Cardiac Neural Crest Expression of Hand2 Regulates Outflow and Second Heart Field Development

Yuka Morikawa, Peter Cserjesi

Abstract—The cardiac neural crest (cNC) lineage plays key roles in heart development by directly contributing to heart structures and regulating development of other heart lineages. The basic helix-loop-helix factor Hand2 regulates development of cardiovascular structures and NC-derived tissues including those that contribute to face and peripheral nervous system. Although Hand2 is expressed in cNC, its role has not been examined because of an early embryonic lethality when Hand2 is deleted in the NC lineage. We find that the lethality is attributable to loss of norepinephrine synthesis that can be overcome by activating adrenergic receptors. In rescued embryos, loss of Hand2 in the NC lineage leads to the misalignment of the outflow tract and aortic arch arteries. Defects include pulmonary stenosis, interrupted aortic arch, retroesophageal right subclavian artery, and ventricular septum defect, which resemble congenital heart defects attributed to defects in the NC. Hand2 functions in part by regulating signaling from the cNC to other cardiac lineages but not by regulating migration or survival of the cNC. Loss of Hand2 in NC also uncovered a novel role for the cNC in regulating proliferation and differentiation of the second heart field–derived myocardium that persists late into development. These results show that the cNC functions as a major signaling center for heart development and Hand2 plays a pivotal role in regulating both cell-autonomous and -nonautonomous functions of the cNC. (Circ Res. 2008;103:1422-1429.)

Key Words: neural crest • Hand2 • hypertrabeculation

Development of the mammalian heart occurs through complex interactions between numerous lineages regulated by multiple signaling pathways that coordinate these interactions. A subset of neural crest (NC) cells, the cardiac NC (cNC) cells, invade the outflow tract (OFT) and are essential for septation of the conotruncus into the aortic and pulmonary arteries.1,2 These cells arise from the level of the otic vesicle through the third somite, migrate through the pharyngeal arches, and populate the aorticopulmonary septum and conotruncal cushion2 and contribute to the smooth muscle of aortic arch arteries (AAAs). In addition to the direct contribution to the OFT vasculature, cells of the cNC lineage participate in a number of morphological events in a cell nonautonomous manner. Although the NC lineage makes a minor direct contribution to the heart,4,5 defects in the cNC lineage have a profound effect on patterning the heart and accounts for a large numbers of human congenital heart defects (CHDs) including OFT and AAA defects such as persistent truncus arteriosus and tetralogy of Fallot.1,2

The involvement of cNC in the development of non-NC–derived tissue has been well documented.1,2 For example, the cNC, OFT, and endothelia interact, and this interaction is mediated by the semaphorin family of ligands and their receptors.6–8 Furthermore, a role for cNC in regulating the cells of the second heart field (SHF) has also been suggested. During early embryogenesis, ablation of the cNC results in a shortened OFT caused by an inability of the SHF cells to migrate into the primary heart tube.9 Although the mechanism underlying the signaling process remains unclear, members of the fibroblast growth factor10 and transforming growth factor-β families11 have been implicated.

The basic helix-loop-helix transcription factor Hand2 is expressed in the SHF and cNC during heart development.12 Deletion of Hand2 has shown that it is essential for right ventricle (RV) development and the survival of the embryos beyond 10.5 days postconception (dpc).13 In Hand2 mutant embryos, the RV forms but undergoes apoptosis,14 suggesting that Hand2 is required for survival, but not specification, of the cardiomyocytes. An essential role for Hand2 in cNC was also suggested from our previous study showing that deletion of Hand2 specifically in NC resulted in pooling of blood in the peripheral vasculature and liver of mutant embryos, which suggests a cardiovascular defect.15

Previously, it was not possible to examine the role of Hand2 specifically in cNC because of an early lethality in either systemic or NC-specific conditional knockout (cKO) embryos.13,15 We developed a pharmacological approach to rescue the embryonic lethality associated with the loss of Hand2 in NC, allowing an analysis of Hand2 function in cNC. We find that deletion of Hand2 in the NC lineage...
results in the misalignment of the OFT and AAA and defects that resemble those observed in CHDs associated with NC defects.1,2 In addition, we find that Hand2 expression in the NC lineage regulates development of the SHF-derived myocardium. The defects observed in the SHF-derived heart structures suggest that NC has a much larger impact on heart development than previously suspected.

