Available online at www.sciencedirect.com

SCIENCE DIRECT

Developmental Biology 278 (2005) 301–309

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

Mouse *hesr1* and *hesr2* genes are redundantly required to mediate Notch signaling in the developing cardiovascular system

Hiroki Kokubo[a,b,*], Sachiko Miyagawa-Tomita[c], Makoto Nakazawa[c], Yumiko Saga[a,b], Randy L. Johnson[d]

[a]*Division of Mammalian Development, National Institute of Genetics, Mishima, Japan*
[b]*Department of Genetics, The Graduate University for Advanced Studies (Sokendai), Mishima, Japan*
[c]*Pediatric Cardiology, The Heart Institute of Japan, Tokyo Women's Medical University, Japan*
[d]*Department of Biochemistry and Molecular Biology, University of Texas, M.D. Anderson Cancer Center, Houston, Texas, USA*

Received for publication 15 March 2004, revised 4 August 2004, accepted 29 October 2004
Available online 26 November 2004

Abstract

Notch signaling is required for multiple aspects of cardiovascular development, including arterial-venous differentiation, septation and cushion formation. Despite recognition of the importance of the Notch pathway in normal cardiovascular development, the proximate downstream effectors are not yet known. Likely candidate effectors are members of the hairy and enhancer of split related (hesr) family of bHLH transcription factors. However, mutational analysis of individual *hesr* genes has so far failed to elucidate their role in all Notch-mediated cardiovascular signaling events. An example of this is evident for mutants of *gridlock*, the zebrafish counterpart of mouse *hesr2*, which have vascular defects, whereas mouse *hesr2* mutants have only cardiac defects. One possible explanation for these differences could be functional redundancy between *hesr* family members. Here, we report that mice lacking the *hesr1* gene are viable and fertile, whereas knockout mouse of both *hesr1 and hesr2* is embryonic lethal at 11.5 days postcoitum (dpc) and recapitulates most of the known cardiovascular phenotypes of disrupted Notch pathway mutants including defects in arterial-venous specification, septation and cushion formation. Taken together, our results demonstrate a requirement for hesr1 and hesr2 in mediating Notch signaling in the developing cardiac and vascular systems.

Keywords: *hesr1*; *hesr2*; Cardiovascular system

Introduction

The cardiovascular system is the first organ system to form during embryogenesis and the cardiac and vascular endothelia play essential roles in this process. During cardiac development, the inner cardiac endothelium regulates the maturation of the outer myocardium, which is required for trabecular formation (Brutsaert et al., 1998). Coalescence of trabeculations at the apex of the heart results in the formation of a muscular ventricular septum that divides the primitive ventricle into the right and left ventricles early in development (Ben-Shachar et al., 1985). Luminal endothelial cells in the regions of both the atrioventricular (AV) cushion and outflow tract undergo epithelial–mesenchymal transformation and participate in the formation of valves and membranous septa (Runyan and Markwald, 1983). During vascular development, after a primary vascular plexus has formed, more vascular endothelial cells are generated, and these form new capillaries by sprouting or splitting from their vessel of origin in a process termed angiogenesis (reviewed in Risau, 1997). Reciprocal interactions between vascular endothelial cells, vascular mesenchymal cells and the surrounding matrix are essential in this process.

Notch signaling is essential for cardiac and vascular morphogenesis in mice. In the heart, endothelial cells,

* Corresponding author. Department of Mammalian Development, National Institute of Genetics, 1111 Yata, Mishima, Shizuoka, 411–8540, Japan. Fax: +81 55 981 6828.
E-mail address: hkokubo@lab.nig.ac.jp (H. Kokubo).

0012-1606/$ - see front matter © 2004 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2004.10.025

especially in the AV canal region, express *Notch1*. Evidence for a requirement of Notch signaling during cardiovascular development came from studies of mice that contain a processing-deficient allele of *Notch1*. These mice exhibit heart anomalies, including a distended pericardium (Huppert et al., 2000; Timmerman et al., 2004). Furthermore, a transcriptional co-factor of Notch, RBPJk, expressed in cardiac endothelial cells, has been shown to be essential for epithelial to mesenchymal transformation (EMT) (Timmerman et al., 2004). Additionally, hypomorphic mutants of *Notch2*, which is expressed in the myocardium, exhibit a thinning of the myocardial wall (McCright et al., 2002). Moreover, mice mutants for the Notch ligand, Jagged1, which causes the autosomal dominant disorder Alagille syndrome in humans (Li et al., 1997; Oda et al., 1997), exhibit embryonic lethality and vascular defects (Xue et al., 1999) and *Jagged1* and hypomorphic *Notch2* double heterozygous mutants show further defects in heart, eye and kidney development, which parallel the defects observed in Alagille syndrome in humans (McCright et al., 2002). Additional evidence supporting a role for notch signaling in vascular development stems from the observations that *Notch1*, *Notch3*, *Notch4*, *Delta4*, *Jagged1* and *Jagged2* are all expressed in arterial tissues (Krebs et al., 2000; Villa et al., 2001) and mutations in these genes results in vascular defects (Huppert et al., 2000; McCright et al., 2002; Shutter et al., 2000; Xue et al., 1999).

The hesr family of bHLH transcription factors was identified through its similarity to the *hairy and enhance of split* (*hes*) family of genes, which are downstream targets of Notch signaling in *Drosophila*. In both mouse and human, there are three *hesr* genes, *hesr1-3*, (also known as *hey*, *hrt*, *chf*, *gridlock*, ""or *herp*) that form a distinct subfamily of bHLH-containing transcriptional repressors from the *hes* genes. Previous studies of mouse knockouts for *hesr2* have shown phenotypes that display dysplastic AV valves, a perimembranous ventricular septal defect (VSD), and a secundum atrial septal defect (ASDII), (Donovan et al., 2002; Gessler et al., 2002; Kokubo et al., 2004; Sakata et al., 2002). Transthoracic echocardiographies revealed that AV valve regurgitations, due to AV valve hypoplasia, were most likely responsible for heart dysfunction in *hesr2* homozygous mutant mice (Kokubo et al., 2004). However, *hesr2*-null mice do not show vascular defects, in contrast to mutants of zebrafish *gridlock,* a homologue of *hesr2*, which show with coarctation of the aorta (Zhong et al., 2000). Morpholino knock-down experiments also indicate a role for *gridlock* in arterial specification (Zhong et al., 2001). Expression of *hesr1* and *hesr2* in both the cardiac and vascular endothelium overlap at early stages of the development (Fischer and Gessler, 2003; Iso et al., 2003) (our unpublished data), suggesting possible functional redundancy. We have now generated *hesr1* homozygous mutant mice, but they are viable and fertile, and show no obvious phenotype (data not shown). We therefore hypothesized that the phenotypic difference between the mouse and zebrafish *hesr2* mutants might be due to differences in the compensatory actions of other *hesr* gene family members.

