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Role of *Hand1/eHAND* in the Dorso-Ventral Patterning and Interventricular Septum Formation in the Embryonic Heart

Kiyonori Togi,[1] Takahiro Kawamoto,[1] Ryoko Yamauchi,[2] Yoshinori Yoshida,[2] Toru Kita,[2] and Makoto Tanaka[1,3]*

Department of Geriatric Medicine[1] and Department of Cardiovascular Medicine,[2] Graduate School of Medicine, Kyoto University, and Department of Social Service, Kyoto University Hospital,[3] Kyoto, Japan

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Molecular mechanisms for the dorso-ventral patterning and interventricular septum formation in the embryonic heart are unknown. To investigate a role of *Hand1/eHAND* in cardiac chamber formation, we generated *Hand1/eHAND* knock-in mice where *Hand1/eHAND* cDNA was placed under the control of the *MLC2V* promoter. In *Hand1/eHAND* knock-in mice, the outer curvature of the right and left ventricles expanded more markedly. Moreover, there was no interventricular groove or septum formation, although molecularly, *Hand1/eHAND* knock-in hearts had two ventricles. However, the morphology of the inner curvature of the ventricles, the atrioventricular canal, and the outflow tract was not affected by *Hand1/eHAND* expression. Furthermore, expression of *Hand1/eHAND* in the whole ventricles altered the expression patterns of *Chisel*, *ANF*, and *Hand2/dHAND* but did not affect *Tbx5* expression. In contrast, the interventricular septum formed normally in transgenic embryos overexpressing *Hand1/eHAND* in the right ventricle but not in the boundary region. These results suggested that *Hand1/eHAND* is involved in expansion of the ventricular walls and that absence of *Hand1/eHAND* expression in the boundary region between the right and left ventricles may be critical in the proper formation of the interventricular groove and septum. Furthermore, *Hand1/eHAND* is not a master regulatory gene that specifies the left ventricle myocyte lineage but may control the dorso-ventral patterning in concert with additional genes.

In vertebrate cardiac development, dorso-ventral (DV) patterning, as well as antero-posterior (AP) patterning, plays an essential role in the transformation of the linear heart tube into the four-chambered heart (7, 9). The linear heart tube is polarized along the AP axis, composed of five primordial segments: inflow tract (IFT), common atrium, atrioventricular canal (AVC), primitive ventricle, and outflow tract (OFT). Each segment is controlled by different developmental programs, characterized by specific gene expression profiles (2, 9, 20). In addition to the AP polarity, DV patterning has recently received attention. During cardiac looping, the ventricular chambers expand from the ventral surface of the heart tube (8). Trabeculae form only at the outer curvature of the heart tube, whereas the inner curvature remains smooth walled (7). Christoffels et al. and Lamers and Moorman proposed a two-step model for chamber formation in the embryonic heart (7, 13). First, the primary myocardium is induced, which makes up the linear heart tube. Second, the chamber myocardium is specified on the ventral side of the heart tube, acquiring an additional and presumably more advanced transcriptional program. In the looping heart, the specified chamber myocardium located at the outer curvature expands rapidly while the myocardium of the inner curvature, as well as the IFT, AVC, and OFT, retains the functional and molecular properties of the primary myocardium that has a limited proliferative capacity (7, 13). As a result, the atrial and ventricular chambers balloon out along the outer curvature (9). This ballooning model provides a view that DV, as well as AP, patterning information is critical for chamber specification. However, molecular mechanisms for the expansion of the chamber walls and the DV patterning of the embryonic heart are unknown.

Hand1/eHAND is a potential candidate gene. *Hand1/eHAND* is expressed on the ventral surface in the caudal half of the linear heart tube and predominantly at the outer curvature of the left ventricle (LV) in the looping heart while the gene is absent at the inner curvature (1, 21, 24). Therefore, its expression is highly restricted along the DV as well as the AP axis. Tetraploid-rescued *Hand1/eHAND* null embryos displayed a single ventricle, suggesting that the gene may play a critical role in specification or proliferation of LV myocytes during ballooning (17).

Moreover, the ballooning of chamber walls may be closely related to the formation of the interventricular septum (IVS). In the ballooning model, the structures flanking the atrial and ventricular chambers do not expand and retain the tubular shape, contributing to the proper AV septation and alignment of the IFT and OFT (7, 13). However, in this model, it is not clear what determines the boundary between the right ventricle (RV) and LV. The myocardium at the interventricular groove (IVG) is not the primary but working myocardium according to this model, but this region does not expand. It is totally unknown what molecular mechanisms determine the location of the IVS and IVG.

In this study, we examined a role of *Hand1/eHAND* in the DV patterning of the embryonic heart and the IVS formation. For this purpose, we knocked in the *Hand1/eHAND* gene to

* Corresponding author. Mailing address: Department of Social Service, Kyoto University Hospital, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. Phone: 81-75-751-3465. Fax: 81-75-751-3574. E-mail: makoto@kuhp.kyoto-u.ac.jp.

the mouse myosin light chain 2V (*MLC2V*) locus. We demonstrated that *Hand1*/*eHAND* enhanced expansion of chamber walls and that absence of *Hand1*/*eHAND* expression in the IVG may be critical in the proper formation of the IVS.

MATERIALS AND METHODS

Gene targeting. From a 129SvJ bacterial artificial chromosome library, 3-kb upstream and 6-kb downstream fragments of the coding region of the *MLC2V* gene were isolated. The upstream 3-kb fragment, FLAG-tagged mouse *Hand1*/*eHAND* cDNA, the human growth hormone poly(A) signal, and the 6-kb downstream fragment were ligated into pPNTloxPneo (15). The targeting vector was linearized with NotI for transfection.

RW4 embryonic stem (ES) cells (Incyte Genomics, St. Louis, Mo.) isolated from the 129SvJ strain were cultured on mouse embryonic fibroblast feeder layers in high-glucose Dulbecco's modified Eagle medium containing 20% fetal calf serum and 10^3 U of leukemia inhibitory factor/ml. ES cells (1.0×10^7) were electroporated with 30 μg of the linearized targeting vector. Electroporated ES cells were cultured on neomycin-resistant feeder cells with 300 μg of G418/ml and 2 μM ganciclovir for 8 days. Two hundred eight drug-resistant colonies were isolated, and Southern hybridization demonstrated that four clones contained the correctly targeted allele at the *MLC2V* locus.