Materials and Methods

Generation of Conditional Knockout Embryos
To generate embryos containing NC-specific deletion of Hand2 embryos, mice containing the floxed allele of Hand2 were crossed with the Wnt1-Cre line16 as previously described.15 Hand2flox/flox embryos were used as controls. To delete Hand2 in the noradrenergic neurons, we generated a DBH-Cre line by pronuclear injection of a construct containing 5.8 kb upstream region of the human DBH gene linked to myc-nuclear-Cre (gift from David Weisenthaler, Emory University School of Medicine). Transgenic lines were tested for Cre activity by crossing the conditional β-galactosidase mouse line R26R [Gt(ROSA)26Sortm1Sto]17 to obtain embryos with NC ablation of Hand2 older than 12.5 dpc, the water of pregnant dams was supplemented with 200 μg/mL isoproterenol hydrochloride (Sigma) and 2.5 mg/mL ascorbic acid starting at 7.5 dpc. To delete Hand2 in myocardium and extraembryonic membranes, Hand2 cKO line was crossed with the cTnt-Cre18 and HoxB6-Cre19 lines, respectively.

In Situ Hybridization
Whole mount in situ hybridization was carried out with digoxigenin-labeled probes as previously described.15,20 Plasmids used to generate these probes, nppa, PlxnA2, and Sema3C were obtained from mouse embryonic cDNA (Ambion) by PCR amplification and cloning of the PCR products into pBluescriptSKII.

Immunohistochemistry
Immunohistochemistry was performed as described previously.15,21 Antibodies used in this study were mouse monoclonal antibody against α-smooth muscle actin (diluted 1:500, Sigma) and rabbit polyclonal antibody against Ki-67 (diluted 1:100, Abcam). Secondary antibodies were labeled with Alexa 555 (Invitrogen) for fluorescence detection.

Cell proliferation was monitored by 5-bromodeoxyuridine (BrdU) incorporation as described previously.22 Proliferation rate was measured by counting BrdUrd-positive cells as a percentage of DAPI-positive nuclei. Sample means were compared using the Student’s t test.

Visualization of the OFT Vasculature
Blue latex (Connecticut Valley Biological Supply Co) was injected into the left ventricles of 16.5 dpc embryos. Immediately after injection, embryos were fixed in 70% ethanol, dehydrated in 100% ethanol, and cleared with benzyl benzoate: benzyl alcohol (2:1) solution.

Quantitative PCR Analysis
Total RNA was extracted and purified from the OFT of 12.5 dpc embryos using an RNA easy (Qiagen) kit. cDNA was synthesized using Superscript III (Invitrogen), and quantitative PCR analysis was carried out using iQ CybrGreen Supermix (Bio-Rad) chemistry in an iCycler iQ system (Bio-Rad). Three replicates of 4 samples were analyzed. Sema3C and PtxnA2 were quantified relative to β-actin using 2-ΔΔCT to calculate relative levels.23

Results

Activation of Noradrenergic Receptor Rescues the Embryonic Lethality of Hand2 cKO Embryos
Hand2 regulates development of numerous NC-derived tissues,15,22 but its function in cNC has not been examined because of early embryonic lethality with both systemic13 and NC-specific deletion of Hand2.15 When Hand2 is deleted in NC by crossing our conditional knockout (cKO) Hand2 mouse line with Wnt1-Cre, embryos die before complete development of the cardiovascular system (Table 1) and exhibit pooling of blood in the peripheral vasculature and liver.15 The phenotype resembles that seen in other embryos with cardiovascular defects resulting from NC-specific gene deletions5,11,24 but differs in that cNC defects generally do not affect embryonic viability. To analyze how Hand2 functions in cNC, we developed a strategy to rescue the early lethality.

