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Hesr1 and Hesr2 regulate atrioventricular boundary formation in the developing heart through the repression of Tbx2

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The establishment of chamber specificity is an essential requirement for cardiac morphogenesis and function. *Hesr1* (*Hey1*) and *Hesr2* (*Hey2*) are specifically expressed in the atrium and ventricle, respectively, implicating these genes in chamber specification. In our current study, we show that the forced expression of *Hesr1* or *Hesr2* in the entire cardiac lineage of the mouse results in the reduction or loss of the atrioventricular (AV) canal. In the *Hesr1*-misexpressing heart, the boundaries of the AV canal are poorly defined, and the expression levels of specific markers of the AV myocardium, *Bmp2* and *Tbx2*, are either very weak or undetectable. More potent effects were observed in *Hesr2*-misexpressing embryos, in which the AV canal appears to be absent entirely. These data suggest that *Hesr1* and *Hesr2* may prevent cells from expressing the AV canal-specific genes that lead to the precise formation of the AV boundary. Our findings suggest that *Tbx2* expression might be directly suppressed by Hesr1 and Hesr2. Furthermore, we find that the expression of *Hesr1* and *Hesr2* is independent of Notch2 signaling. Taken together, our data demonstrate that *Hesr1* and *Hesr2* play crucial roles in AV boundary formation through the suppression of *Tbx2*.

KEY WORDS: Hesr1 (Hey1), Hesr2 (Hey2), Heart, Notch signaling

INTRODUCTION

The heart is the first functional organ to be established during embryogenesis. The formation of the atrial and ventricular chambers is one of the most important processes during the development of the complex morphology and physiology of the heart. These chambers become morphologically distinguishable after the looping of the cardiac tube at embryonic day (E) 9.5 in the developing mouse embryo. Atrial and ventricular cardiomyocytes, expressing distinct subsets of cardiac muscle genes, develop pectinated muscles in the atrium and trabeculae in the ventricle, which confer the contractile, electrophysiological and pharmacologic properties unique to each chamber. Separation of the cardiac tube into the atrium and ventricle is accomplished by cardiac cushions, which form as a regional swelling of the cardiac jelly (Eisenberg and Markwald, 1995). Delamination and migration of endothelial cells to these cushions occurs by an epithelial-to-mesenchymal transformation (EMT). As the cushions expand, anlages of the septal and valvular structures are formed to demarcate the developing chambers.

Several transcription factors have now been implicated in both atrial and ventricular chamber formation in the mouse. Knockout (KO) studies in mice for the *Nkx2-5*, *Tbx5*, *Mef2c*, *Hand2*, and *Tbx20* genes indicate that these factors may be involved in chamber specification. The continuous expression of these factors throughout the cardiac tube, however, has not resulted in the identification of the genes responsible for specification. In addition, although the expression of *Irx4* is restricted to the ventricular chamber (Yamagishi et al., 2001), *Irx4*-deficient mice are viable at adulthood, although they develop impaired contractile function (Bruneau et al., 2001). *Hand1* and *Cited1* are expressed in a ventricle-specific manner at an early stage of development (Carotta et al., 1998; Thomas et al., 1998), and *Hand1* has been suggested to be required for the proper formation of the outer curvature of the ventricle and interventricular septum (Togi et al., 2004), but not the atrioventricular (AV) boundary. Hence, little is known about the mechanisms that control the differentiation of the cardiogenic precursors and their acquisition of atrial and ventricular chamber-specific properties.

The myocardium of the AV canal (AV myocardium) is important for the development of the AV cushion and AV node. Bone morphogenetic protein 2 (*Bmp2*) is expressed in the AV myocardium and conditional KO studies of the *Bmp2* gene in the mouse cardiac lineage suggest that it is involved in cardiac cushion development, including the induction of EMT, the accumulation of cardiac jelly and the establishment of AV canal specificity (Zhang and Bradley, 1996; Ma et al., 2005). Tbx2, a T-box transcription factor, is also expressed in the AV myocardium. Tbx2 is induced by chicken Bmp2 beads implanted in the AV myocardium in chick, and shows reduced expression in *Bmp2*-null mice. These observations indicate that Bmp2 establishes the AV cushions via the induction of *Tbx2* (Yamada et al., 2000; Ma et al., 2005). *Tbx2*-null mice also show morphological defects in the AV canal and allow the expression of chamber differentiation markers in the AV myocardium, indicating that Tbx2 is required to repress chamber differentiation in the AV canal region (Harrelson et al., 2004). However, the mechanisms underlying the formation of the boundaries between the chambers and the AV canal remain unclear.

The *Hesr* genes (also known as *Hey*, *Hrt*, *Chf*, *Herp* or *Gridlock*) were identified through their similarities to the hairy and enhancer of split (*Hes*) family of genes (Kokubo et al., 2005a). Several previous studies have also now shown that the *Hesr* genes are putative direct targets of Notch signaling (Iso et al., 2003). Because the myocardial expression of *Hesr1* (*Hey1*) or *Hesr2* (*Hey2*) is

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restricted to the atrium and ventricle, respectively, it has been speculated that these genes might regulate atrial or ventricular specification. However, *Hesr1*-null mice show no obvious phenotype in their cardiac development and *Hesr2*-null mice display no anomalies during chamber specification, although several defects do occur in these animals, including dysplastic AV valves, a perimembranous ventricular septal defect, and a secundum atrial septal defect (Donovan et al., 2002; Gessler et al., 2002; Sakata et al., 2002; Kokubo et al., 2004). Even in *Hesr1-Hesr2* double-null mice, the atria and ventricles seem to be properly formed, although they do show some defects during cardiac development, including few EMT cells in the AV cushions and abnormal apoptosis in the trabecular layer of the ventricle.

To further clarify the function of Hesr1 and Hesr2 during cardiac development, we have established a system whereby *Hesr1* and *Hesr2* are constitutively expressed in the cardiac lineage in mouse. This enables us to analyze the functions of Hesr1 and Hesr2 during chamber specification. Marker analyses reveal a significant reduction in and loss of the AV canal in the *Hesr1*- and *Hesr2*-misexpressing (ME) hearts, respectively. Furthermore, by analysis of the respective KO animals, as well as of the corresponding ME hearts, we find that Hesr1 and Hesr2 can directly repress the expression of *Tbx2*. These observations indicate that Hesr1 and Hesr2 play essential roles during AV boundary formation through the repression of *Tbx2*.