These clones were electroporated with 5 μg of the Cre-expressing vector pCre-Pac (KURABO, Osaka, Japan). After electroporation, cells were cultured on feeder cells with 1.7 μg of puromycin/ml for 2 days. Single colonies were picked up in duplicate, and neomycin-sensitive colonies were amplified and genotyped by Southern blotting. Two correctly targeted clones were injected into blastocysts from C57BL/6J mice. Male chimeras were bred with female C57BL/6J mice to test for germ line transmission. All animal procedures were approved by the Animal Research Committee, Graduate School of Medicine, Kyoto University.

Genotyping of progeny. DNA was isolated from tail biopsy specimens of weaned mice, yolk sacs, or placentas. PCR and Southern hybridization were performed to genotype embryos and mice. The primers used for detection of the targeted allele were 5′-TCCGCCTCACCTACAACTGC-3′ and 5′-ACAGAAG GGGGTCACCGTGG-3′.

Generation of transgenic mice. The 250-bp rat MLC2V promoter (10) was synthesized by PCR and was ligated to FLAG-tagged mouse *Hand1*/*eHAND* cDNA with the human growth hormone poly(A) signal. The identity of the synthesized promoter was confirmed by DNA sequencing. The creation of transgenic mice was done in a standard manner. F_0 embryos were dissected at embryonic day 11.5 (E11.5), and genotyping was performed by PCR on DNA isolated from the yolk sacs. PCR primer pairs used for detection of the transgenes were 5′-TGCTGTCAGCCCAATTAG-3′ and 5′-GGCTGCAGTCCTC CTCTTCCTCCCCCTC-3′.

In situ hybridization. In situ hybridization was performed as described previously (23). Briefly, embryos were fixed in 4% paraformaldehyde at 4°C overnight, dehydrated through graded ethanol and xylene, and embedded in paraffin wax. Sections of 6-μm thickness were hybridized with [^{35}S]CTP-labeled riboprobe at 55°C overnight. After hybridization, they were treated with RNase A, washed, and dehydrated through graded ethanol, and emulsion autoradiography was performed. Probes for α-cardiac actin, atrial natriuretic factor (*ANF*), *Hand1*/*eHAND*, *Hand2*/*dHAND*, *MEF2C*, *TEF-1*, *Nkx2.5*, and N-*myc* were described previously (23). An EagI-EcoRI fragment of the 3′ untranslated region (3′ UTR) of *Hand1*/*eHAND* was used to detect endogenous *Hand1*/*eHAND* expression. A probe for *Tbx5* (3) was kindly provided by Benoit G. Bruneau (University of Toronto, Toronto, Canada). A probe for *Chisel* (16) was synthesized by reverse transcription-PCR. The identity of the probe was confirmed by DNA sequencing.

Immunohistochemistry. After rehydration, paraffin sections of embryos were autoclaved in 10 mM EDTA (pH 8.0) at 121°C for 10 min, blocked with an avidin-biotin blocking kit (Vector, Burlingame, Calif.), and incubated with biotinylated mouse anti-FLAG M2 monoclonal antibody (Sigma, St. Louis, Mo.) (1:200) overnight at 4°C. After incubation, sections were washed and incubated with streptavidin-horseradish peroxidase (Nichirei, Tokyo, Japan), and peroxidase activity was detected with 3,3′-diaminobenzidine.

RESULTS

Generation of *Hand1*/*eHAND* KI mice. To investigate a role of *Hand1*/*eHAND* in the DV patterning of the embryonic heart, we generated mice expressing *Hand1*/*eHAND* in the whole ventricles. For this purpose, we employed a knock-in (KI) strategy to place *Hand1*/*eHAND* cDNA into the genomic locus of *MLC2V*, since this gene is expressed in ventricular myocytes throughout development, and heterozygous knockout mice for *MLC2V* were reported to display no obvious phenotype (5). After the first round of homologous recombination, the FLAG-tagged *Hand1*/*eHAND* cDNA and the *pgk-neo* cassette flanked by two *loxP* sites were inserted into the *MLC2V* locus (Fig. 1A). Four correctly targeted clones were identified (Fig. 1B). We then removed the *pgk-neo* cassette by transiently expressing the Cre recombinase (Fig. 1C). After the second round of recombination, two ES clones were injected into C57BL/6 blastocysts. We crossed male chimeras with female C57BL/6 to check for germ line contribution of ES cells by screening for the presence of agouti offspring. Two germ line chimeras were obtained, but none of their offspring carried the KI allele (0 of 20 agouti offspring), indicating that *Hand1*/*eHAND* KI mice were embryonically lethal.

Morphological and histological analysis of *Hand1*/*eHAND* KI embryos. To investigate the timing of lethality, we examined litters from a germ line chimera, all of whose offspring had agouti coat color. At E9.5 and E10.5, *Hand1*/*eHAND* KI embryos were indistinguishable from wild-type littermates. However, *Hand1*/*eHAND* KI embryos showed slight growth retardation at E11.5 and were severely retarded at E12.5, and PCR analysis of the placenta of absorbed embryos at E14.5 revealed that all absorbed embryos carried the KI allele. Viable embryos at E14.5 were all wild type. These results indicated that *Hand1*/*eHAND* KI embryos died between E12.5 and E14.5.

Histological examination at E9.5 revealed that trabeculation and endocardial cushion formation occurred normally in the hearts of *Hand1*/*eHAND* KI embryos. *Hand1*/*eHAND* KI and wild-type embryos were indistinguishable except that there was no IVG in *Hand1*/*eHAND* KI hearts (Fig. 2A and B). At E10.5, ventricular chambers, particularly the RVs, balloon out more markedly in *Hand1*/*eHAND* KI embryos, although their ventricles were single chambers, lacking the IVG and IVS (Fig. 2D and F). In contrast, IVS formation was clearly observed in wild-type littermates (Fig. 2C and E). The morphology of the inner curvature, AVC, and OFT was comparable between *Hand1*/*eHAND* KI and wild-type embryos (Fig. C, D, G, and H). At E11.5, no IVG or IVS formation was observed in *Hand1*/*eHAND* KI embryos (Fig. 2J and L), whereas the IVS was well developed in wild-type hearts (Fig. 2I and K). The compact zone myocardium was thinner in *Hand1*/*eHAND* KI embryos, suggesting that the embryonic lethality may be due to heart failure caused by poor development of the compact zone myocardium.