Hand2 is required for the expression of norepinephrine (NE) biosynthetic enzymes in the developing sympathetic nervous system (SNS),15 and loss of NE in the SNS leads to embryonic lethality.25 To determine whether this alone can account for the embryonic lethality, we deleted Hand2 specifically in noradrenergic neurons using a dopamine β-hydroxylase (DBH) Cre mouse line (Figure I, A through C, in the online data supplement, available at http://circres.ahajournals.org). When Hand2 is deleted by DBH-Cre, no mutant embryos were recovered past 12.5 dpc (Table 1), and all embryos collected at 12.5 dpc show pooling of blood in the liver and peripheral vasculature (supplemental Figure II, A and B), resembling the phenotype of the Hand2flox/flox;Wnt1-Cre embryos. No other morphological defects were observed demonstrating that Hand2 maintains embryonic viability by regulating noradrenergic differentiation in the SNS.

Noradrenergic deficiency can be overcome by activation of adrenoceptors.25,27 When the β-adrenoceptor agonist isoproterenol was fed to pregnant mothers, approximately one-half of the Hand2flox/flox;Wnt1-Cre cKO embryos survived to 15.5 dpc (Table 1), with a comparable number of these cKO embryos surviving to birth. This shows that embryonic lethality is attributable to a lack of adrenoceptor activation through loss of NE synthesis.

Hand2 Is Required for the Development of cNC
The main contribution of NC to the cardiovascular system is to the OFT and AAA. Using isoproterenol to rescue embryonic lethality, we examined the consequences of deleting Hand2 in the cNC on the development of OFT and AAA (Figure 1 and supplemental Figure III). Defects caused by loss of Hand2 included pulmonary stenosis (Figure 1D), interrupted aorta (IAA) type B (Figure 1B and 1C), anomalous origin of the right subclavian artery (ARSA) including a retroesophageal right subclavian artery (Figure 1B and 1C), and membranous ventricular septum defects. The rates at which these defects occur are summarized in Table 2. In addition to the arteries, the thymus is colonized by a sub-

### Table 1. Survival Rate of Cre;Hand2flox/flox cKO Embryos at 15.5 dpc

<table>
<thead>
<tr>
<th>Cre Line</th>
<th>Isoproterenol</th>
<th>Mutant Embryos*</th>
<th>Total Embryos</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wnt1-Cre</td>
<td>−</td>
<td>0/13</td>
<td>53</td>
</tr>
<tr>
<td>DBH-Cre</td>
<td>−</td>
<td>0/7</td>
<td>35</td>
</tr>
<tr>
<td>Wnt1-Cre</td>
<td>+</td>
<td>7/15</td>
<td>52</td>
</tr>
</tbody>
</table>

*All embryos, including those undergoing reabsorption, were collected and genotyped. Numbers represent the no. of mutants embryos living over total.
population of cNC, and thymus abnormalities are often associated with CHDs.1,28,29 The thymus is normally located on the ventral side of the OFT close to the heart (Figure 1E).

One mechanism by which deficiencies in cNC development can disrupt OFT and AAA development is through defective colonization by the NC. The distribution of cNC in the AAA was examined by lineage tracing using the R26R mouse line17 combined with cKO of Hand2 (Figure 1E and 1F). The tracing results show that NC contribution to the AAA is unaffected in mutant embryos.

Postmigratory cNC cells also differentiate into smooth muscle cells of the pharyngeal arch arteries.30 To determine whether Hand2 is required for smooth muscle differentiation, we analyzed the Hand2 cKO embryos for expression of the smooth muscle marker α-smooth muscle actin by immunohistochemistry. The expression of α-smooth muscle actin in the vessel walls of Hand2 cKO was unaffected (data not shown), indicating that Hand2 is not required for differentiation of the cNC into smooth muscle. Taken together, our results show that cNC expression of Hand2 is required for the patterning of the pharyngeal arch arteries and thymus but not for cNC migration or smooth muscle differentiation.