MATERIALS AND METHODS

Generation of transgenic mouse lines

The transgene vectors, CAG-lox-*CAT*-lox-*Hesr1*, *-Hesr2*, *-Tbx2* and *-Notch2-ICD* were constructed by inserting *Hesr1*, *Hesr2*, *Tbx2* or *Notch2-ICD* cDNAs, respectively, into the CAG-*CAT*-(cDNA insert)-polyA cassette (Sakai and Miyazaki, 1997). The *lacZ* expression vectors, *Tbx2-D3-nlacZ* and *Tbx2-Xho-nlacZ*, were constructed by insertion of the 6083 bp (*Hin*dIII-*Nco*I) or 2712 bp (*Xho*I-*Nco*I) upstream region of the *Tbx2* gene, derived from the BAC clone RP23-48A17, into a *lacZ* cassette (Kokubo et al., 2005b). These constructs were injected into fertilized eggs to generate permanent transgenic lines by standard methods. Each transgenic line was then crossed with a *Mesp1-Cre* mouse line (Saga et al., 1999) to obtain embryos expressing the inserted cDNAs in the cardiac lineage. We refer to such gene-misexpressing mice as ME mice.

Histological analysis

Histological observations, Hematoxylin and Eosin staining, and transmission electron microscopic analyses were carried out as described previously (Miyagawa-Tomita et al., 1996). The InsituPro system (M&S Instruments) was used for whole-mount in situ hybridizations according to the manufacturer's instructions. Section in situ hybridizations were performed using 20 μm frozen sections. Immunohistochemistry was performed using anti-myosin (Skeletal, Slow; Sigma) and anti-α-smooth muscle actin (IA4; Sigma) antibodies with 6 μm paraffin sections using standard protocols.

Quantification of relative expression domains

The ratio of the *Bmp2*-expressing to non-expressing area in the heart was determined from a lateral side view using the WinRoof program (Mitani Corp, Japan) (n=4).

RT-PCR analysis

Total RNA was extracted from mouse hearts (n=10) using a mini-extraction kit (Qiagen). Real time quantitative PCR (RT-PCR) was then performed using the ExTaq-RPCR kit (Takara) with the MiniOpticon RT-PCR system (Bio-Rad). The primers and PCR conditions for the genes under study have been described previously (Watanabe et al., 2006).

Luciferase assay

For luciferase reporter analysis of the 6 kb (*Hin*dIII-*Nco*I) and 2.7 kb (*Xho*I-*Nco*I) *Tbx2* upstream enhancer regions, reporter constructs (200 ng) were individually transfected with or without expression vectors for the constitutively active or kinase-dead forms of Alk3 (50 ng), 3×Flag-Smad5 (20 ng), 6×Myc-tagged Hesr1 (10-50 ng) or 6×Myc-tagged Hesr2 (10-50 ng). Transfections of NIH3T3 cells (0.25×10^5 cells per well in 24-well plates) were then performed using Lipofectamine Plus (Invitrogen), according to the manufacturer's instructions. The vector containing the Renilla luciferase gene under the control of the thymidine kinase promoter (10 ng) was used as an internal standard to normalize for transfection efficiency. After 36 hours of further culturing, cell lysates were prepared and the luciferase activities measured using the Dual Luciferase Assay Kit (Promega).

RESULTS

The atrial myocardial-specific and ventricular myocardial-specific expression of *Hesr1* and *Hesr2*

It is known that the *Hesr1* and *Hesr2* genes are specifically expressed in the atrial and ventricular chamber, respectively, but their exact expression boundaries within the atrium, AV canal and ventricle have not yet been identified. Hence, we first ascertained these expression boundaries by in situ hybridization analyses. Using *Hesr1* and *Hesr2* probes either individually or in combination at E9.5, we confirmed that the expression of the *Hesr1* and *Hesr2* genes is specific to the myocardium of the atrium and the ventricle, respectively, but is not detectable in the AV myocardium (Fig. 1A-F).

Expansion of the AV canal in *Hesr1* and *Hesr2* individual- and double-KO hearts

Given that the members of the Hesr family function as transcriptional repressors, we hypothesized that both Hesr1 and Hesr2 would suppress genes that are essential for AV canal formation. Because *Bmp2* is specifically expressed in the AV myocardium in wild-type embryos and its conditional KO results in the reduced formation of the AV canal (Ma et al., 2005), we speculated that Hesr1 and Hesr2 might function in the formation of the AV boundaries by repressing *Bmp2*. If this proved to be the case, we predicted that in the absence of Hesr1 or Hesr2, *Bmp2* expression would be expanded to the atrium or ventricle. In our *Hesr1*-null embryos, the expression of *Bmp2* was specifically detected in the AV canal, and was expanded compared with the wild-type heart (Fig. 1, compare H with G). The *Bmp2* expression area representing the AV canal was also increased by approximately 11%, judged from a lateral side view (Fig. 1K). This finding was supported by the expression pattern of *Anf* (*Nppa* – Mouse Genome Informatics), which is a chamber-specific marker (Fig. 1, compare M with L). Similarly, an expanded AV canal showing *Bmp2* and *Anf* expression was observed in both *Hesr2*-null and *Hesr1-Hesr2* double-null embryos (Fig. 1, compare I,J,N,O with G,L). However, this expansion showed no tendency towards the atrium or ventricle in either the *Hesr1*- or *Hesr2*-null heart.

We also examined whether the expression of *Tbx2*, which is a known downstream target of Bmp2, is affected in these KO mutants. *Tbx2* was specifically detected in the AV myocardium in wild-type embryos, but was found to be slightly expanded to the atrium of the *Hesr1* KO hearts (arrowhead in Fig. 1Q). *Tbx2* is weakly expressed but was found to be extended to the ventricle in the *Hesr2* KO hearts (arrowhead in Fig. 1R). In addition, ambiguous boarders for the AV canal were observed in the double-KO heart (arrowheads in Fig. 1S). These observations suggest that Hesr1 and Hesr2 are involved in AV canal specification, although no definitive conclusions could be drawn in this regard from these loss-of-function studies.

