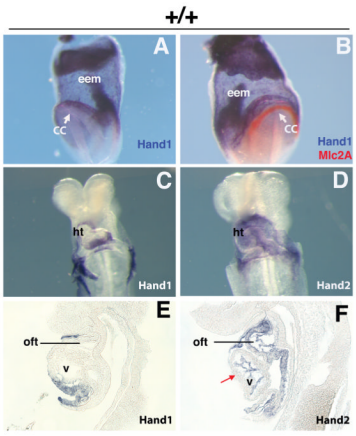

Figure 2.

Hand factor expression is distinct. Single and double-labeled *Mlc2a* and *Hand1* wholemount 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, B). E8.5 wholemounts of *Hand1* and *Hand2* reveal complimentary expression during cardiac development (C-F). *Hand2* expression is confined to the SHF and endocardium (black arrow). cc, cardiac crescent; eem, extraembryonic mesoderm; ht, heart tube; lm, lateral mesoderm; oft, outflow tract; v, ventricle.

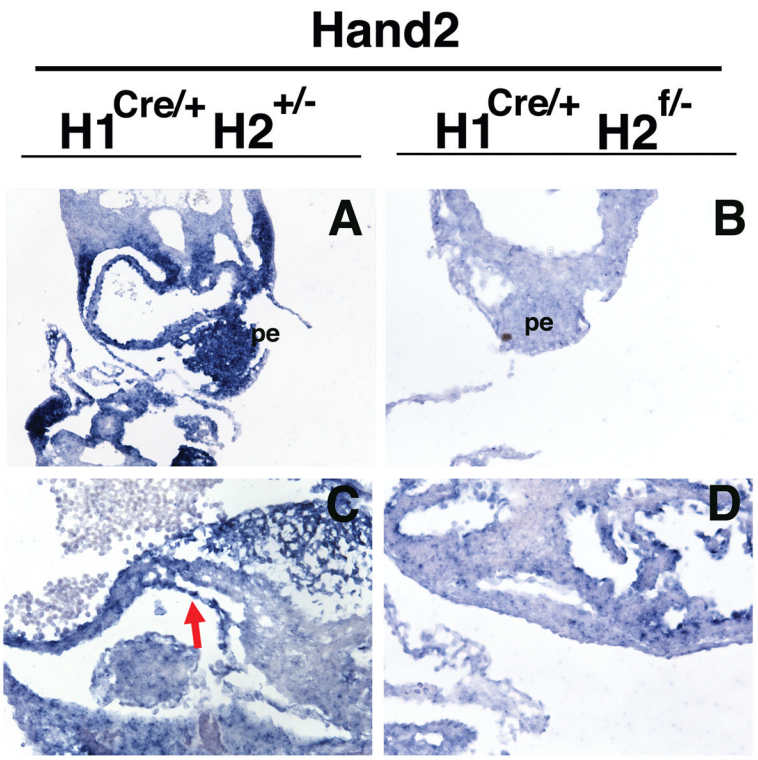

Figure 3.
Inactivation of *Hand2* within the *Hand1* lineage RNA ISH at E9.5 (A, B) shows *Hand2* expression throughout the PE and in the epicardium (red arrow) at E10.5 (C, D). Consequently, *Hand2* expression is ablated in the presumptive epicardial mesothelium in *H2CKOs*; pe, proepicardium.

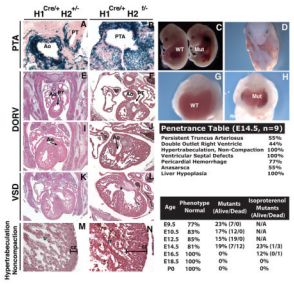

Figure 4.

Conditional *Hand2* deletion within the *Hand1* lineage results in extensive embryonic and cardiovascular defects. Histological (A, B, E, F, I-N) and wholemount (C, D, G, H) analysis of E14.5.*H2CKO* embryos. Mutants display pericardial hemorrhaging (C) and anasarca (D). Compared to WT mice (G), *H2CKOs* have hypoplastic livers with extensive hemorrhaging (H).. *H2CKOs* display either PTA or DORV (A, B, E, F, I, J). All *H2CKOs* exhibit VSDs (K, L). Myocardial examination shows abnormal trabeculae and non-compaction (M, N). ao, dorsal aorta; cz, compact zone; pt, pulmonary trunk; pta, persistent truncus arteriousus; tr, trabeculae.