Semaphorin Signaling Pathway Is Downstream of Hand2

Misalignment of the vessels derived from the fourth pharyngeal arch arteries leads to IAA type B and ARSA, both of which are found at high frequency when Hand2 is deleted in NC (Table 2). These defects are also found in embryos lacking member of the semaphorin family ligand, semaphorin3C (Sema3C),6 and defects in cNC can lead to downregulation of Sema3C.11,31 To determine whether Hand2 regulates the expression of Sema3C or its receptor PlxnA2, we examined their expression by in situ hybridization in cKO embryos (Figure 2A through 2D). At 12.5 dpc, Sema3C is expressed in the myocardium of the OFT of control hearts (Figure 2A) and expression is not affected by loss of Hand2 (Figure 2B). However, the expression of PlxnA2 in NC-derived cells in the OFT of control hearts (Figure 2C) is downregulated in cKO littermates (Figure 2D). The levels of Sema3C and PlxnA2 messages were quantified by quantitative PCR analysis (Figure 2E). Consistent with the in situ results, Sema3C expression is not affected by Hand2 loss, whereas the expression of PlxnA2 in the OFT of cKO embryos are reduced to approximately 50% of control OFT.

A possible explanation for the decrease in PlxnA2 expression is that cNC cells fail to fully populate the OFT. We examined if Hand2 is required for the migration of cNC cells into the OFT by lineage tracing (Figure 2E through 2H). At 10.5 dpc, cNC cells are migrating through pharyngeal arch arteries and migratory patterns are comparable between control and cKO embryos (Figure 2F and 2I). At 12.5 dpc, cNC cells are detected in the OFT of both control and cKO embryos (Figure 2G and 2J) and histological analysis of the OFT shows that NC distribution within the OFT is not affected by loss of Hand2 (Figure 2H and 2K). Thus, Hand2-deficient cNC cells may be incapable of responding to Sema3C signaling from the myocardium and display a phenotype similar to the Sema3C null embryos.

The NC Lineage Regulates Proliferation of SHF-Derived Myocardium

A remarkable and novel consequence of Hand2 loss in the NC lineage is the development of an enlarged RV (Figure 3A

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**Table 2. Abnormalities Found in NC Deletion of Hand2**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>Occurred (n=8), %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pulmonary stenosis</td>
<td>37.5</td>
</tr>
<tr>
<td>IAA (type B)</td>
<td>62.5</td>
</tr>
<tr>
<td>Anomalous origin of RSA</td>
<td>100</td>
</tr>
<tr>
<td>Retroesophageal RSA</td>
<td>87.5</td>
</tr>
<tr>
<td>Membranous VSD</td>
<td>100</td>
</tr>
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RSA indicates right subclavian artery; VSD, ventricular septum defect. The organization of the OFT and AAA and the presence of an interventricular septum in Hand2-Wnt1-Cre cKO embryos were examined at 16.5 or 18.5 dpc.
Histological analysis revealed that the enlargement is attributable to hypertrabeculation, suggesting an overproliferation of the cardiomyocytes (Figure 3C and 3D). This overproliferation could be regulated by either shortening of the cell cycle or a failure to terminally differentiate and exit the cell cycle. To determine whether the proliferation rate of cells in the trabecular zone is increased in Hand2 cKO hearts, we labeled cardiomyocytes with BrdUrd (Figure 3E). In the trabecular zone of the RV of 13.5 and 15.5 dpc cKO hearts, the proportion of labeled cells increased 2-fold over control (Figure 3E). Significantly, increased proliferation was specific to the SHF derivatives in the trabecular zone of the RV with neither the compact zone of the heart (supplemental Figure IV) nor the trabecular zone of the left ventricle (Figure 3E) showing an increase in proliferation rates. To determine whether this represents an increase in the rate of the cell cycle or in the proportion of cycling cardiomyocytes, we examined the expression of the cell cycle marker Ki-67 (Figure 3F and 3G). The proportion of Ki-67–expressing cells increased 2-fold in the trabecular zone of the RV (Figure 3G), showing a doubling in the proportion of cycling cells. During normal growth of the trabecular myocardium, only myocardium at the base of the trabeculae proliferate (Figure 3H). An examination of the distribution of proliferating cells in the trabecular zone of the RV and 3B).
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To determine whether loss of Hand2 in the myocardium is responsible for the lethality at 10.5 dpc seen in the systemic knockout of Hand2, we deleted Hand2 only in the myocardium. Unlike the systemic knockout of Hand2 that results in embryonic lethality, we deleted Hand2 only in the myocardium. Neither viability drops until 12.5 dpc, after which time no viable embryos were recovered (Figure 5A). A morphological examination of 10.5 dpc mutant embryos with severe phenotypes showed that embryos have a single clearly defined ventricle (Figure 5B through 5E). The phenotype of these cTnt-Cre deletions of Hand2 strongly resembles the Hand2 systemic knockout, suggesting that Hand2 expression in SHF cardiomyocytes is required for their survival. The hearts of cKO embryos that survive until 12.5 dpc also show hypoplastic RV and OFT (supplemental Figure V). The variability in survival may be attributable to variability in the level and timing of Hand2 recombination.