Here, we report that mouse embryos lacking both *hesr1* and *hesr2* are embryonic lethal due to severe cardiovascular malformations. Instead of forming paired left and right ventricles, *hesr1/2* double mutants have a single large ventricle, indicating that ventricular septum formation is blocked in the absence of both *hesr1* and *hesr2*. In addition, few cells underwent endocardial to mesenchymal transitions in the developing AV cushions. Finally, arterial differentiation is also defective in *hesr1/2* double mutants. These results highlight that essential roles of the *hesr* genes in the control of critical early events in cardiovascular development and suggest that they are essential downstream targets of Notch signaling in the cardiovascular system.

Material and methods

Knockout mice

To disrupt the *hesr1* gene, a targeting vector was constructed with a complete deletion of the bHLH region from exon1 through exon4, (see Supplementary Figure) using *hesr1* containing BAC clones derived from the RPCI-22 mouse BAC library (BACPAC resources, CA). The targeting vector was then electroporated into AB1 embryonic stem (ES) cells. Colonies resistant to both neomycin (positive selection) and FIAU (negative selection) were screened by Southern blotting to identify correctly targeted clones. The recombinants were subsequently injected into C57B1/6 blastocysts to generate chimeras. Two independent lines were finally obtained that transmitted the targeted allele through the germ line. For genotyping, a PCR method was developed to detect wild-type alleles, using a 3′ forward: CCCTCCCCTCCGTGCTTCTAACCTCAT/3′ reverse: CTCTCCCCACCCCACAAAGCAAAGCAG, primer set, and for the targeted allele a 3′ reverse/Neo: CGACCACCAAGGCGAAACATC primer set. The neo cassette was removed by crossing the mice with CAG-Cre mice and the genotype was determined using the 3′ reverse/lacZ: CTCTGTGTCCTCATAAACCCTAACCTCCTT primer set. Mice containing a disrupted *hesr2* gene have been described in a separate manuscript (Kokubo et al., 2004).

Analysis of double mutants

All analyses were conducted on mice with a C57B1/6/129Sv mix genetic background. Animals and embryos were genotyped by PCR and in situ hybridizations were performed using a whole mount in situ hybridization system (M&S Instruments). Hematoxylin–eosin (HE) staining and transmission electron microscopic observations were carried out using standard methods (Miyagawa-Tomita et al., 1996). We used the ApopTag Kit (Intergen, GA) to detect apoptotic cells. Immunohistochemistry was performed using frozen

sections with anti-ephrinB2 (R and D), anti-CD31 (PharMingen), or anti-α-smooth muscle actin (SIGMA) primary antibodies, and with alexa468-conjugated anti-rat or mouse IgG (Molecular Probes) as a secondary antibody. The corresponding signals were observed with an Olympus fluorescence microscope system.

Results

The hesr1$^{-/-}$/2$^{-/-}$ shows embryonic lethal at around 10.5 dpc

To investigate the respective roles of *hesr1* and *hesr2* in cardiovascular development in the mouse, we intercrossed *hesr1*$^{+/-}$/2$^{+/-}$ mice. At weaning, however, no double homozygous mutant was found (Table 1), suggesting that combined homozygosity at the *hesr1* and *hesr2* loci results in embryonic lethality. Although the number of *hesr1*$^{+/-}$/2$^{-/-}$ mice obtained was significantly reduced, some were found to be both viable and fertile, as was previously observed for *hesr2*$^{-/-}$ single knockout mice. To define the stage at which the double mutant lethality was occurring, timed pregnant matings were set up and *hesr1*$^{-/-}$/2$^{-/-}$ embryos were isolated at the expected frequency until 10.5 dpc (Table 1). No *hesr1*$^{-/-}$/2$^{-/-}$ embryos were observed beyond 11.5 dpc, indicating that the double mutants die prior to this stage. At 10.5 dpc, *hesr1*$^{-/-}$/2$^{-/-}$ homozygous embryos were easily recognized by their poorly formed telencephalic regions and small first and second pharyngeal arches, as well as by heart malformations and hemorrhaging (Figs. 1A and B). These defects are therefore likely to be responsible for the embryonic lethality observed in *hesr1*$^{-/-}$/2$^{-/-}$ mice.

The hesr1$^{-/-}$/2$^{-/-}$ embryos display anomalies in heart development

Since previous studies of knockout mice have shown that *hesr2* has an important role in cardiac development, particularly in the development of cardiomyocytes and in AV valve formation, we first focused our analysis on heart anomalies in *hesr1*$^{-/-}$/2$^{-/-}$ embryos. At the gross level *hesr1*$^{-/-}$/2$^{-/-}$ hearts had only a single ventricle, whereas *hesr1*$^{+/-}$/2$^{+/-}$ hearts became to be divided into two ventricles (Figs. 1C and D). Histological examination also revealed that the wall of the ventricle was thin and that the trabecular zones and AV cushions had not properly formed (Figs. 1C–F). At this stage, wild type, *hesr1*, *hesr2* single mutant embryos have undergone initial stage of chamber formation, resulting in well-formed left and right atria and ventricles (Figs. 2C and E). In contrast, *hesr1*$^{-/-}$/2$^{-/-}$ mice have a single open atria and ventricle, showing hypoplastic myocardial trabeculae (Figs. 2D and F). During chamber formation, the ventricular wall, accompanied by the proliferation and accumulation of cardiomyocytes, gives rise to two layers corresponding to the sub-epicardial compact and sub-endocardial trabecular zones. Until 9.5 dpc, *hesr1*$^{-/-}$/2$^{-/-}$ hearts with d-ventricular looping showed a normal-sized ventricular chamber containing both the compact and trabecular zones (Figs. 1G–J). These observations suggested that in hesr1/2 double mutants, the cardiomyocytes in the ventricle could successfully divide into compact and trabecular zones, but that trabecular zone cells are selectively lost at a later stage. We thus speculated that apoptosis might be a contributing factor to this phenotype. Indeed, many TUNEL-positive cells were found in the trabecular zone but not in the compact zone at 10.0 dpc (Figs. 1K and L), although TUNNEL-positive cells were not detected earlier at 9.5 dpc (data not shown). To further characterize the nature of these cardiomyocyte defects, the microstructure of the cardiomyocytes was further analyzed via transmission electron microscopy. Disorganized cardiomyocytes with fewer myofibrils, and with swollen and irregular-shaped mitochondria, were observed in *hesr1*$^{-/-}$/2$^{-/-}$ mutants (Figs. 1M and N). These observations suggest that poor trabecular formation results in defects in cardiac chamber development.