Gene expression in *Hand1*/*eHAND* KI heart. We first examined expression of *Hand1*/*eHAND* in *Hand1*/*eHAND* KI and wild-type embryos. In wild types, *Hand1*/*eHAND* was expressed in the outer curvature of the LV and the OFT at E9.5 and E10.5 (Fig. 3A and C). Weak expression of *Hand1*/*eHAND* was also observed in the outer curvature of the RV. At E11.5, *Hand1*/*eHAND* expression was down-regulated (Fig. 3E). Notably, *Hand1*/*eHAND* expression was absent at the IVG and IVS throughout development in wild-type embryos (Fig. 3A, C, and E). In contrast, *Hand1*/*eHAND* was expressed in the whole ventricle as well as in the AVC and OFT in *Hand1*/*eHAND* KI embryos (Fig. 3B, D, F, and H). The expression level was still

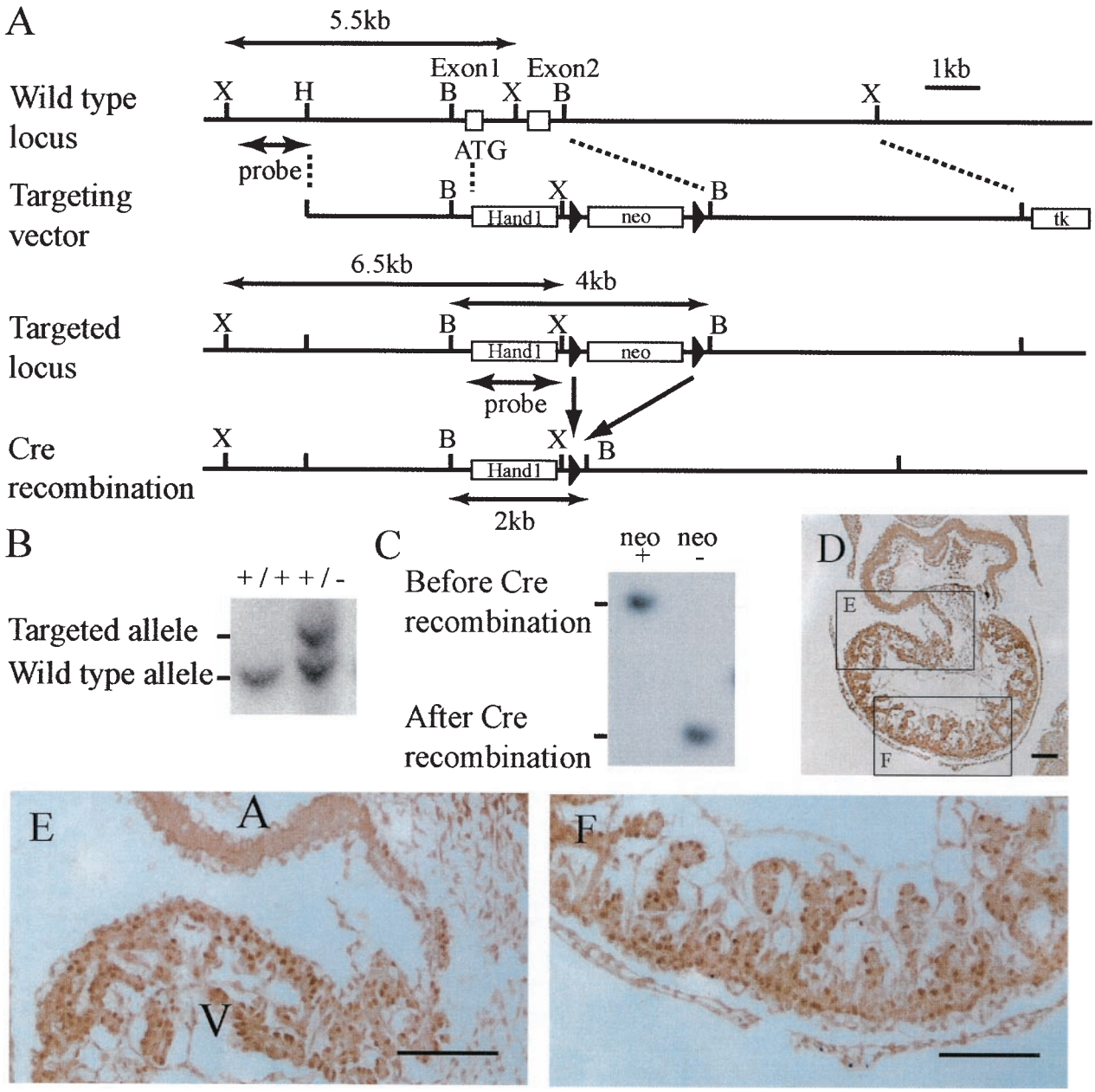

FIG. 1. (A) Targeting strategy. The structure of the *MLC2V* locus and the targeting vector are shown first and second, respectively. The mutated locus after homologous recombination is shown third, and the modified locus by Cre recombination is shown at the bottom. ATG is the transcriptional start site. The closed arrowheads represent the *loxP* sites. B, BamHI; H, HindIII; X, XbaI. (B) Genotyping of ES cell clones after homologous recombination. Genomic DNA was digested with XbaI and analyzed by Southern blotting. The 5′ probe (a BamHI-HindIII fragment) was used. Hybridization with the 5′ probe revealed the expected 5.5- and 6.5-kb fragments from the wild-type and targeted alleles, respectively. (C) Genotyping of ES clones after Cre recombination. Genomic DNA was digested with BamHI and analyzed by Southern blotting. *Hand1/eHAND* cDNA was used as a probe. The expected 4-kb fragment from the original targeted allele and the 2-kb fragment from the Cre mutated allele were revealed. Fragments from the wild-type allele for *Hand1/eHAND* were also detected (not shown on this figure). (D, E, and F) Immunohistochemistry with an anti-FLAG antibody. FLAG-tagged Hand1/eHAND protein was expressed in the nuclei of ventricular cells, whereas the expression was not detected in atrial cells in *Hand1/eHAND* KI embryos (E). FLAG-tagged Hand1/eHAND protein was expressed in the whole ventricle (F). A, atrium; V, ventricle. Bars, 100 μm.

high at E11.5 (Fig. 3F). To confirm the expression of the FLAG-tagged Hand1/eHAND protein, we also performed immunohistochemistry with an anti-FLAG antibody. The FLAG-tagged Hand1/eHAND protein was detected in cardiac myocytes of the whole ventricle in *Hand1/eHAND* KI embryos (Fig. 1D, E, and F) but not in wild types (data not shown).