Although embryonic lethality caused by systemic loss of Hand2 appears to be attributable to loss of Hand2 in the heart, it has also been shown to play a role in extraembryonic vasculature development. To determine whether the cause of lethality in Hand2-null embryos is attributable to a role in heart or extraembryonic mesoderm, we deleted Hand2 in the extraembryonic and lateral mesoderm by crossing with the HoxB6-Cre transgene. Mutant embryos are viable to birth and did not exhibit visible defects in extraembryonic mesoderm or blood vessel formation (Figure 5F through 5I). However, the mutant neonates exhibit severe limb deformities (Figure 5J and 5K). Loss of Hand2 leads to down regulation of Shh expression in the early limb, accounting for the limb phenotype. The deletion of Hand2 in extraembryonic membranes shows that it is not required for extraembryonic mesoderm or vasculature development, but its role in the SHF is essential for embryonic viability.

**Discussion**

In this study, we show that Hand2 plays numerous roles in cardiovascular development both in the NC and SHF lineages (Figure 6). In the NC lineage, Hand2 regulates embryonic survival through synthesis of NE in SNS. In the SNS, Hand2 is required for expression of the NE biosynthetic enzymes tyrosine hydroxylase and DBH. Activation of adrenergic receptors by NE is required for embryonic survival and myocardial growth. The targets of NE that are required for survival are not known with certainty but activation of adrenergic receptors is required during the time that the embryo transitions from a diffusable supply of oxygen and nutrients to dependence on an active circulatory system. The essential function of NE during development is likely to be the cardiovascular system.

In addition to its role in the NC, it has been reported that Hand2 expression is essential for extraembryonic vasculature development and survival of SHF-derived myocardium. Because defects in either of these tissues could lead to the lethality at 10.5 dpc seen in the systemic knockout of Hand2, we examined the function of Hand2 in the individual tissues. Deletion of Hand2 in the extraembryonic mesoderm did not result in a defective vasculature suggesting that the previously observed defects may have resulted as a secondary defect caused by defects within the embryo. To determine whether loss of Hand2 in the myocardium is responsible for embryonic lethality, we deleted Hand2 only in the myocardium.
lethality by 10.5 dpc, cKO embryos begin to die at 10.5 dpc with approximately 30% of mutant embryos surviving to 12.5 dpc. This enhanced viability may result from the timing and efficiency of recombination.

Within the NC lineage, loss of Hand2 leads to multiple defects resembling human CHDs including IAA type B and ARSA. These defects are also found in the embryo lacking Sema3C, suggesting an interaction between Hand2 and this signaling pathway. We show that a downstream target of Hand2 in cNC is the Sema3C receptor, PlxnA2. PlxnA2 is expressed in cNC and is often downregulated in embryos with cNC defects, suggesting that Sema3C may signal to cNC through PlxnA2. However, it has not been shown that PlxnA2 functions in cNC during heart development.