Endocardial cushion development is essential for AV valve formation. In the AV cushions, cardiac endothelial cells undergo epithelial–mesenchymal transformation and participate in the formation of valves and membranous septa. Our analysis of *hesr2* single mutants showed that AV valve regurgitations due to hypoplasia of the valves were most the likely cause of heart dysfunction in these mutant mice, suggesting that hesr2 functions in either the formation or maintenance of the AV valve. Our current studies favor the former possibility. At 9.5 dpc, epithelial–mesenchymal transformations in the AV canal occurred normally and on schedule in the *hesr*$^{+/-}$/2$^{+/-}$ embryo (Fig. 1O). In contrast,

Table 1
Numbers of progeny obtained by intercrossing *hesr1* and *hesr2* mutants

Age (*hesr1,2*)	+/+, +/+	+/+, +/−	+/+, −/−	+/−, +/+	+/−, +/−	+/−, −/−	−/−, +/+	−/−, +/−	−/−, −/−	ND	Total
10 dpp (+/−, +/− × +/−, +/−)	12 (9)	25 (18)	4 (9)	23 (18)	37 (36)	10 (18)	17 (9)	16 (18)	0 (9)	12	155
10.5 dpc (+/−, −/− × −/−, +/−)					19 (22)	20 (22)		21 (22)	23 (22)	7	90

Genotypes of the progeny from the intercross between *hesr1*$^{+/-}$/2$^{+/-}$ mice, or between *hesr1*$^{-/-}$/2$^{+/-}$ and *hesr1*$^{+/-}$/2$^{-/-}$ mice were examined at day 10 postpartum (dpp) or at day 10 postcoitum (dpc), respectively. *hesr1*$^{-/-}$/2$^{-/-}$ mice were not detected at 10 dpp, but were evident at 10 dpc as expected. The predicted numbers are parenthesized in the panel.

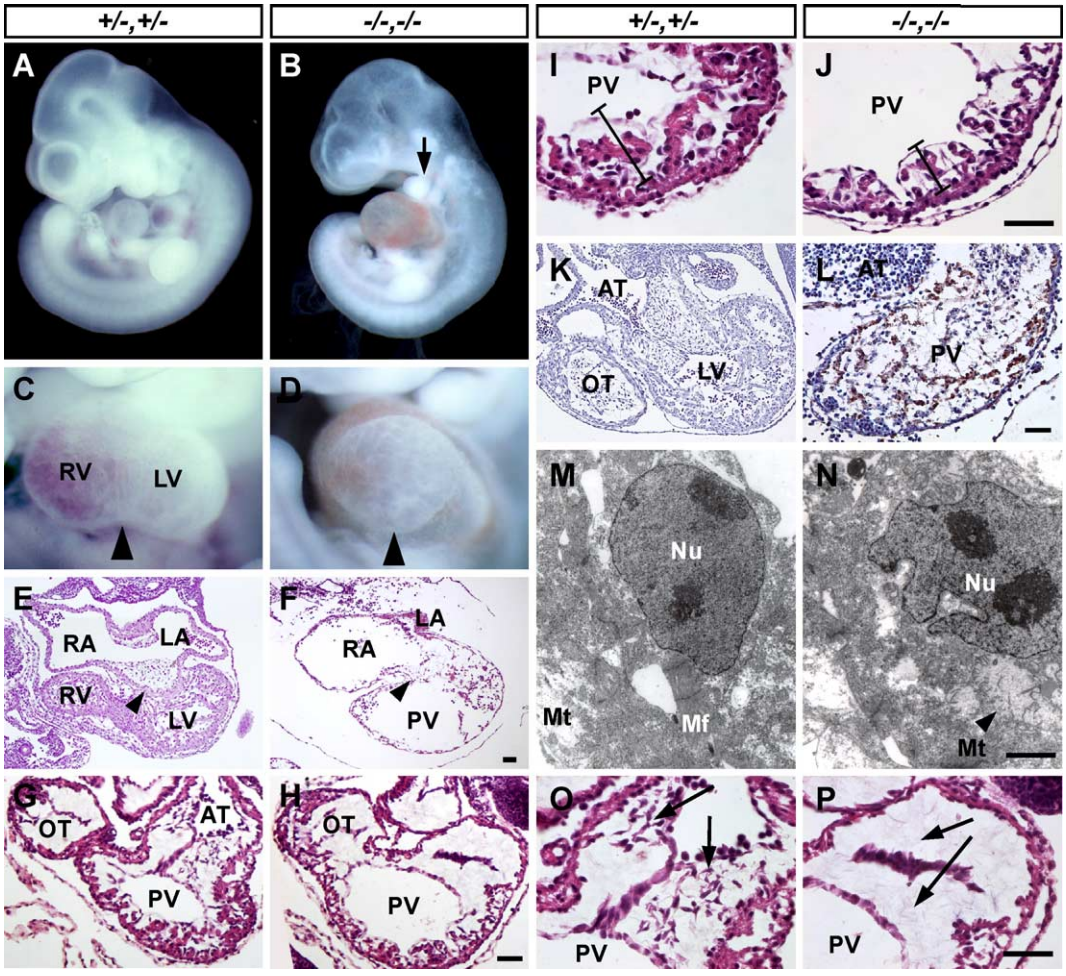

Fig. 1. Gross morphology and heart abnormalities in the *hesr1*$^{-/-}$/*2*$^{-/-}$ embryo. (A, B) Comparison of a *hesr1*$^{+/-}$/*2*$^{+/-}$ embryo (A) and a *hesr1*$^{-/-}$/*2*$^{-/-}$ embryo (B) at 10.5 dpc *hesr1*$^{-/-}$/*2*$^{-/-}$ embryo shows small pharyngeal arch (arrow). (C, D) Frontal view of the hearts shown in A and B at higher magnification. The *hesr1*$^{+/-}$/*2*$^{+/-}$ heart is clearly divided into a primitive right ventricle (RV) and primitive left ventricle (LV) at the center of the chamber (arrowhead). In contrast, the *hesr1*$^{-/-}$/*2*$^{-/-}$ heart has a single chamber. Arrows indicate the center of the chamber. (E, F) HE-stained sections of 10.5 dpc embryonic hearts. The primitive four chambers are well formed in the *hesr1*$^{+/-}$/*2*$^{+/-}$ heart (E). AV cushion tissue is also well developed (arrowhead). A single primitive ventricle (PV) and right (RA) and left (LA) atrium can be seen in the *hesr1*$^{-/-}$/*2*$^{-/-}$ heart (F); the atrial septum is formed, but there is no interventricular septum. AV cushion tissue (arrowhead) and the myocardial trabeculae of the ventricle are also not well formed. (G, H) HE-stained sections at 9.5 dpc. A primitive atrium (AT) and ventricle (PV) are formed in both the *hesr1*$^{+/-}$/*2*$^{+/-}$ (G) and the *hesr1*$^{-/-}$/*2*$^{-/-}$ embryonic hearts (H), both hearts show normal d-ventricular looping. OT, outflow tract. (I, J) Magnified view of the ventricular wall shown in G and H. The trabecular zone of the *hesr1*$^{-/-}$/*2*$^{-/-}$ heart (indicated bar in panel J) appears thinner than that of the *hesr1*$^{+/-}$/*2*$^{+/-}$ heart (indicated bar in panel I). (K, L) Many apoptotic cells are detected by a TUNEL assay in the trabecular zone of the primitive ventricle in the *hesr1*$^{-/-}$/*2*$^{-/-}$ embryo (L) at 10.5 dpc. (M, N) TEM analysis, indicating that well-organized myofibrils (Mf) and mitochondria (Mt) are evident at 10.5 dpc in the ventricle of the *hesr1*$^{+/-}$/*2*$^{+/-}$ embryo (M). In contrast, disorganized myofibrils and swollen mitochondria (arrowhead) are observed in the *hesr1*$^{-/-}$/*2*$^{-/-}$ embryo (N). Nu, nucleus. (O, P) Magnified HE section at the atrioventricular cushion of G and H. Transformed mesenchymal cells (arrows) from the endocardium are observed in the *hesr1*$^{+/-}$/*2*$^{+/-}$ embryo (O), whereas few are seen in the *hesr1*$^{-/-}$/*2*$^{-/-}$ embryo (P).

hesr1$^{-/-}$/*2*$^{-/-}$ embryos failed to undergo cardiac endothelial cell transformation although the AV cushion was enriched in extracellular matrix (Fig. 1P). These observations suggest that both *hesr1* and *2* play redundant roles in epithelial–mesenchymal transformation during AV valve formation.