We next examined endogenous *Hand1/eHAND* expression using the 3′ UTR of *Hand1/eHAND* as a probe, since the 3′ UTR is not included in the FLAG-tagged *Hand1/eHAND* cDNA. Endogenous *Hand1/eHAND* expression was confined to the left side of the single ventricle in *Hand1/eHAND* KI embryos, and the expression level was comparable to that in

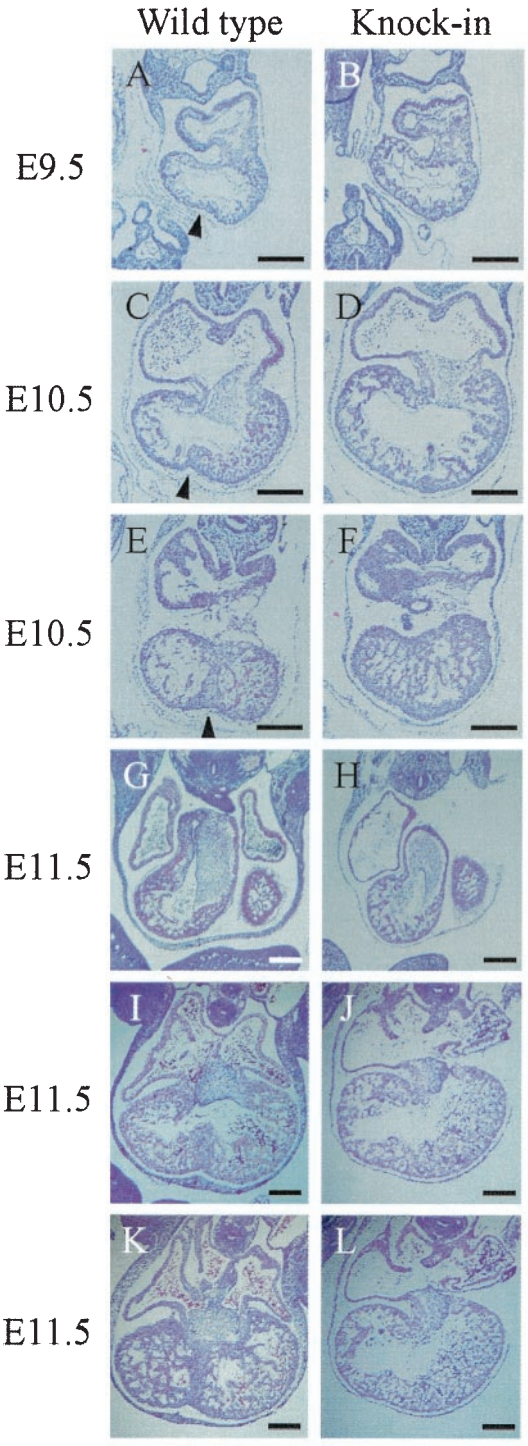

FIG. 2. Histological analysis of wild-type and *Hand1/eHAND* KI embryos from E9.5 to E11.5. Hematoxylin- and eosin-stained sections of wild-type (A, C, E, G, I, and K) and *Hand1/eHAND* KI (B, D, F, H, J, and L) embryos are shown. At E9.5, trabeculation, endocardial cushion formation, and looping normally occurred in *Hand1/eHAND* KI embryos (B). Note the absence of the IVG in *Hand1/eHAND* KI embryos. The IVG can be observed in wild-type embryos (arrowhead in panel A). At E10.5, the outer curvature expanded more markedly in *Hand1/eHAND* KI embryos (C and D). The difference was more evident in the RV. There was no IVG or IVS formation in *Hand1/eHAND* KI embryos (D and F). At E11.5, *Hand1/eHAND* KI embryos exhibited a single ventricle with complete absence of the IVS and IVG (J and L). Endocardial cushion formation in the OFT was comparable between wild-type (G) and *Hand1/eHAND* KI (H) embryos. In KI embryos, the development of the AVC was disturbed (J and L). The arrowheads in panels A, C, and E indicate the IVG. Bars, 200 μm.

wild-type embryos (Fig. 4A and B), indicating that there was a clear distinction between the left and right sides of the ventricle at the molecular level, although there was no IVG or IVS formation. Moreover, the left-side expression of *Tbx5* was not disturbed in *Hand1/eHAND* KI embryos (Fig. 4C and D), further supporting the observation that the right and left sides of the ventricle were molecularly distinctive in *Hand1/eHAND* KI embryos. Furthermore, endogenous *Hand1/eHAND* expression was detected in the AVC in *Hand1/eHAND* KI embryos (Fig. 4B), suggesting that a positive feedback regulation of *Hand1/eHAND* may exist in the AVC.

Chisel and *ANF* are regarded as molecular markers for the working myocardium (7). *Chisel* was expressed in the atrium and the outer curvature of the ventricle but was absent in the inner curvature and the AVC in wild-type embryos (Fig. 4I) (7, 16). Interestingly, in *Hand1/eHAND* KI embryos, *Chisel* was also expressed in the inner curvature and AVC, suggesting that *Chisel* expression was dependent on *Hand1/eHAND* (Fig. 4J). In wild-type embryos, *ANF* was strongly expressed in the trabecular layer of the LV and weaker expression was observed in the atrium and the trabecular layer of the RV. *ANF* expression was not observed in the inner curvature, the AVC, or the IVG in wild-type embryos (Fig. 4K). In *Hand1/eHAND* KI embryos, *ANF* expression in the RV was up-regulated and the expression was also detected in the inner curvature but not in the AVC, indicating that *ANF* expression in the RV and the inner curvature was regulated by *Hand1/eHAND* (Fig. 4L).