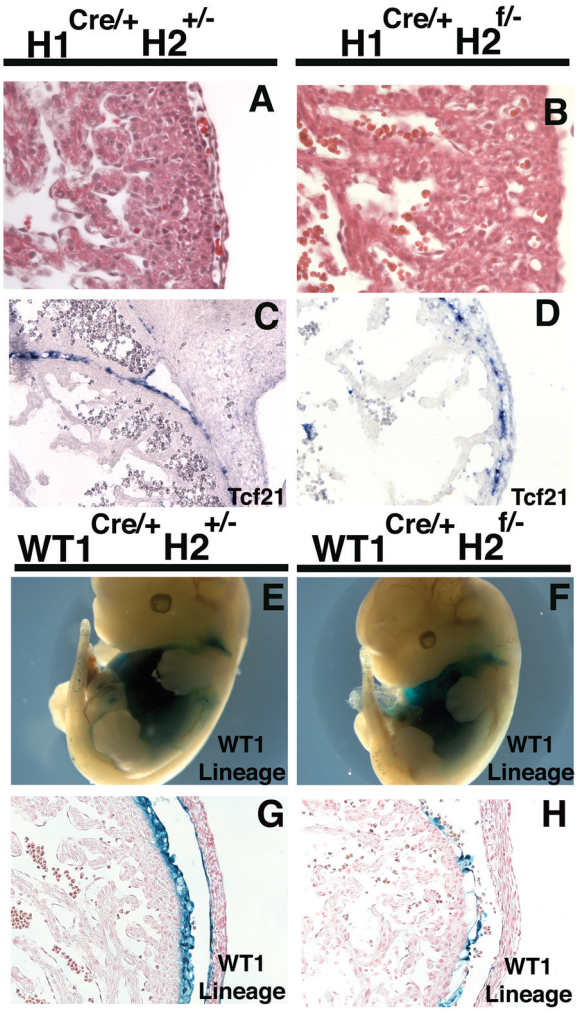

Figure 5.

H2CKOs display epicardial phenotypes. Histological examination at E14.5 shows a lack of epicardium and compaction abnormalities in *H2CKOs* (A, B). RNA *ISH* for *Tcf21* at E12.5 shows initial establishment of the epicardium (C, D). X-gal staining and conditional deletion of *Hand2* within the *WT1* lineage at E13.5 photocopies *H2CKO* epicardial defects (E-H).