Left–right ventricle identity is unaffected in the hesr1$^{-/-}$/*2*$^{-/-}$ *heart*

In the *hesr1*$^{-/-}$/*2*$^{-/-}$ mutant hearts, only a single ventricle was formed at the gross level. This lack of left–right ventricular formation could be due to either an inability to specify either the left or right ventricles. Alternatively, the ventricular defects could be due to a failure to undergo morphogenetic changes required for ventricular septation. To test these possibilities, we examined the expression of marker genes for cardiac development and for ventricular right–left polarity. Expression of *nkx2.5*, the earliest gene expressed in the cardiac lineage (Harvey, 1996; Lyons et al., 1995), was normal in *hesr1*$^{-/-}$/*2*$^{-/-}$ hearts (Figs. 2A and B), indicating that general cardiac cell type specification was unaltered. Additionally, the expression of *ehand*, which is normally only detected in the left ventricle at 10.5 dpc

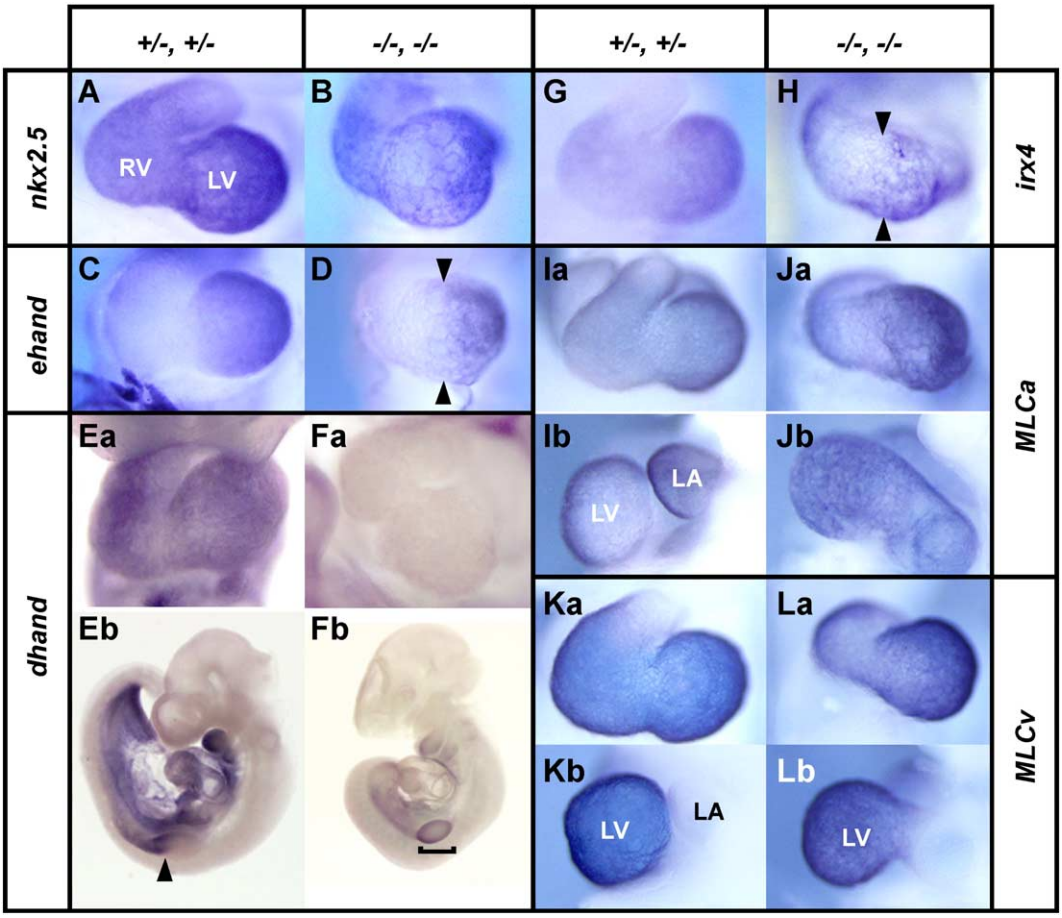

Fig. 2. In situ hybridization analyses using ventricular and atrial markers in 10.5 dpc hearts. (A, B) *nkx2.5* is expressed in both the right (RV) and left (LV) ventricles of the *hesr1*$^{+/-}$/*2*$^{+/-}$ (A) and *hesr1*$^{-/-}$/*2*$^{-/-}$ embryos (B). A frontal view is shown. (C, D) In the *hesr1*$^{+/-}$/*2*$^{+/-}$ heart (C), *ehand* expression is normally restricted to the left ventricle but in the *hesr1*$^{-/-}$/*2*$^{-/-}$ heart (D), is restricted to the left half of the single ventricle (arrows indicate the edge of the zone of expression). (E, F) *dhand* expression in the *hesr1*$^{-/-}$/*2*$^{-/-}$ heart (Fa) is reduced over the whole ventricle compared to the *hesr1*$^{+/-}$/*2*$^{+/-}$ heart (Ea). Expression patterns of *dhand* in the whole embryos are shown in Eb (*hesr1*$^{+/-}$/*2*$^{+/-}$) and Fb (*hesr1*$^{-/-}$/*2*$^{-/-}$). Signal is normally restricted in the posterior region of limb (arrowhead in Ea), but in the *hesr1*$^{-/-}$/*2*$^{-/-}$ limb, signal is expanded to the anterior region (indicated bar in Fb). (G, H), *Irx4* is expressed in the ventricles of the *hesr1*$^{+/-}$/*2*$^{+/-}$ (G) and the *hesr1*$^{-/-}$/*2*$^{-/-}$ (H) embryonic hearts (arrows indicate the border of the different expression levels). (I, J) Expression levels of the myosin light chain atrium (*MLCa*) gene are shown in frontal (a) and left lateral (b) views. The *MLCa* gene is normally expressed in both embryonic atria and ventricles and, the pattern of expression is normal in the *hesr1*$^{-/-}$/*2*$^{-/-}$ embryos. (K, L) Expression of the myosin light chain ventricle (*MLCv*) gene is shown in frontal (a) and left lateral (b) views. *MLCv* gene expression is restricted in the ventricles of both the *hesr1*$^{+/-}$/*2*$^{+/-}$ and the *hesr1*$^{-/-}$/*2*$^{-/-}$ embryos.