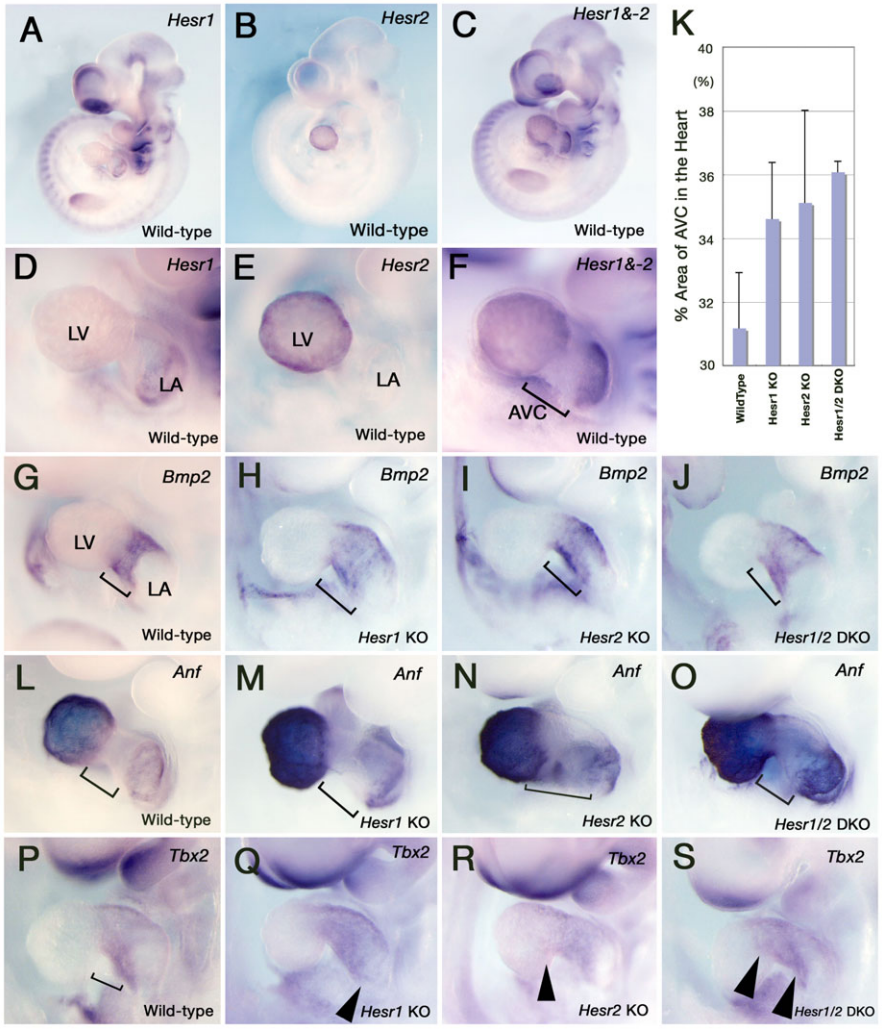

Fig. 1. The possible involvement of Hesr1 and Hesr2 in AV boundary formation. (**A-F**) Expression patterns of *Hesr1* and *Hesr2* in wild-type mouse embryos at E9.5 revealed by whole-mount in situ hybridization. The specific expression of *Hesr1* in the atria (A,D) and of *Hesr2* in the ventricles (B,E) can be seen, but neither gene is found to be expressed in the AV canal (AVC) (C,F). The heart regions in A-C are magnified in D-F, respectively. (**G-J**,**L-S**) The expression patterns of AV boundary markers in wild-type (G,L,P), *Hesr1*-null (H,M,Q), *Hesr2*-null (I,N,R) and *Hesr1-Hesr2* double-KO (J,O,S) mouse embryonic hearts at E9.5. To demarcate the boundaries between both the atrium and ventricular chambers and the AV canal, the AV myocardial markers *Bmp2* (G-J) and *Tbx2* (P-S), and the chamber marker *Anf* (L-O), were used as probes. (**K**) The ratio of the AV canal area was calculated by determining the *Bmp2* expression area in comparison with the entire heart area from a lateral side view (n=4, P<0.005, t-test). The brackets in G-J and L-P indicate the AV canal. The arrowheads in Q-S indicate the affected AV boundaries. The gene probes used (upper right) and genotypes examined (lower right) are indicated in each panel. LA, left atrium; LV, left ventricle.

Generation of mice that express *Hesr1* and *Hesr2* in the entire cardiac lineage

As an alternative method of revealing the possible functions of Hesr1 and Hesr2 during regional specification and/or AV boundary formation, we attempted the forced expression of these genes throughout the entire mouse heart. We created the CAG-lox-*CAT*-lox-*Hesr1* and -*Hesr2* transgenic lines, which begin expressing these genes under the control of the CAG promoter upon excision of the *CAT* gene by Cre recombinase. For this purpose, we crossed these transgenic lines with an *Mesp1*-Cre knock-in mouse line, which shows transient expression of *Cre* in cardiovascular precursor cells at the cylinder stage (E6.5). Using this transgenic strategy, we successfully obtained embryos showing the expression of *Hesr1* (Fig. 2A) or *Hesr2* (Fig. 2B) throughout the cardiac lineage. Section in situ analysis confirmed the expression of these genes in the myocardium of the entire heart, although this was not observed clearly in the endocardial cells (see Fig. S1 in the supplementary material). We used RT-PCR to measure the expression levels of the *Hesr1* and *Hesr2* genes using RNA prepared from pooled hearts (n=10) generated from two independent transgenic lines for each gene. We found that the expression levels were elevated 2.5- to 5.3-fold relative to wild type (Fig. 2C). As the gross morphological phenotypes were similar in the two lines for each gene, we utilized *Hesr1*-ME-Line#2 and *Hesr2*-ME-Line#2 for further analysis.

We initially examined whether the ectopic expression of either *Hesr1* or *Hesr2* would influence the endogenous expression pattern of each other. However, the respective ventricular- and atrial-specific expression patterns of *Hesr2* and *Hesr1* were observed to be normal in the respective ME embryos (Fig. 2D,E). These observations suggest that the mechanisms regulating *Hesr1* and *Hesr2* are not interdependent, and that these genes do not alter the atrial or ventricular chamber identities.

Until stage E9.5, both *Hesr1*- and *Hesr2*-ME embryos appear to develop normally. However, the *Hesr1*-ME embryos die by E11.5, showing heart malformations. *Hesr2*-ME embryos die at around E10, harboring not only cardiac defects but also vascular defects (data not shown). Histological examination of wild-type embryos at E9.5 shows that the atrium, the AV canal, and the ventricle of the heart are well developed, and that the endocardium of the inferior- and superior-AV cushions are attached to each other and begin undergoing EMT (Fig. 2F). In the *Hesr1*-ME embryo, however, the atrium and the ventricle appear to be normal but the width of the AV canal, recognized by the characteristic constriction between the atrium and the ventricle, is found to be small (bracket in Fig. 2G). Despite the small width of the AV canal in the transgenic embryo, the endocardial cushion tissue is still formed, and mesenchymal cells that have undergone EMT are detectable. Strikingly, in the *Hesr2*-ME embryos, AV canal constriction is rarely observed and the atrium and ventricle seemed to be directly connected (arrowhead in

Fig. 2H). In this region, however, neither the accumulation of cardiac jelly nor EMT were observed, indicating that the myocardium of the AV canal is not formed.