We further examined expression of transcription factors known to play critical roles in cardiac development. While expression of *Nkx2.5* (Fig. 4G and H) and *MEF2C* (data not shown) was comparable, *Hand2/dHAND* expression in the RV was down-regulated in *Hand1/eHAND* KI embryos (Fig. 4E and F), suggesting that *Hand1/eHAND* may suppress *Hand2/dHAND* expression. What is the molecular mechanism for thin myocardium in *Hand1/eHAND* KI embryos? Inactivation of N-*myc* or *TEF-1* in mice resulted in thin myocardium (4, 6, 14), but these genes were normally expressed in *Hand1/eHAND* KI embryos (data not shown). Homozygous Splotch mutant mice that lack the transcription factor *Pax3* also showed thin myocardium. *p57*, a cyclin-dependent kinase inhibitor normally expressed in the trabecular layer, was also expressed in the compact zone layer in the mutant embryos, suggesting precocious cardiomyocyte differentiation in Splotch mutants (11). We thus investigated expression of *p57* in *Hand1/eHAND* KI embryos, but *p57* expression was detected only in the trabecular layer both in *Hand1/eHAND* KI and wild-type embryos (Fig. 4M and N).

Normal IVS formation in transgenic embryos overexpressing *Hand1/eHAND* in the RV. The defect in the IVS formation in *Hand1/eHAND* KI embryos may be due to nonspecific effects of *Hand1/eHAND* overexpression. To further examine the significance of the absence of *Hand1/eHAND* expression in the boundary region, we generated and analyzed transgenic em-

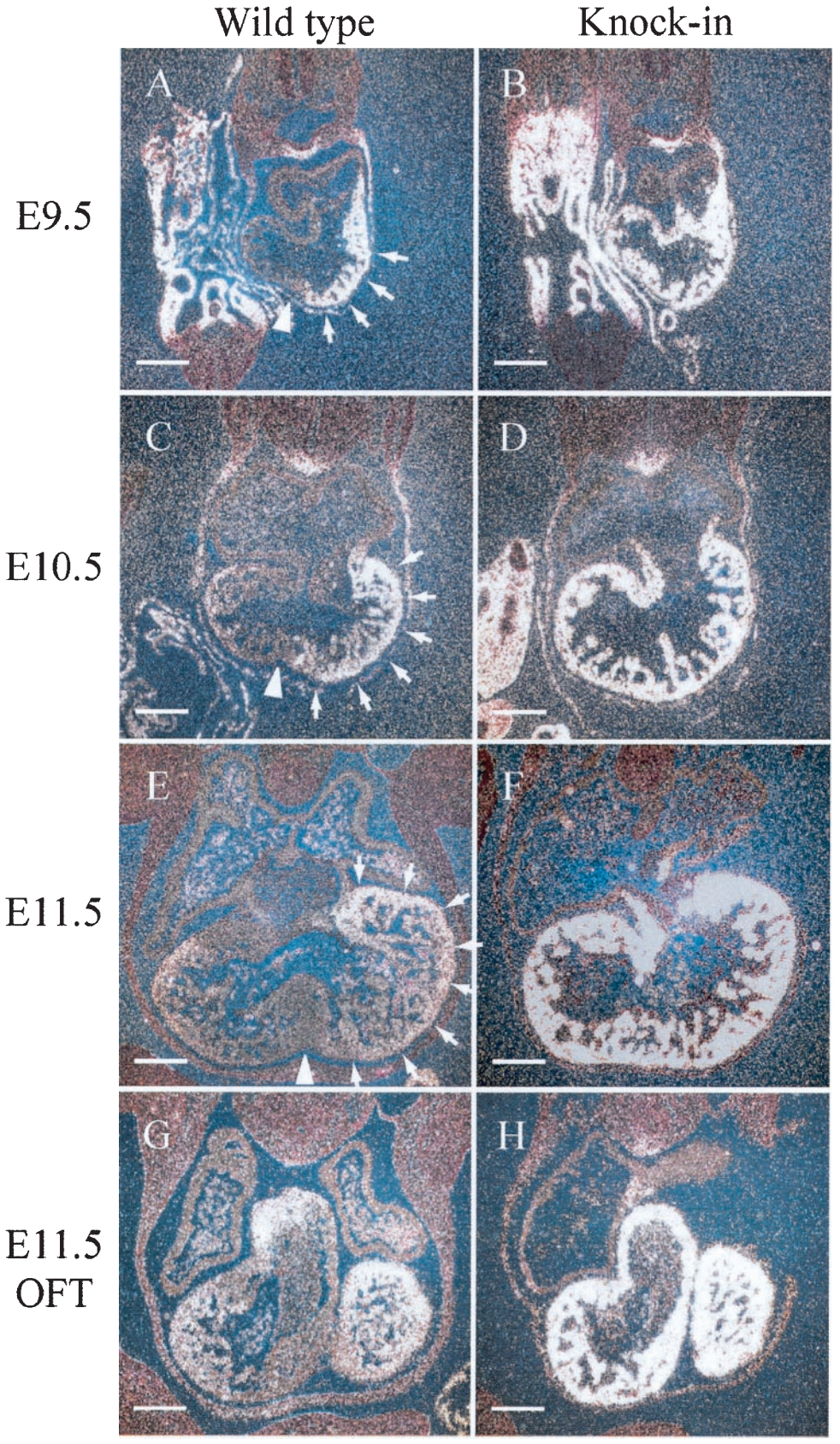

FIG. 3. Expression of *Hand1*/*eHAND* in wild-type and *Hand1*/*eHAND* KI embryos. In wild-type embryos, *Hand1*/*eHAND* was expressed in the outer curvature of the LV at E9.5 (arrows in panel A). Weak expression was observed in the outer curvature of the RV at E10.5 and E11.5 (C, E, and G). *Hand1*/*eHAND* expression was also detected in the distal part of the OFT (G). Note the absence of *Hand1*/*eHAND* expression in the IVG (arrowheads in panels A, C, and E). In *Hand1*/*eHAND* KI embryos, *Hand1*/*eHAND* was expressed in the whole ventricle, including the inner curvature, as well as in the AVC and the proximal part of the OFT (B, D, F, and H). In spite of the ectopic *Hand1*/*eHAND* expression, the inner curvature, AVC, or OFT did not expand outwards in *Hand1*/*eHAND* KI embryos (D, F, and H). Bars, 200 μm.