One of the remarkable findings is that loss of Hand2 in the NC results in hypertrabeculation of the RV myocardium. Hypertrabeculation/ventricular noncompaction (VHT) is a congenital disorder that has been considered rare, yet recent improvements in imaging technology have revealed it occurs more often than previously suspected. Its cause is not understood; however, because VHT is often associated with facial dysmorphism and other NC-associated cardiac defects, it may be attributable, in part, to defects in NC lineage development.

The RV is derived from the anterior region of the SHF, which migrates into the primitive heart formed by the primary heart field. The regions formed by the SHF does not contain a significant population of NC, suggesting that an interaction between the 2 lineages occurs before, or during, migration of the SHF cells. Such an interaction has been suggested from cNC ablation studies in chick embryos. Ablation of cNC...
results in a failure of the SHF to migrate into the developing heart and to overproliferation of the SHF precursors. This interaction between cNC and the SHF occurs before the two lineages migrate into the primary heart field. Loss of Hand2 in cNC does not affect migration of the SHF into the developing heart but leads to their aberrant development after differentiation into cardiomyocytes. Our results suggest that transient interaction with cNC is sufficient to permanently alter development of SHF-derived myocardium. We speculate that this interaction must take place early in development, while the SHF and cNC are in close proximity. To our knowledge, this is the first report that cNC cells regulate later steps in cardiomyocyte differentiation and thus represents a new paradigm in heart development.

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Disclosures

None.

References


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Supplement Material

Online Figure I

Online Figure II
Online Figure III
Online Figure IV

Online Figure V
Online Figure Legends

Online Figure I. Cre expression in the DBH-Cre line is restricted predominantly to noradrenergic neurons. (A-C) Expression of Cre was monitored by crossing DBH-Cre and R26R lines. DBH-Cre is active in noradrenergic neurons (A) including those in the sympathetic trunk where expression starts at 10.5 dpc (C) but not in the heart (B). drg: dorsal root ganglia, en: enteric neurons, on: oculomotor nerve, m: facial mesenchyme, mes: rostral metencephalon, met: metencephalon, spc: spinal code, st: sympathetic trunk, tg: trigeminal ganglion.

Online Figure II. Hand2 in noradrenergic neurons are required for embryonic survival. (A, B) Deletion of Hand2 in noradrenergic neurons using DBH-Cre leads to embryonic lethality at 12.5 dpc with embryos exhibiting pooling of blood in the liver and peripheral vasculature (B).

Online Figure III. Histological analysis of hearts from embryos lacking Hand2 in the NC lineage. Embryos were collected at 18.5 dpc from an isoproterenol-fed mothers and sectioned. (A-D) Control heart sections showing the same plane as the mutant hearts. (A´-D´) Sections through a Hand2fx/-; Wnt1-Cre cKO heart with an interrupted aortic arch. Panel A´ and B´ show a large pulmonary trunk which is fused to the descending aorta. Panel C´ shows that this heart also has tricuspid atresia. (A”-D”) Sections through a Hand2fx/-; Wnt1-Cre cKO heart with pulmonary stenosis. Panel A” shows the pulmonary stenosis and panel C” shows the also has a membranous VSD. AA: aortic artery, LA: left atria, LV: left ventricle, MV: mitral valve, PA: pulmonary artery, RA: right atria, RV: right ventricle, TV: tricuspid valve, VSD: ventricular septum defect.
Online Figure IV. Loss of Hand2 in the cNC does not affect proliferation in the myocardial compact zone. The proliferation rate of the myocardial compact zone was examined by BrdU labeling at 13.5 and 15.5 dpc in WT and Hand2flo; Wnt1-Cre cKO embryos. L: left ventricle, R: right ventricle.

Online Figure V. Loss of Hand2 in myocardium show hypoplastic right ventricle. (A, B) Morphological analysis of control (A) and Hand2flo; cTnt-Cre (B) hearts shows that loss of Hand2 in differentiating myocardium leads to hypoplastic right ventricle and thin OFT myocardium.