It is also noteworthy that the trabeculation of the ventricle is not well developed in the *Hesr2*-ME embryo (Fig. 2H). We therefore performed TEM analysis of myocardium in the *Hesr1*- and *Hesr2*-ME embryos at E9.5. At this stage, in the wild-type embryo the myocardial cells develop thick myofibrils with clear sarcomere structures, in which the Z bands were clearly visible (Fig. 2I). In both *Hesr1*- and *Hesr2*-ME myocardial cells, however, only disorganized thin myofibrils develop (Fig. 2J,K), in which the Z bands were often barely detectable, indicating immature myocardial differentiation in both transgenic mouse types. We next analyzed the expression of early markers for myocardial differentiation – myosin light chain (*Mlc2v*; *Myl2* – Mouse Genome Informatics), *Mlc2a* (*Myl7* – Mouse Genome Informatics), α-smooth muscle actin, and myosin heavy chain – and *Bmp10* for ventricular trabeculation. None of these markers were found to be altered at the E9.5 stage in the *Hesr1*- or *Hesr2*-ME hearts (data not shown), indicating that early myocardial differentiation and trabeculation occur normally in these ME embryos.

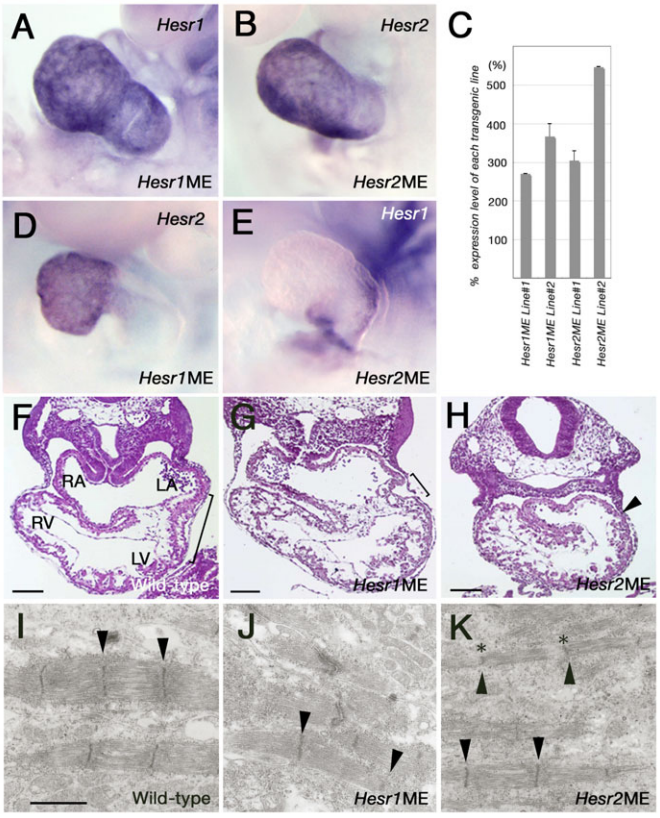

Fig. 2. Misexpression of *Hesr1* and *Hesr2* in the entire embryonic mouse heart at E9.5. (**A-E**) The expression of *Hesr1* (A,E) and *Hesr2* (B,D) was examined by whole-mount in situ hybridization of double-transgenic mouse embryos. The embryos were produced by crossings between *Mesp1-Cre* and either CAG-lox-*CAT*-lox-*Hesr1* (A,D) or CAG-lox-*CAT*-lox-*Hesr2* (B,E) mice. (C) Expression levels of *Hesr1* and *Hesr2* in two independent lines for each gene derived from these misexpressing mouse embryonic hearts (n=10). Both *Hesr1* and *Hesr2* are strongly induced (2.5-5.5 fold) compared with the wild-type heart. (**F-H**) Sections stained with Hematoxylin and Eosin showing a normal morphology for the atrium, AV canal (bracket), and the ventricle in the wild-type embryonic heart (F). The width of the AV canal (bracket) is smaller in the *Hesr1*-ME heart (G). The atrium and ventricle seem to be directly connected (arrowhead) in the *Hesr2*-ME heart (H). (**I-K**) Analysis of cardiomyocytes in the left ventricles of the mouse embryonic heart by transmission electron microscopy. Myofibrils were observed to be thin and disorganized in the *Hesr1*-ME (J) and *Hesr2*-ME (K) hearts, compared with wild type (I). In the *Hesr2*-ME hearts, Z bands (arrowhead) were sometimes barely discernible (asterisks). Scale bars: 100 μm in F-H; 1 μm in I-K. RA, right atrium; RV, right ventricle; LA, left atrium; LV, left ventricle.

The boundaries of the AV canal do not properly form in either the *Hesr1*- or *Hesr2*-misexpressing heart

We next investigated whether the atrium, ventricle or AV canal were properly formed in the *Hesr1*- or *Hesr2*-ME mouse heart. First, we examined the expression of the ventricle-specific transcription factors, *Hand1* (Fig. 3A-C) and *Cited1* (see Fig. S2A-C in the supplementary material). Both genes were found to be specifically expressed in the left ventricle of the *Hesr1*-ME, *Hesr2*-ME, and wild-type heart. However, we note that the expression of *Cited1* in the *Hesr1*-ME embryo appeared to have expanded weakly to the AV canal [see Fig. S2B (arrow) in the supplementary material], and that the expression of *Hand1* was downregulated in the *Hesr2*-ME embryo (Fig. 3C). *Irx4*, which encodes a homeobox-containing transcription factor, was also found to be expressed in the ventricles of the *Hesr1*- and *Hesr2*-ME embryos, as in wild type (Fig. 3D-F). However, whereas the expression of this factor in the AV canal was detectable in the wild-type embryo, it was barely evident in either the *Hesr1*- or *Hesr2*-ME embryos.