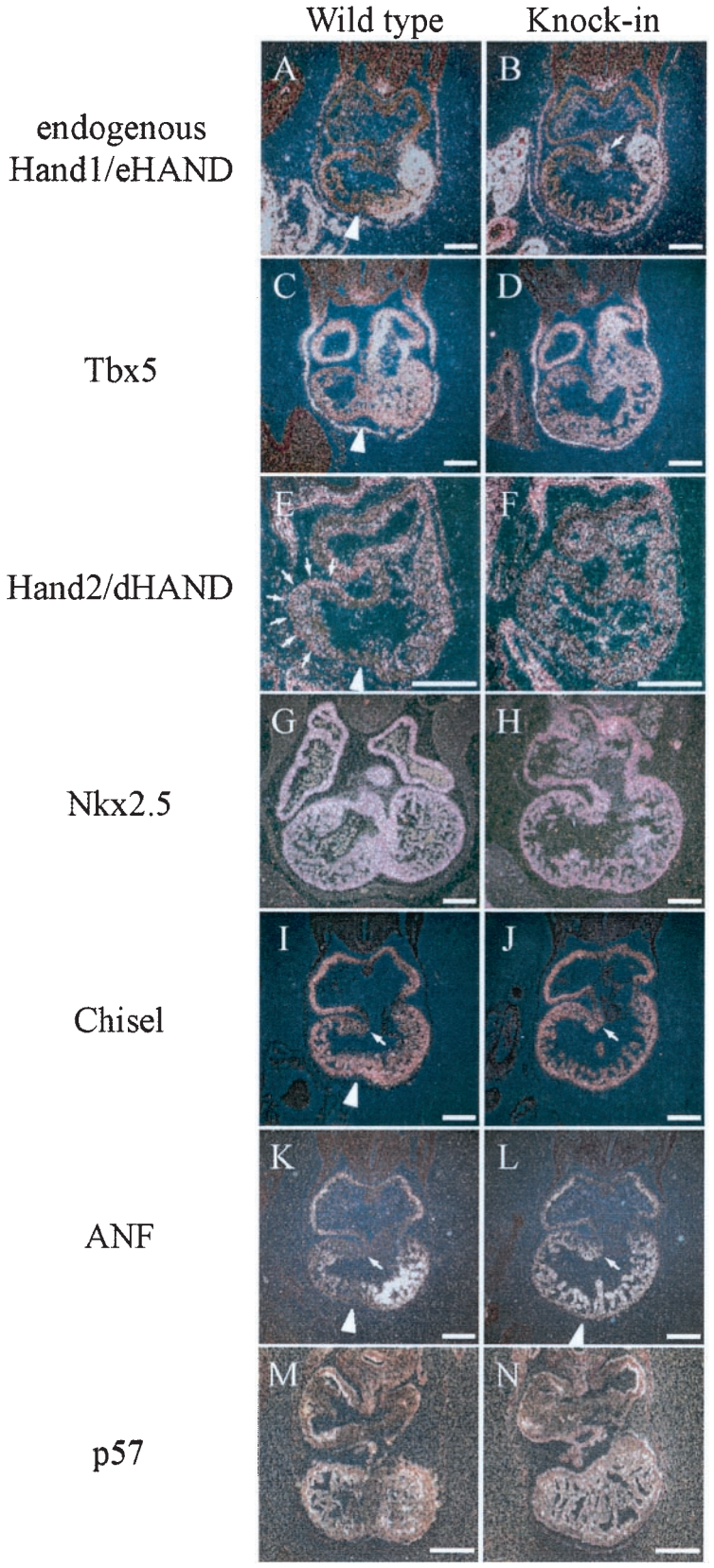

FIG. 4. Expression of cardiac transcription factors and molecular markers for the chamber myocardium. Expression of endogenous *Hand1/eHAND* (A and B), *Tbx5* (C and D), *Hand2/dHAND* (E and F), *Nkx2.5* (G and H), *Chisel* (I and J), *ANF* (K and L), and *p57* (M and N) are shown. Endogenous *Hand1/eHAND* expression was only detected in the left half of the ventricle in *Hand1/eHAND* KI embryos (B). The *Tbx5* expression

bryos overexpressing *Hand1/eHAND* in the RV by using the *MLC2V* promoter (Fig. 5A and B) (10, 18). As expected, the IVS formed normally in these transgenic embryos (Fig. 5D). Immunohistochemistry revealed FLAG-tagged Hand1/eHAND expression in the RV but not in the boundary region (Fig. 5C). These results indicated that the absence of *Hand1/eHAND* expression in the boundary region was critical for the proper formation of the IVS.

DISCUSSION

Septum formation is one of the critical steps in the transformation of a linear heart tube into a four-chambered heart. Morphologically, it has been pointed out that the boundary region between the LV and RV does not expand during the formation of the muscular IVS (Fig. 6A) (19). When the outer curvatures on each side of the narrow boundary region keep expanding, the two walls will eventually fuse, forming a septum (19). However, the molecular mechanism for expansion of the ventricular walls has never been elucidated. *Hand1/eHAND* KI embryos had a morphologically single ventricle, but there were distinctive LV and RV at the molecular level. Therefore, forced expression of *Hand1/eHAND* in the whole ventricle resulted in expansion of the entire ventricular wall including the boundary region (Fig. 6B). Although it is possible that overexpression of *Hand1/eHAND* may have affected the phenotype, the absence of *Hand1/eHAND* expression in the boundary region was critical in the development of the IVG and IVS (Fig. 6A) because transgenic embryos expressing *Hand1/eHAND* in the RV and LV, but not in the boundary region, exhibited normal formation of the IVS (Fig. 6C).