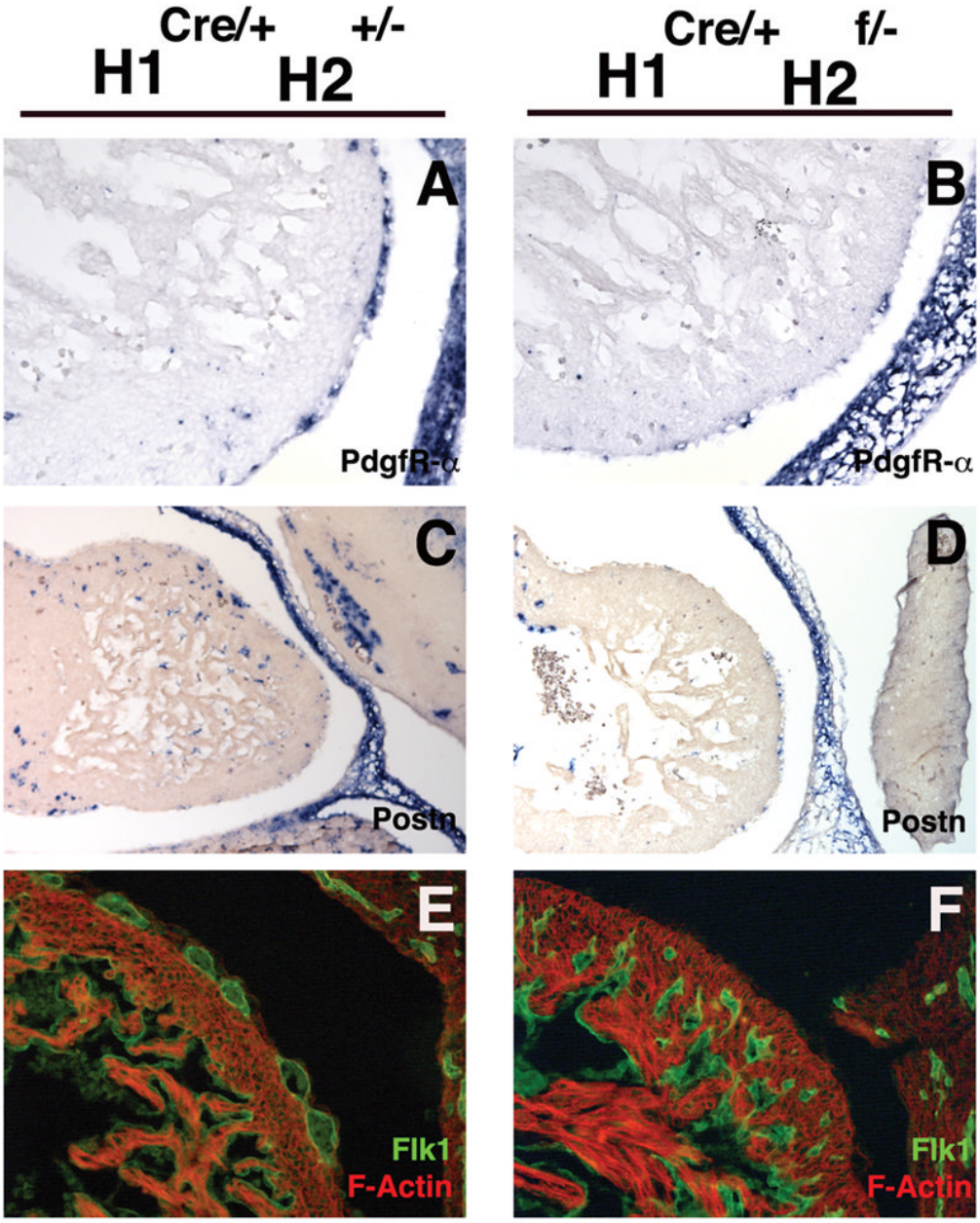

Figure 6.

Fibroblast lineage and secondary coronary defects in *H2CKOs.* RNA *ISH* at E12.5 for *Pdgfrα a*nd *Postn* (A-D). A decrease in cardiac fibroblasts is observed within the compact zone of *H2CKOs*. Immunohistochemistry for Flk1 (green) and F-actin (red) at E12.5 (E, F). A decrease in patent coronary vasculature is observed in *H2CKOs*.

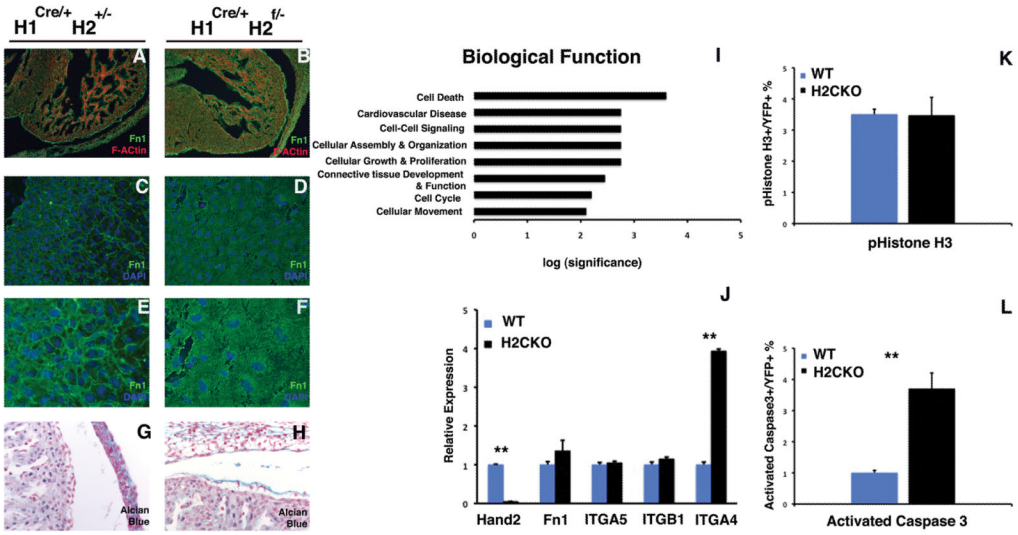

Figure 7.

Abnormal Fn1 matrix assembly and function results in ECM related defects and impaired epicardial development. Immunohistochemistry at E12.5 for *Fn1* (green) and F-actin (red) showing Fn1 expression in the epicardium and absence of patent coronary vessels (A, B). Primary epicardial cells isolated from H2CKOs show a disorganized Fn1 localization, where Fn1 appears to be distributed uniformly throughout the cells, further suggesting migratory defects in *H2CKO* epicardial cells (D, F). In contrast, wild type primary epicardial cells show Fn1 distributed in a series of well-organized bundles (C, E). An increase in Alcian Blue staining within the epicardium indicates increased deposition of ECM (G, H). Gene ontology analysis of microarray data from primary epicardial cells showing affected gene pathways within *H2CKO* isolated epicardium (I). qRT-PCR on wild type (blue bar) and *H2CKO* (black bar) RNA isolated from primary epicardial cells; n=4 (J). Cell proliferation (K) and cell apoptosis (L) on wild type and *H2CKO* embryos at E12.5; n=3. Asterisks indicate statistical significance: *p < 0.05; **p <0.01.