A similar phenomenon was also observed for the chamber-specific genes, connexin 40 (*Cx40*; *Gja5* – Mouse Genome Informatics) (Fig. 3G-I), *Anf* (see Fig. S2D-F in the supplementary material) and *Chisel* (*Smpx* – Mouse Genome Informatics) (see Fig. S2G-I in the supplementary material), which are normally expressed in both the atrium and ventricle at this stage in the wild-type embryo (Fig. 3G, and see Fig. S2D,G in the supplementary material). In the *Hesr1*-ME heart, a normal expression pattern for these genes was observed in both the atrium and ventricle, but the expression borders along the AV canal become ambiguous (arrowheads in Fig. 3H, and see Fig. S2E,H in the supplementary material). Strikingly, in the *Hesr2*-ME heart, the expression of each of these genes was found to be continuous from the atrium to the ventricle (arrowheads in Fig. 3I, and see Fig. S2F,I in the supplementary material). These observations suggest that the boundaries between the AV canal and the chambers are not clearly established in the *Hesr1*-ME heart, and that the AV canal does not form in the *Hesr2*-ME heart.

Bmp2 and *Tbx2* are downregulated in both the *Hesr1*- and *Hesr2*-misexpressing hearts

To next investigate the formation of the AV canal in greater detail in the *Hesr1*- and *Hesr2*-ME hearts, we examined the expression of the AV myocardial factors *Bmp2* and *Tbx2* (Fig. 4A-F). In *Hesr1*-ME embryos, *Bmp2* is weakly expressed (Fig. 4B) and *Tbx2* is strongly downregulated (Fig. 4E), suggesting that the suppression of *Tbx2* is one of main causes of the AV cushion phenotype in these embryos. Interestingly, the *Hesr2*-ME embryos showed loss of both *Bmp2* and *Tbx2* expression in the AV canal (Fig. 4C,F), indicating that the repression of not only *Tbx2* but also *Bmp2* may lead to loss of the AV canal in these embryos.

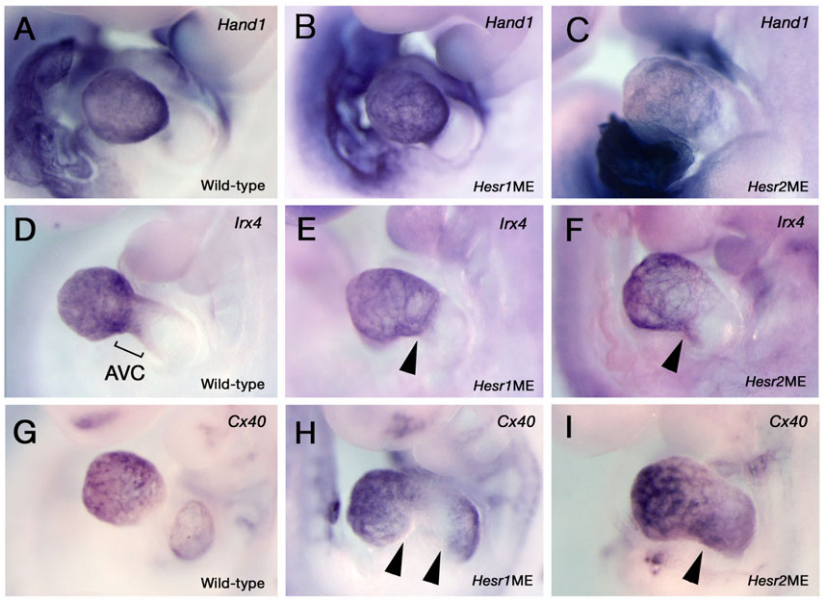

Fig. 3. Defects in AV boundary formation in both the *Hesr1*- and *Hesr2*-misexpressing heart. Chamber identities were examined by whole-mount in situ hybridization of E9.5 embryos. *Hand1* (**A-C**) and *Irx4* (**D-F**) were used as markers for the ventricular chamber. *Cx40* (**G-I**) was used to demarcate both the atrial and ventricular chambers. Boundaries between the chambers and AV canal are less evident in the *Hesr1*-ME heart (B,E,H) and appear to be lost in the *Hesr2*-ME heart (C,F,I), as compared with the wild-type embryo (A,D,G). The bracket in D indicates the AV canal (AVC) and the arrowheads in E,F,H,I indicate the AV boundaries for the atrium and/or ventricle.

Hesr1 and Hesr2 may directly repress *Tbx2* expression

Because our misexpression studies strongly suggest that *Tbx2* expression is suppressed by both Hesr1 and Hesr2, we performed experiments to determine the location of a *Tbx2* enhancer likely to be involved in this mechanism. We created two kinds of LacZ-reporter transgenic line, containing either 2.7 kb (*Tbx2-Xho-nlacZ*) or 6 kb (*Tbx2-D3-nlacZ*) of the region upstream of the *Tbx2* translational start site (Fig. 4O). Two independent *Tbx2-Xho-nlacZ* lines were found to show no β-gal expression in the AV canal (Fig. 4G,H). By contrast, two independent *Tbx2-D3-nlacZ* lines showed positive expression in not only the AV canal, but also in the outflow tract and eyes (Fig. 4I,J). Moreover, this latter expression pattern is similar to that of the endogenous *Tbx2* gene. We next examined the expression of a *Tbx2* transgene in both the *Hesr1*- and *Hesr2*-ME background by generating triple transgenic mice (*Tbx2-D3-nlacZ*/*Mesp1-Cre*/CAG-*CAT-Hesr1* or -*Hesr2*). As expected, the β-galactosidase expression pattern was greatly reduced in the AV canal, but not in the eyes, of both *Hesr1*- and *Hesr2*-ME embryos (Fig. 4K-N), indicating that the cis-regulatory elements that are required for the repression of *Tbx2* by Hesr1 and/or Hesr2 exist in the *Hin*dIII-*Xho*I upstream region of this gene. However, we could not exclude the possibility that the downregulation of *Tbx2* is a consequence of the suppression of *Bmp2*, particularly in the *Hesr2*-ME embryos. To address this possibility, we established a luciferase reporter assay system using NIH3T3 cells and a reporter construct containing the 6 kb upstream region of *Tbx2* (*Tbx2-D3-Luc*). This reporter showed little response to a constitutively active form of Alk3 (Bmpr1a – Mouse Genome Informatics), a receptor of Bmp2, but exhibited upregulation upon the addition of Smad5, which is suppressed by both Hesr1 and Hesr2 (Fig. 4P). These data suggest that *Tbx2* transcription, which is upregulated by Bmp signaling, is suppressed by both Hesr1 and Hesr2. However, it has been suggested that several putative Smad-binding sites, but not Hesr-binding sites, are localized within a ~400 bp stretch of the *Hin*dIII-*Xho*I upstream region, and this possibly serves as the enhancer of *Tbx2* that drives AV canal restricted expression (M. Shirai, personal communication). This observation suggests that the *Hesr* genes repress *Tbx2* expression through mechanisms that are independent of DNA binding.