Ectopic expression of *Hand1/eHAND* in the entire RV resulted in more marked expansion of the outer curvature of the RV. Together with the result that *Hand1/eHAND* expression caused expansion of the boundary region, it is likely that *Hand1/eHAND* is involved in expansion of the ventricular walls. Then, which gene(s) regulate ballooning of the RV during normal cardiac development? Specific hypoplasia of the RV soon after cardiac looping in *Hand2/dHAND* knock-out embryos suggested a role of *Hand2/dHAND* in the expansion of the RV (21). Notably, *Hand2/dHAND* expression was also absent in the boundary region (Fig. 4E), thus suggesting a possibility that absence of *Hand1/eHAND* and *Hand2/dHAND* expression in the boundary region may be essential for the IVG and IVS formation (Fig. 6A).

Does *Hand1/eHAND* control the DV patterning of the embryonic heart? Interestingly, *Chisel* and *ANF*, molecular markers for the working myocardium (7), were ectopically expressed in the inner curvature and/or the AVC in *Hand1/eHAND* KI embryos. However, the inner curvature or the AVC did not expand morphologically. These results indicated that *Hand1/*

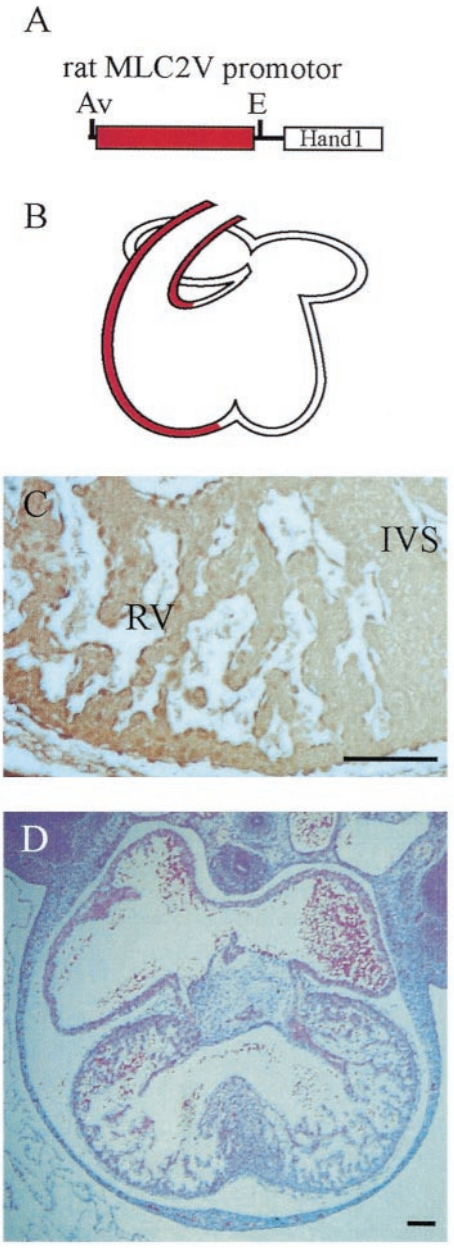

FIG. 5. *MLC2V-Hand1/eHAND* transgenic mice. (A) Schematic representation of the transgene. (B) The *MLC2V* promoter drives transgene expression in the RV and OFT but not in the boundary region. (C) Immunohistochemistry with an anti-FLAG antibody revealed FLAG-tagged Hand1/eHAND protein expression in the RV but not in the IVS. (D) In *MLC2V-Hand1/eHAND* transgenic embryos, the IVS formed normally. Av, AvaII; E, EcoRI. Bars, 100 μm.

gradient with higher expression in the LV was not disturbed in *Hand1/eHAND* KI embryos (D). Expression of *Hand2/dHAND* in the RV (E) was almost abolished in *Hand1/eHAND* KI embryos (F). Note the absence of *Hand2/dHAND* expression in the IVG in wild-type embryos (arrowhead in panel E). *Nkx2.5* expression was comparable between wild-type (G) and *Hand1/eHAND* KI (H) embryos. *Chisel* expression was also detected in the inner curvature and AVC in *Hand1/eHAND* KI embryos (J). Note the absence of *ANF* expression in the IVG (arrowhead in panel K) and inner curvature (arrow in panel K) in wild-type embryos (K). *ANF* expression was up-regulated in the RV and inner curvature in *Hand1/eHAND* KI embryos (L). *ANF* was also expressed at the region where the IVS was expected to form (arrowhead in panel L). Expression of *p57* was detected only in the trabecular layer both in wild-type and *Hand1/eHAND* KI embryos (M and N). Bars, 200 μm.