(Bao et al., 1999; Srivastava et al., 1997), was restricted to the left side of the ventricle in the *hesr1/2* double knockout mice (Figs. 2C and D), indicating the left side of the ventricle was correctly specified. Transcription of *dhand* normally occurs in both ventricles and is then enhanced in the right ventricle (Srivastava et al., 1995; Yamagishi et al., 2000), but was significantly reduced in the *hesr1*$^{-/-}$/*2*$^{-/-}$ mice (Figs. 2Ea and Fa). In addition to the heart, *dhand* expression is reduced even other regions including the limb bud, in which the normal posterior restricted domain of *dhand* expression is not observed in the *hesr1*$^{-/-}$/*2*$^{-/-}$ mutant embryos. These results may indicate that Notch signaling and/or hesr1/2 function is involved in the AP specification of limb bud (Figs. 2Fb and Eb). In the *hesr1*$^{-/-}$/*2*$^{-/-}$ heart, *Irx4*, a ventricle-specific homeobox gene, was normally expressed (Figs. 2G and H), and expression of the atrial and ventricular muscle-specific differential markers, *MLCa* and *MLCv* (Kubalak et al., 1994), was also normal, indicating that atrial and ventricular specification and terminal differentiation were unaffected (Figs. 2I–L). We conclude therefore that *hesr1* and *hesr2* are essential for myocardial trabecular proliferation of the ventricle rather than for the establishment of the right–left polarity of the cardiac chamber.

The hesr1$^{-/-}$/*2*$^{-/-}$ *embryos display vascular defects*

The *hesr2* single mutants do not show vascular defects (Donovan et al., 2002; Gessler et al., 2002; Kokubo et al., 2004; Sakata et al., 2002). On the other hand, mutation of zebrafish *gridlock,* a homologue of *hesr2*, is associated with coarctation of the aorta (Zhong et al., 2000). Expression of

hesr1 and *hesr2* in the vascular endothelium is found to overlap at an early stage of development (Fischer and Gessler, 2003; Iso et al., 2003) (our unpublished data). We thus hypothesized that the phenotypic difference between the mouse and zebrafish mutants was due to differences in the compensatory actions of the *hesr* gene family members.

Defects in blood vessel development were also evident in *hesr1*$^{-/-}$/*2*$^{-/-}$ embryos. In yolk sacs, large blood vessels were not formed in the double mutants (Fig. 3B). In contrast, in wild-type embryos and in single knockout *hesr1* or *hesr2* mutants at E10.5, remodeling of vessels occurs to form larger vessels and blood circulation commences in the yolk sac (Fig. 3A). In the *hesr1/2* double mutants, small blood vessels in the yolk sac itself were present (Fig. 3D), suggesting that *hesr1* and *hesr2* are redundantly required for the remodeling of vessels in the yolk sac. Additionally, in the *hesr1/2* double mutant embryos, as seen for *hesr1* or *hesr2* single mutants, large vessels, including cardiac vein and dorsal aorta, were normally positioned following ink injection and HE staining analysis (Figs. 3E–H). However, ink injection into both the vitelline artery and veins could not directly reach the cardinal vein in the double mutants but instead, the ink filled several small vessels connecting the dorsal aorta and cardinal vein (red arrow in Fig. 3F).

Histologically, the cell layers surrounding the arteries were thinner in the double mutants than in the corresponding double heterozygotes, which prompted us to speculate that *hesr1/2* double mutants have arterial specification defects. To test this possibility, we examined the expression of Ephrin-B2 and smooth muscle actin (SMA), both of which are expressed in the mesenchyme surrounding the endothelium of developing arteries, but not in developing veins at this stage of embryogenesis and thus can distinguish arteries and veins. In *hesr1/2* double mutants, we observed a large vessel in the position of the dorsal aorta that is prominently stained by PECAM-1 (Figs. 3I, L). Unlike wild-type or single mutant embryos, *hesr1/2* double homozygous mutants do not express either SMA or Ephrin-B2, indicating that arterial formation requires either *hesr1* or *hesr2* activity. Hence, the roles of the *hesr* gene family in arterial-venous differentiation are conserved between zebrafish and mouse.