The expression of *Hesr1* and *Hesr2* is unaffected by Tbx2

It was recently reported that Tbx2 functions as a repressor of chamber-specific gene expression (Harrelson et al., 2004). We thus examined whether Tbx2 would downregulate the expression of *Hesr1* and/or *Hesr2* in the AV myocardium, and thereby form a negative-feedback system that would establish the precise boundaries for the atrium and ventricle in mice. To address this possibility, we established a *Tbx2*-misexpression transgenic mouse line. However, our observations revealed a normal expression pattern for *Hesr1* and *Hesr2* in the *Tbx2*-ME heart (Fig. 5A-C), suggesting that *Hesr1* and *Hesr2* are not repressed by Tbx2.

The myocardial expression of *Hesr1* and *Hesr2* is not regulated by Notch2

Hesr1 and *Hesr2* are thought to be direct downstream targets of Notch signaling in culture systems (Iso et al., 2003). However, the expression of Notch1 and Notch4 is restricted to the endocardium and does not occur in the myocardium, suggesting that the myocardial expression of *Hesr1* and *Hesr2* is unlikely to be regulated by either of these Notch factors. However, as Notch2 is expressed in the myocardium of the heart we examined whether it might be an upstream regulator of *Hesr1* and/or *Hesr2*. A normal expression pattern for *Hesr1* and *Hesr2* was found specifically in the atrium and ventricle of the *Notch2*-KO mouse (Fig. 5D,E). We also generated transgenic lines for the misexpression of *Notch2* activated form, which express only the intracellular domain (IDC) of *Notch2* (Fig. 5F,I), using a similar strategy to that mentioned above. However, both the atrial- and ventricular-specific expression patterns of *Hesr1* or *Hesr2* were unaffected in the *Notch2-ICD*-ME hearts (Fig. 5G,H). These data suggest that the expression of *Hesr1* and *Hesr2* is regulated in the myocardium through a Notch2-independent pathway.

DISCUSSION

In the present study, we investigated the function of Hesr1 and Hesr2 during cardiac development by their forced expression in the entire cardiac lineage of the mouse embryo. Our findings provide strong genetic evidence that the *Hesr1* and *Hesr2* genes play crucial roles during both AV boundary formation and myocardial differentiation.

We furthermore find that either a reduction in or loss of the AV canal is accompanied by the suppression of the AV myocardial-specific expression of *Tbx2*, or of both *Bmp2* and *Tbx2*, in the *Hesr1*- and *Hesr2*-ME hearts, respectively. In addition, our finding of the expansion of the AV canal in individual- as well as double-KO mice suggests that Hesr1 and Hesr2 are required to maintain the identity of each chamber by preventing differentiation into the AV myocardium. Our data reveal that myofibrils are not well developed in the *Hesr1*- and *Hesr2*-ME heart. Taken together, our results suggest that Hesr1 and Hesr2 may function coordinately to establish the boundary between the atrium, ventricle and AV canal.

Hesr1 and Hesr2 function in AV boundary formation but not in chamber specification

As *Hesr1* and *Hesr2* are specifically expressed in the atrium or ventricle of the forming heart, we expected that these proteins might be involved in the specification of these structures. However, upon ectopic expression of *Hesr1* or *Hesr2* in the entire mouse embryonic heart, the atrium and ventricle seem to develop normally, indicating that Hesr1 and Hesr2 do not function in the determination of chamber identities. We do provide evidence, however, that Hesr1 and Hesr2 have repressive effects upon AV cushion formation and our current working model of this is summarized in Fig. 6. The Bmp2 signaling pathway is thought to induce EMT, enhance cardiac jelly accumulation in the AV cushion, and prevent the chamber-specific differentiation in the AV myocardium via the upregulation of Tbx2 (Harrelson et al., 2004). In *Hesr1*- or *Hesr2*-KO mice, and in the *Hesr1-Hesr2* double-KO mice, an expanded AV canal was observed as an extended expression region of *Bmp2*, although this expansion did not have any bias to either the atrial or ventricular chamber. These data suggest that neither Hesr1 nor Hesr2 is the direct repressive regulator of *Bmp2*. However, *Tbx2* expression was found to be expanded to the atrial side in the *Hesr1*-null embryos and to the ventricular side in the *Hesr2*-null embryos (Fig. 1Q,R).