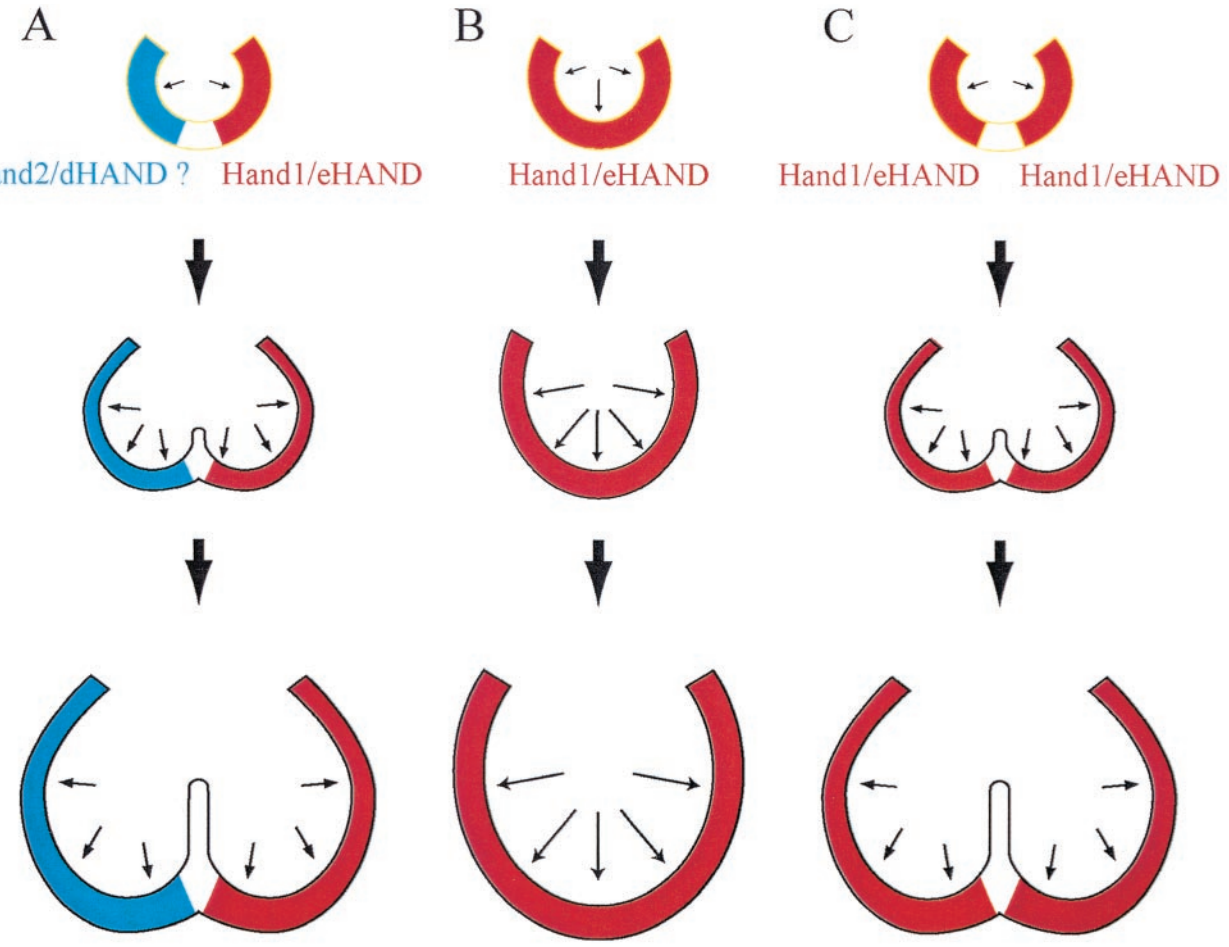

FIG. 6. Schematic presentation of ventricular expansion and IVS formation. (A) IVS formation in normal hearts. The outer curvatures of the LV and RV expand outwards. For the proper formation of the IVS, the boundary region should not expand. *Hand1/eHAND* and *Hand2/dHAND* may regulate expansion of the LV and RV, respectively. Note the absence of *Hand1/eHAND* and *Hand2/dHAND* expression in the boundary region. (B) In *Hand1/eHAND* KI hearts, the boundary region also expanded outward. As a result, the IVS did not form properly. (C) In *MLC2V-Hand1/eHAND* transgenic embryos, while the *Hand1/eHAND* transgene was expressed in the RV and endogenous *Hand1/eHAND* was expressed in the LV, *Hand1/eHAND* expression was absent in the boundary region. In these transgenic embryos, the boundary region did not expand outwards and the IVS formed normally.

eHAND regulated expression of molecular markers for the working myocardium but that additional gene(s) normally expressed in the outer curvature may be required for expansion of the chamber walls together with *Hand1/eHAND*.

The results of this study also gave insight into the hierarchical and combinatorial molecular cascade that controls cardiac development. Forced expression of *Hand1/eHAND* in the RV down-regulated *Hand2/dHAND* expression. It is possible that expression of *Hand2/dHAND* in the LV may be suppressed by high expression of *Hand1/eHAND* in the normal embryonic heart. However, *Hand1/eHAND* expression in the whole ventricles did not disturb the *Tbx5* expression gradient or endogenous *Hand1/eHAND* expression. Therefore, it is unlikely that *Hand1/eHAND* is the most upstream gene that specifies the LV myocyte lineage.

Between E10.5 and E11.5, cardiac myocytes undergo rapid cell division, resulting in doubling of cardiac mass (11). By E10.5, *Hand1/eHAND* KI embryos were indistinguishable from wild-type embryos except that they lacked the IVG and IVS. At E11.5, the compact zone layer of the *Hand1/eHAND* KI hearts were thin, suggesting that heart failure due to poor development of the compact zone layer may have caused the embryonic lethality. What is the mechanism for thin myocardium in *Hand1/eHAND* KI embryos? N-*myc*, *TEF-1*, and *p57* were normally expressed in *Hand1/eHAND* KI embryos, suggesting that there may be other mechanism(s). At E11.5, *Hand1/eHAND* expression was obviously down-regulated in wild-type embryos, while strong expression of *Hand1/eHAND* persisted in *Hand1/eHAND* KI embryos. Thus, down-regulation of *Hand1/eHAND* at the mid-stage of cardiac development may be important for the proper formation of the compact zone myocardium. Although it may seem inconsistent that *Hand1/eHAND* enhanced expansion of the ventricular chambers at E10.5 but that overexpression of *Hand1/eHAND* at E11.5 disturbed proliferation of the compact zone myocardium, fine-tuning of *Hand1/eHAND* expression at each stage may be required for the proper development of the embryonic heart. It is also possible that different mechanisms may exist to regulate expansion of the ventricular chambers and thickening of ventricular walls.

Recently, Takeuchi et al. reported that *Tbx5* may determine the position of the IVS in chicken and mouse embryonic hearts (22). When *Tbx5* was overexpressed in the whole ventricles, the *Hand1/eHAND* expression domain was expanded to the RV, resulting in a lack of IVS formation. Their study suggested that *Tbx5* may control *Hand1/eHAND* expression and that in the chicken heart, the boundary of the *Tbx5* and *Tbx20* expression domains may determine the position of the IVS (22). Together with the results of our study, it was likely that the function of *Tbx5* in the expansion of the ventricular walls and the IVS formation in murine hearts was mediated through eHAND. Moreover, since *Tbx20* is uniformly expressed in the LV and RV (12) and *Tbx5* is not expressed in the boundary region between the LV and RV in the normal murine hearts, the absence of *Tbx5* and *Hand1/eHAND* expression in the boundary region may be critical in the proper formation of the IVS in murine cardiac development.

In summary, expression of *Hand1/eHAND* enhanced expansion of chamber walls, and absence of *Hand1/eHAND* expression in the boundary region may be essential for the proper formation of the IVG and IVS. Moreover, additional factors normally expressed in the outer curvature may determine the DV patterning of the embryonic heart in concert with *Hand1/eHAND*.

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