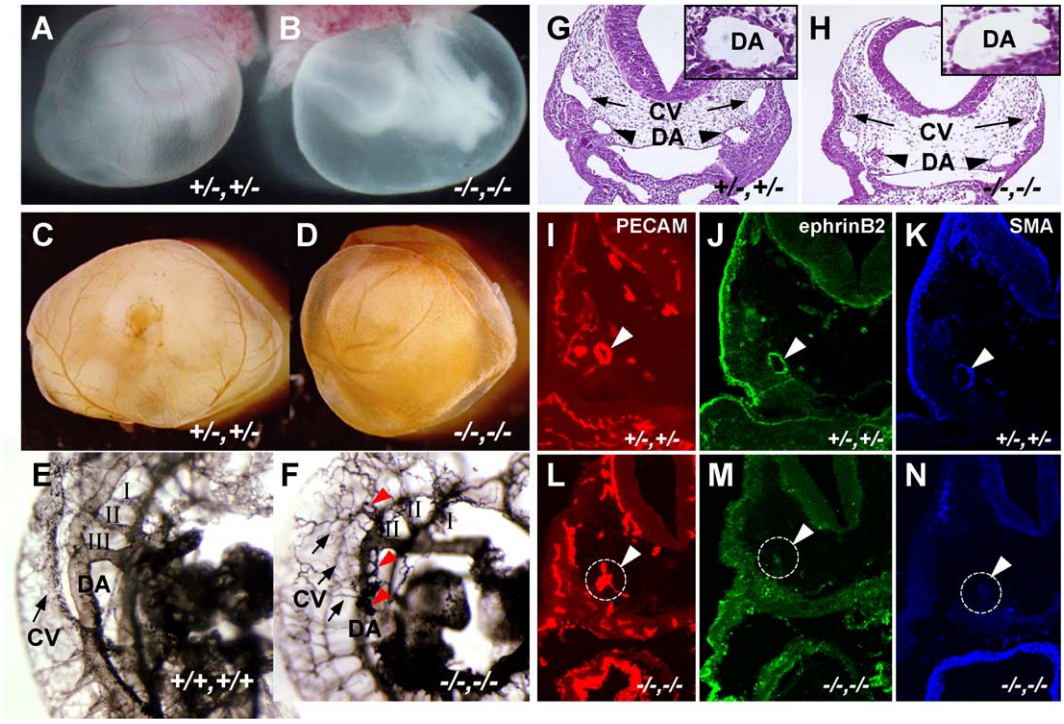

Fig. 3. Vascular defects in the *hesr1*$^{-/-}$/*2*$^{-/-}$ mutant. (A, B) Yolk sacs of *hesr1*$^{+/-}$/*2*$^{+/-}$ (A) and *hesr1*$^{-/-}$/*2*$^{-/-}$ embryos (B) at 10.5 dpc. The absence of major vessels is evident in the freshly dissected *hesr1*$^{-/-}$/*2*$^{-/-}$ yolk sac. (C, D) Whole-mount immunohistochemical staining for PECAM-1. Large and small vessels are seen in the *hesr1*$^{+/-}$/*2*$^{+/-}$ yolk sac whereas only small capillaries are evident in the *hesr1*$^{-/-}$/*2*$^{-/-}$ yolk sac. (E, F) Major vasculature in the trunk region is visualized by ink-injection of the 10.0 dpc embryos showing that rostral is up and dorsal is left. The anterior cardinal vein (CV) and dorsal aorta (DA) running in parallel along the sides of the body, and also the first, second and third aortic arches (I, II, III) can be seen in the wild-type embryo (E). The anterior cardinal vein (arrows), dorsal aorta, and aortic arches are formed in the *hesr1*$^{-/-}$/*2*$^{-/-}$ embryo, but the anterior cardinal vein is connected to the dorsal aorta by multiple vascular branches (red arrowheads). Although the right sinus horn appears normal, the size of the common cardinal vein is reduced. A pair of dorsal aorta in the *hesr1*$^{-/-}$/*2*$^{-/-}$ embryo shows a smaller size compared to that of the *hesr1*$^{+/-}$/*2*$^{+/-}$ embryo and fails to be assembled in the common dorsal aorta at the caudal level (data not shown). (G, H) HE-stained cross-sections in the head region. The anterior cardinal vein and dorsal aorta are present in both the *hesr1*$^{+/-}$/*2*$^{+/-}$ and *hesr1*$^{-/-}$/*2*$^{-/-}$ embryos. The dorsal aortas are magnified in the right upper panels. The cell layer surrounding the artery in the double mutant is thinner than in *hesr1*$^{+/-}$/*2*$^{+/-}$ embryos. (I–N) Antibody staining of adjacent sections of the head region for PECAM-1 (I, L), ephrin-B2 (J, M), and α-smooth muscle actin (K, N). Expression levels of ephrin-B2 (M) and α-smooth muscle actin (N) are decreased in the *hesr1*$^{-/-}$/*2*$^{-/-}$ embryo compared with the *hesr1*$^{+/-}$/*2*$^{+/-}$ embryo (J and K), whereas PECAM expression appears unaffected (compare I with L). The dorsal aorta is indicated by arrowheads.

Discussion

Our results indicate that the Notch signaling pathway, via the hesr1 and hesr2 effectors, plays an important role in cardiac morphogenesis and in angiogenesis. *hesr1*$^{-/-}$/*2*$^{-/-}$ mice have defects in myocardial trabeculation and cardiac endothelial cell transformation, as well as in angiogenesis. Although there are arguments as to whether the cardiac defects or the vascular defects are primary or secondary to each other, both defects could occur independently. In support of a primary role for hesr1 and hesr2 in vascular development, small blood vessels were present in the yolk sac, and blood vessels for great arteries or veins themselves are formed in the *hesr1*$^{-/-}$/*2*$^{-/-}$ embryos, and blood circulation was detected up to at least 10.0 dpc. The major defect we found in the vasculature of the *hesr1*$^{-/-}$/*2*$^{-/-}$ embryos was in the specification of artery vs. vein, which is detected as early as 9.5 dpc in wild-type or single-mutant embryos. The early development of the cardiac and vascular systems occurs independently until circulation starts about at 9.0 dpc. Thus, we think that it is unlikely that the defect in arterial-venous specification that we observe in the *hesr1*$^{-/-}$/*2*$^{-/-}$ mutants is secondary to cardiac defects. With respect to whether the cardiac defects we observe in the *hesr1*$^{-/-}$/*2*$^{-/-}$ mutants are direct or indirect, it should be noted that since endocardial transformation and myocardial differentiation occur after cardiac contractions initiate, it is therefore possible that these processes may be affected indirectly by defects in blood circulation. However, we have observed cardiac anomalies in *hesr2*-single knockout mice without any obvious defects in the vasculature. Collectively favors the idea that the defects we observed in the heart and the arteries of the *hesr1*$^{-/-}$/*2*$^{-/-}$ mutants are primary defects in each system. However, we cannot exclude the possibility that some defects are secondary and we will require tissue-specific deletion of *hesr* genes in cardiac and/or vascular tissues to address the possibility that the phenotypes we observe are indirect.

Synergistic functions of hesr1 and hesr2 in heart development

The *hesr1*$^{-/-}$/*2*$^{-/-}$ embryonic hearts had little EMT in the AV cushion tissue, whereas the *hesr1* single mutant displayed no heart anomalies (our unpublished data), although some *hesr2* single mutants showed dysfunction of the AV valves (Kokubo et al., 2004). This indicates that *hesr1* and *hesr2* are necessary for EMT, but also they can compensate for each other in this process. *TGFβ2*, which is restricted to the AV canal myocardium, was shown to be downregulated in the *RBPJk* mutants (Timmerman et al., 2004). In *hesr1*$^{-/-}$/*2*$^{-/-}$ embryonic hearts, expression of *TGFβ2* is reduced (Supplemental Fig. S2), suggesting that endocardial Notch activity somehow affects myocardial *TGFβ2* expression, likely through *hesr1* and *hesr2*. However, *hesr2* and/or *hesr1* are unlikely to be sufficient to induce EMT in the AV cushion tissue as this transformation fails to occur in *RBPJk* mutants, even though *hesr2* expression is maintained in this mutant (Timmerman et al., 2004). We speculate that other gene(s) may be activated by Notch1/RBPJk that, together with hesr1 and hesr2, are necessary for the induction of EMT. In this context, the zinc finger transcription factor snail is a likely candidate since the expression is known to be down-regulated in RBPJk-null embryonic heart and it can repress E-cadherin expression to induce EMT in epithelial tumor cells (Timmerman et al., 2004). In addition, BMP2 is also know to be involved in EMT (Keyes et al., 2003; Sugi et al., 2004) and cross talk with Notch and BMP2 signaling has been suggested in this context (Takizawa et al., 2003). Recent studies have shown that *hesr1* expression could be synergistically activated by BMP and Notch signaling (Itoh et al., 2004). Expression of *bmp2* is normally detected in the atrium through the AV canal in both *hesr1*$^{+/-}$/*2*$^{+/-}$ and *hesr1*$^{-/-}$/*2*$^{-/-}$ embryonic hearts (Supplemental Fig. S2), however, this BMP signal may not be processed properly in the absence of *hesr1* and *hesr2*, which might result in the suppression of EMT. Further studies will be required to elucidate the molecular mechanism underlining EMT with regard to Notch and BMP signaling and *hesr1/2* function.

