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

Hiroki Kokubo1,2,*, Sachiko Tomita-Miyagawa3, Yoshio Hamada4 and Yumiko Saga1,2,*

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, anlagen 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, Hand1, 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 (Ito et al., 2003). Because the myocardial expression of Hesr1 (Hey1) or Hesr2 (Hey2) is...
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-lacZ and Tbx2-Xho-lacZ, were constructed by insertion of the 6083 bp (HindIIICol) or 2712 bp (XhoI-Ncol) 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 embryo 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 InstiPro system (MI&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-RPC 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 (HindIII-Ncol) and 2.7 kb (XhoI-Ncol) 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 Akt3 (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.
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 myofibris 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.

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 (Smox – 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.
Hesr1 and Hesr2 may directly repress Tbx2 expression

Because our expression 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. 4LJ). 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 transgenic mice (Tbx2-D3-nlacZ/Hesp1-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 HindIII-XhoI 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 HindIII-XhoI 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 canal, 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-IDC-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 Hesr1-null 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).

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. 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.

**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.
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 RBPJΔ (Rbpsuh – Mouse Genome Informatics) -null mutant mice (Timmerman et al., 2004), indicating at least that Hesr1 but not Hesr2 is regulated by RBPJΔ-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.

We thank Hiroaki Nagao for undertaking the transmission electron microscopic analysis at Tokyo Women’s Medical University; Dr Manabu Shirai at the National Cardiovascular Center Research Institute for generously providing us with the Tbx2 probe and also for critical discussion; Dr Takashi Imamura at the JCR Cancer Institute for kindly providing the Smad5 and ALK3 constructs; Yuka Satoh and Yuki Takahashi for their technical assistance and also each of JFCR Cancer Institute for kindly providing the Smad5 and ALK3 constructs; National Cardiovascular Center Research Institute for generously providing us with the Tbx2 probe; and the BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan.

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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