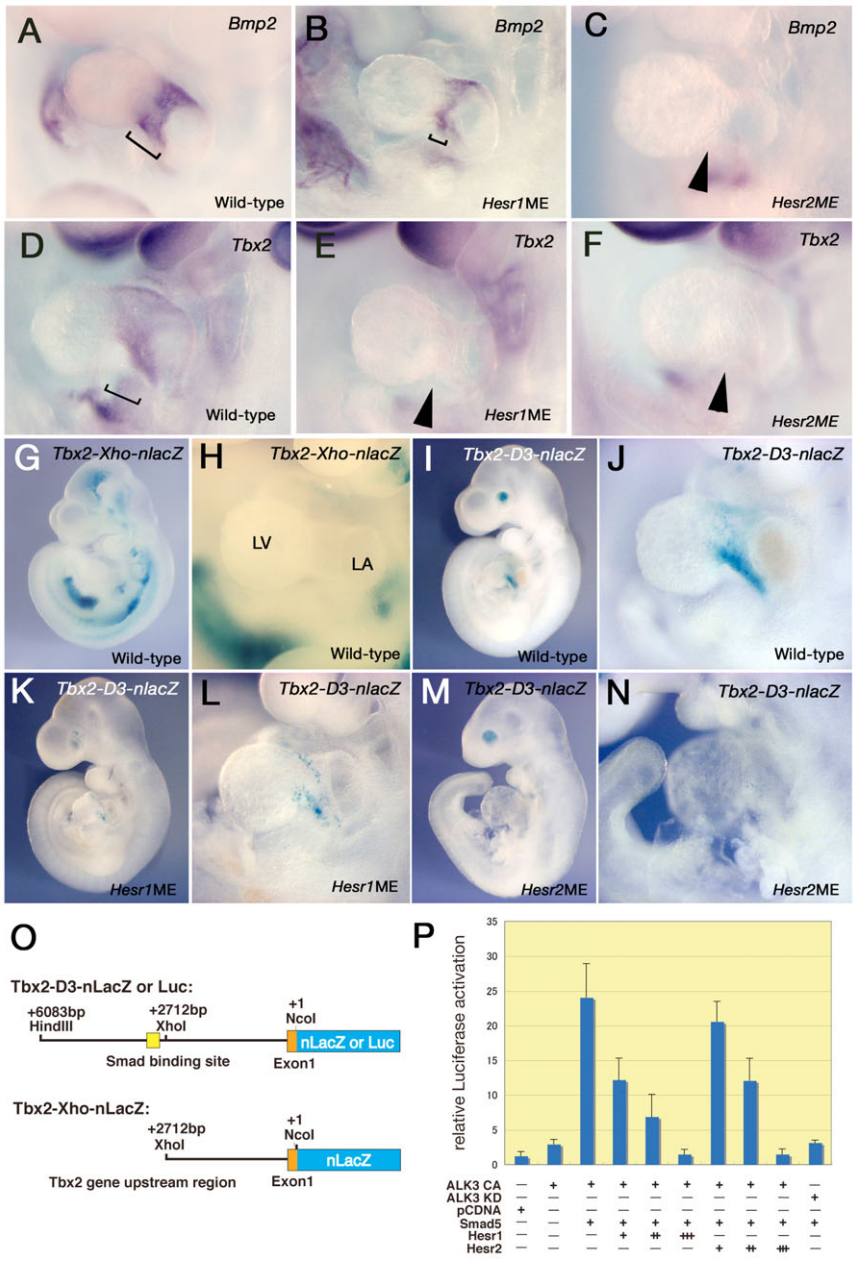

Fig. 4. The gene expression pattern underlying the regulation of AV canal formation and the possible regulation of *Tbx2* by both Hesr1 and Hesr2. (**A-F**) Expression of the AV myocardium-specific markers, *Bmp2* (A-C) and *Tbx2* (D-F), were examined in wild-type (A,D), *Hesr1*-ME (B,E) and *Hesr2*-ME (C,F) mouse embryos (E9.5) by in situ hybridization. The reduction or lack of an AV canal was evident in these misexpressing hearts (B,C,E,F). (**G-N**) X-Gal staining of transgenic embryos containing either a 2.5 kb (*Tbx2-Xho-nlacZ*) (G,H) or 6 kb (*Tbx2-D3-nlacZ*) (I-N) upstream region of *Tbx2* in wild-type (G-J), *Hesr1*-ME (K,L) or *Hesr2*-ME (M,N) mouse hearts. The heart regions shown in G,I,K,M are magnified in H,J,L,N, respectively. The brackets in A,B,D indicate the AV canal and the arrowheads in C,E,F indicate the AV boundaries. LA, left atrium; LV, left ventricle. (**O**) Schematic of the luciferase or LacZ reporter constructs harboring the *Tbx2* upstream regions. A region containing Smad-binding sites is present ~2.9 kb upstream from the first ATG of the *Tbx2* gene. (**P**) Reporter assay using the Tbx2-D3 luciferase construct (shown in P) in NIH3T3 cells. Luciferase activity was assessed with or without the empty vector (pCDNA), 6×Myc-Hesr1 or 6×Myc-Hesr2, in the presence or absence of the constitutive active (CA) or kinase-dead (KD) form of Alk3 and Smad5.

We also observed repressive effects of both Hesr1 and Hesr2 in a luciferase reporter assay using upstream *Tbx2* enhancer sequences. We thus speculate that the Bmp proteins secreted by the AV myocardium induce *Tbx2* expression in this structure and also in the surrounding cells, if the *Hesr* genes are not expressed. Hesr1 or Hesr2 must therefore suppress *Tbx2* expression in the atrium or ventricle to define the boundaries between the atrium and the AV canal (by Hesr1), and ventricle and AV canal (by Hesr2), during normal development. However, the repression of *Tbx2* is not the only mechanism responsible for the lack of an AV canal because the AV canal is present in the *Tbx2*-null heart (Harrelson et al., 2004).

Similarly, the heart-specific knockout of *Bmp2* results in an abnormal AV canal constriction morphology, but the AV canal itself is not completely lost (Ma et al., 2005). The reason for the complete lack of the AV canal in the *Hesr2*-ME heart is currently unknown, yet unidentified upstream regulator(s) for AV canal formation must be regulated by Hesr2. In this regard, the function of Tbx20, which is expressed throughout the entire heart, should be considered. Recently, Tbx20 was reported to be essential for chamber differentiation, as the loss of this gene results in the downregulation of chamber-specific markers, including *Hesr1* and *Hesr2*, whereas the expression of the AV canal marker *Tbx2* was found to be expanded (Cai et al., 2005; Singh et al., 2005; Stennard et al., 2005). Hence, Tbx20 is one of the positive upstream regulators of *Hesr1* and *Hesr2*, although it is not known how *Hesr1* and *Hesr2* are restricted in the atrium or ventricle. There cannot be feedback regulation of *Tbx20* by Hesr1 and Hesr2 as we did not observe any notable changes in its expression pattern in the *Hesr1*- and *Hesr2*-ME hearts (see Fig. S2J-L in the supplementary material).