The *hesr1*$^{-/-}$/*2*$^{-/-}$ hearts displayed a hypomorphic ventricular trabecular zone. Although a mild cardiomyocyte defect was seen in the *hesr2*$^{-/-}$ single mutant, the formation of the trabecular zone itself was not affected in either of the *hesr1* or *hesr2* single knockout mutants. It has been previously suggested that the inner cardiac endothelium regulates maturation of the outer myocardium and that this is required for trabecular formation (Brutsaert et al., 1998). Our studies demonstrate that expression of *hesr1* in endocardial cells and of *hesr2* in myocardial cells are required for trabecular formation. In either Notch1 or RBPJk mutants, a myocardial defect was not reported, although Notch2 is expressed in the myocardial cells and its hypomorphic mutant has been shown to exhibit a thinning of the myocardial wall (McCright et al., 2002). The defect in ventricular formation in the *hesr1*$^{-/-}$/*2*$^{-/-}$ embryonic heart showed a more severe phenotype than this, suggesting that there may be a similar functional role between Notch1 expressed in endocardial cells and Notch2 in myocardial cells.

hesr1 has been reported to be a downstream target of Notch1 signaling through the RBPJk transcription factor in vitro (Iso et al., 2002). Since *Notch1*, *RBPJk* and *hesr1* are co-expressed in the endocardium and *hesr1* expression was absent in the *RBPJk* mutant (Timmerman et al., 2004), this suggested that *hesr1* should be activated by Notch1 through RBPJk in vivo. The regulation of *hesr2*, however, appears not to be solely dependent on *Notch1* and *RBPJk*, and since its expression is not affected in the *RBPJk* mutant, its expression in these tissues is must be controlled via another mechanism. Of particular interest in this regard is the expression of Notch2 in the myocardium (McCright et al.,

2002), which is similar to that of *hesr2,* suggesting that Notch2 might play a role in *hesr2* regulation.

Of further interest is the control mechanism by which hesr proteins modulate cardiovascular development. Information of downstream targets of hesr proteins still remains uncertain. From our studies, one possible downstream target of hesrs is *dhand*, since the expression of *dhand* was reduced in the *hesr1*$^{-/-}$/*2*$^{-/-}$ embryonic heart. Mice lacking *dhand* have a single ventricle that exhibit hypotrabeculation and aortic arch defects and die by 11.0 dpc due to the heart failure (Srivastava et al., 1995; Yamagishi et al., 2000). Therefore, simple explanation for phenotype of the *hesr1*$^{-/-}$/*2*$^{-/-}$ heart might be due reduced *dhand* expression. In this context, it is interesting that the expression level and/or pattern of *dhand* is altered even in other regions such as pharyngeal arch, limb bud and lateral mesoderm. Since hesr proteins have been shown to function in vitro as transcriptional repressors, regulation of *dhand* expression by hesr1 and/or hesr2 may be indirect. Further analysis will be necessary to identify the mechanism of how *dhand* is regulated through either Notch signaling or hesrs proteins.

The synergistic functions of hesr1 and hesr2 in vascular development

The hesr gene family is composed of three members and their expression overlaps in the vasculature, especially in the aorta. In this report, we demonstrate a requirement for *hesr1* and *hesr2* in vascular and cardiac development, raising the question of whether *hesr3* is unable to compensate for the combined loss of *hesr1* and *hesr2*. One possibility is that *hesr3* is unable to substitute for *hesr1 and hesr2* function. Alternatively, the expression of *hesr3* may be altered in *hesr1*$^{-/-}$/*2*$^{-/-}$ mutants. To distinguish between these possibilities, we examined whether *hesr3* is expressed in the *hesr1*$^{-/-}$/*2*$^{-/-}$ embryos by in situ hybridization analysis. We found that the expression of *hesr3* is significantly reduced in the *hesr1*$^{-/-}$/*2*$^{-/-}$ embryos (Supplemental Fig. S3). Although the mechanism of *hesr3* downregulation mediated by hesr1 and/or hesr2 remains uncertain, this downregulation of *hesr3* explain why *hesr3* does not compensate for *hesr1* and *hesr2* in the *hesr1*$^{-/-}$/*2*$^{-/-}$ mutant.

Notch signaling regulates many aspects of cell-fate decisions and of particular relevance to this report plays a critical role in vascular development, especially in remodeling and in arterial-venous specification (Krebs et al., 2000), may act together with ephrinB2 and EphB4 in arterial-venous specification (Lawson et al., 2001; Shawber et al., 2003). Eph receptor tyrosine kinases and their transmembrane ephrinB ligands are expressed in the developing circulatory system, and mice lacking either ephrinB2 or EphB4 have similar phenotypes to *hesr1*$^{-/-}$/*2*$^{-/-}$ mice, including defects in angiogenic remodeling and poor myocardial trabeculation. Additionally, several studies have now suggested that ephrin-Eph signaling is required for angiogenesis (Adams et al., 1999; Gerety and Anderson, 2002; Gerety et al., 1999; Wang et al., 1998). Reduced expression of ephrinB2 was also seen in *hesr1*$^{-/-}$/*2*$^{-/-}$ mice. These findings suggest that ephrin-Eph signaling, particularly ephrinB2 expression, could be regulated, either directly or indirectly, by Notch signaling via the *hesr* genes during cardiovascular development. Further analysis will be necessary to identify the signaling pathways involved in the endothelial cells of the developing cardiovascular system.

Acknowledgments

We thank Hiroaki Nagao for undertaking the transmission electron microscopy analysis and Dr. Toshio Nishikawa in Tokyo Women's Medical University for helpful discussions, concerning mouse pathology. We also thank Yuka Satoh and Yuki Takahashi for technical assistance, Maho Endo MS for histological analyses, and each of the members of Dr. Saga's laboratory in The National Institute of Genetics and of Dr. Johnson's laboratory in The University of Texas M.D. Anderson Cancer Center.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2004.10.025.

References

Adams, R.H., Wilkinson, G.A., Weiss, C., Diella, F., Gale, N.W., Deutsch, U., Risau, W., Klein, R., 1999. Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. Genes Dev. 13, 295–306.

Bao, Z.Z., Bruneau, B.G., Seidman, J.G., Seidman, C.E., Cepko, C.L., 1999. Regulation of chamber-specific gene expression in the developing heart by Irx4. Science 283, 1161–1164.

Ben-Shachar, G., Arcilla, R.A., Lucas, R.V., Manasek, F.J., 1985. Ventricular trabeculations in the chick embryo heart and their contribution to ventricular and muscular septal development. Circ. Res. 57, 759–766.

Brutsaert, D.L., Fransen, P., Andries, L.J., De Keulenaer, G.W., Sys, S.U., 1998. Cardiac endothelium and myocardial function. Cardiovasc. Res. 38, 281–290.

Donovan, J., Kordylewska, A., Jan, Y., Utset, M., 2002. Tetralogy of fallot and other congenital heart defects in hey2 mutant mice. Curr. Biol. 12, 1605.

Fischer, A., Gessler, M., 2003. Hey genes in cardiovascular development. Trends Cardiovasc. Med. 13, 221–226.

Gerety, S.S., Anderson, D.J., 2002. Cardiovascular ephrinB2 function is essential for embryonic angiogenesis. Development 129, 1397–1410.