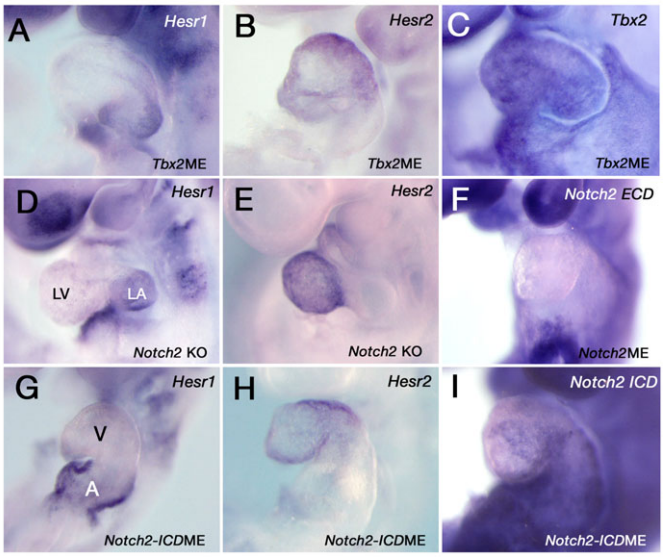

Fig. 5. The expression of *Hesr1* and *Hesr2* is unaffected in *Tbx2*-misexpressed, *Notch2*-KO or *Notch2*-activated hearts. *Hesr1* and *Hesr2* expression was examined in *Tbx2*-ME (**A**,**B**), *Notch2*-KO (**D**,**E**) and *Notch2-ICD*-ME (**G**,**H**) embryos (E9.0). (A,B) The expected expression patterns of *Hesr1* (A) and *Hesr2* (B) were observed in the *Tbx2*-ME hearts. A normal expression pattern for *Hesr1* (D,G) and *Hesr2* (E,H) was observed in *Notch2*-KO (E9.5) and *Notch2-ICD*-ME (E9.0) embryos. (**C**) *Tbx2* was detectable throughout the entire heart in the *Tbx2*-ME embryo. (**F**,**I**) *Notch2* expression was detectable using a probe directed against the *Notch2-ICD* (I), but not with a probe for the extracellular domain (ECD) of *Notch2* (F). A, atrium; V, ventricle; LA, left atrium; LV, left ventricle.

The mechanisms underlying the establishment of the regional specificity and the lineage origin of the cells that eventually form the heart remain to be elucidated. Recently, however, it has been shown that myocardial cells from a second source make an important contribution to the cardiac chambers (Buckingham et al., 2005). Gene expression lineage tracing data show that *Isl1* is expressed in a more anterior/medial field relative to the cells expressing Mlc2a at E7.5, and also that these cells are added mainly to the right ventricle and outflow tract. Although the cells expressing Mlc2a have been suggested to contribute to the left ventricle, the lineage origin of the atrium and AV canal remains ambiguous. Several genes, which have been shown to mark the nascent chamber myocardium, begin to be expressed only after the chambers are clearly visible at E9.5. We therefore expect that future detailed analysis of the early expression of *Hesr1* and *Hesr2*, in comparison with the *Isl1* or *Mlc2a* genes, will provide precise fate-mapping information that could help to delineate the heart chambers.

Functional differences between Hesr1 and Hesr2

Hesr1 and Hesr2 have been suggested to function as transcriptional repressors and we further confirmed their repressive effects on the Tbx2 enhancer in a cell culture system. However, the misexpression of *Hesr1* and *Hesr2* result in the manifestation of different phenotypes during the development of the AV myocardium. Because these proteins have identical amino acid sequences in their basic region, which is responsible for their interaction with DNA, their repressive effects may not differ significantly if they operate through a direct interaction with target DNA sequences. Recently, however, a distinct mechanism was proposed for transcriptional repression by Hesr2, which is mediated by a direct interaction with transcriptional activators including Arnt, myocardin, and Runx2 (Chin et al., 2000; Doi et al., 2005; Garg et al., 2005). Therefore, if Hesr2 but not Hesr1 interacts with such an activator(s), the development of different phenotypes would be expected in transgenic studies. Alternatively, both Hesr1 and Hesr2 might interact with the same activator but with a different affinity, which

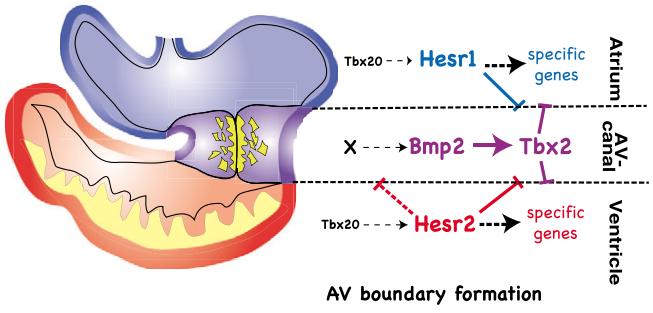

Fig. 6. Model of the role of Hesr1 and Hesr2 in AV boundary formation. Hesr1 and Hesr2 are specifically expressed in the atrium and ventricle but not the AV canal. The AV canal is specified by Bmp2 expression, which induces Tbx2 expression. Bmp2 signals might diffuse to the chamber area and thus induce Tbx2 in the chamber cells. However, because of Hesr1 and Hesr2 expression in the atrium and ventricle, the undesirable expression of *Tbx2* is suppressed in the chamber area, which leads to clear boundary formation between the AV canal and each chamber. Tbx20 is a possible upstream factor in this process as it is known to be required for expression of *Hesr1* and *Hesr2*. Unknown factor (X) could induce *Bmp2* expression, and possibly be regulated by Hesr2. Therefore, Hesr1 and Hesr2 determine the AV boundary by restricting the expression of *Tbx2* to the AV myocardium.

could also account for the different transgenic phenotypes. Further experiments will be necessary to elucidate the functional differences between Hesr1 and Hesr2.

The role of the Notch signaling pathway

The expression of *Hesr1* and *Hesr2* has been shown previously to be regulated by Notch signaling (Maier and Gessler, 2000; Nakagawa et al., 2000; Iso et al., 2003). Notch2 is the only Notch receptor expressed in the myocardium, but in our current study we found that the myocardial expression of *Hesr1* and *Hesr2* is unaffected in both the *Notch2*-KO and *Notch2*-activated embryonic mouse heart. These observations suggest that Hesr1 and Hesr2 play crucial roles in AV boundary formation through a Notch2-independent pathway. In the chick, however, *Hesr1*, but not *Hesr2*, has been reported to respond to Notch2 (Rutenberg et al., 2006). As *Hesr1* expression in the ventricle is observed in the chick but not in mice, the responsiveness of this gene to Notch2 might have diverged during evolution. Previously, we reported that the forced expression of *Notch1-ICD* results in the upregulation of *Hesr1* but not *Hesr2* expression (Watanabe et al., 2006). In addition, *Hesr1* but not *Hesr2* expression in the heart is downregulated in RBPJκ (*Rbpsuh* – Mouse Genome Informatics) -null mutant mice (Timmerman et al., 2004), indicating at least that *Hesr1* but not *Hesr2* is regulated by RBPJκ-dependent Notch1 signaling. However, Notch1, Notch2 and Notch4 are expressed in the endocardial cells of the AV cushion tissue, and expression of *Hesr1* and *Hesr2* also overlaps with this region. Therefore, *Hesr1* and *Hesr2* might be regulated by Notch signaling in the endocardial cells of the AV cushion tissue, and thus might function in the EMT process. Further analyses using cell-type specific KO or misexpression studies will be necessary to gain a greater understanding of these signaling networks.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/4/747/DC1

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