Gerety, S.S., Wang, H.U., Chen, Z.F., Anderson, D.J., 1999. Symmetrical mutant phenotypes of the receptor EphB4 and its specific trans-

membrane ligand ephrin-B2 in cardiovascular development. Mol. Cell 4, 403–414.
Gessler, M., Knobeloch, K., Helisch, A., Amann, K., Schumacher, N., Rohde, E., Fischer, A., Leimeister, C., 2002. Mouse gridlock. No aortic coarctation or deficiency, but fatal cardiac defects in Hey2−/− Mice. Curr. Biol. 12, 1601.
Harvey, R.P., 1996. NK-2 homeobox genes and heart development. Dev. Biol. 178, 203–216.
Huppert, S.S., Le, A., Schroeter, E.H., Mumm, J.S., Saxena, M.T., Milner, L.A., Kopan, R., 2000. Embryonic lethality in mice homozygous for a processing-deficient allele of Notch1. Nature 405, 966–970.
Iso, T., Chung, G., Hamamori, Y., Kedes, L., 2002. HERP1 is a cell type-specific primary target of Notch. J. Biol. Chem. 277, 6598–6607.
Iso, T., Hamamori, Y., Kedes, L., 2003. Notch signaling in vascular development. Arterioscler. Thromb. Vasc. Biol. 23, 543–553.
Itoh, F., Itoh, S., Goumans, M.J., Valdimarsdottir, G., Iso, T., Dotto, G.P., Hamamori, Y., Kedes, L., Kato, M., Dijke Pt, P., 2004. Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. EMBO J. 23, 541–551.
Keyes, W.M., Logan, C., Parker, E., Sanders, E.J., 2003. Expression and function of bone morphogenetic proteins in the development of the embryonic endocardial cushions. Anat. Embryol. (Berl.) 207, 135–147.
Kokubo, H., Miyagawa-Tomita, S., Tomimatsu, H., Nakashima, Y., Saga, Y., Johnson, R.L., 2004. Targeted disruption of hesr2 results in atrioventricular valves anomalies, leading to the heart dysfunction and perinatal lethality. Circ. Res. 95, 540–547.
Krebs, L.T., Xue, Y., Norton, C.R., Shutter, J.R., Maguire, M., Sundberg, J.P., Gallahan, D., Closson, V., Kitajewski, J., Callahan, R., Smith, G.H., Stark, K.L., Gridley, T., 2000. Notch signaling is essential for vascular morphogenesis in mice. Genes Dev. 14, 1343–1352.
Kubalak, S.W., Miller-Hance, W.C., O'Brien, T.X., Dyson, E., Chien, K.R., 1994. Chamber specification of atrial myosin light chain-2 expression precedes septation during murine cardiogenesis. J. Biol. Chem. 269, 16961–16970.
Lawson, N.D., Scheer, N., Pham, V.N., Kim, C.H., Chitnis, A.B., Campos-Ortega, J.A., Weinstein, B.M., 2001. Notch signaling is required for arterial-venous differentiation during embryonic vascular development. Development 128, 3675–3683.
Li, L., Krantz, I.D., Deng, Y., Genin, A., Banta, A.B., Collins, C.C., Qi, M., Trask, B.J., Kuo, W.L., Cochran, J., Costa, T., Pierpont, M.E., Rand, E.B., Piccoli, D.A., Hood, L., Spinner, N.B., 1997. Alagille syndrome is caused by mutations in human Jagged1, which encodes a ligand for Notch1. Nat. Genet. 16, 243–251.
Lyons, I., Parsons, L.M., Hartley, L., Li, R., Andrews, J.E., Robb, L., Harvey, R.P., 1995. Myogenic and morphogenetic defects in the heart tubes of murine embryos lacking the homeobox gene Nkx2–5. Genes Dev. 9, 1654–1666.
McCright, B., Lozier, J., Gridley, T., 2002. A mouse model of Alagille syndrome: Notch2 as a genetic modifier of Jag1 haploinsufficiency. Development 129, 1075–1082.
Miyagawa-Tomita, S., Morishima, M., Nakazawa, M., Mizutani, M.T.K., 1996. Pathological study of Japanese quail embryo with acid alpha-glucosidase deficiency during early development. Acta Neuropathol. 92, 249–254.
Oda, T., Elkahloun, A.G., Pike, B.L., Okajima, K., Krantz, I.D., Genin, A., Piccoli, D.A., Meltzer, P.S., Spinner, N.B., Collins, F.S., Chandrasekharappa, S.C., 1997. Mutations in the human Jagged1 gene are responsible for Alagille syndrome. Nat. Genet. 16, 235–242.
Risau, W., 1997. Mechanisms of angiogenesis. Nature 386, 671–674.
Runyan, R.B., Markwald, R.R., 1983. Invasion of mesenchyme into three-dimensional collagen gels: a regional and temporal analysis of interaction in embryonic heart tissue. Dev. Biol. 95, 108–114.
Sakata, Y., Kamei, C.N., Nakagami, H., Bronson, R., Liao, J.K., Chin, M.T., 2002. Ventricular septal defect and cardiomyopathy in mice lacking the transcription factor CHF1/Hey2. Proc. Natl. Acad. Sci. U. S. A. 99, 16197–16202.
Shawber, C.J., Das, I., Francisco, E., Kitajewski, J., 2003. Notch signaling in primary endothelial cells. Ann. N. Y. Acad. Sci. 995, 162–170.
Shutter, J.R., Scully, S., Fan, W., Richards, W.G., Kitajewski, J., Deblandre, G.A., Kintner, C.R., Stark, K.L., 2000. Dll4, a novel Notch ligand expressed in arterial endothelium. Genes Dev. 14, 1313–1318.
Srivastava, D., Cserjesi, P., Olson, E.N., 1995. A subclass of bHLH proteins required for cardiac morphogenesis. Science 270, 1995–1999.
Srivastava, D., Thomas, T., Lin, Q., Kirby, M.L., Brown, D., Olson, E.N., 1997. Regulation of cardiac mesodermal and neural crest development by the bHLH transcription factor, dHAND. Nat. Genet. 16, 154–160.
Sugi, Y., Yamamura, H., Okagawa, H., Markwald, R.R., 2004. Bone morphogenetic protein-2 can mediate myocardial regulation of atrioventricular cushion mesenchymal cell formation in mice. Dev. Biol. 269, 505–518.
Takizawa, T., Ochiai, W., Nakashima, K., Taga, T., 2003. Enhanced gene activation by Notch and BMP signaling cross-talk. Nucleic Acids Res. 31, 5723–5731.
Timmerman, L.A., Grego-Bessa, J., Raya, A., Bertran, E., Perez-Pomares, J.M., Diez, J., Aranda, S., Palomo, S., McCormick, F., Izpisua-Belmonte, J.C., De La Pompa, J.L., 2004. Notch promotes epithelial–mesenchymal transition during cardiac development and oncogenic transformation. Genes Dev. 18, 99–115.
Villa, N., Walker, L., Lindsell, C.E., Gasson, J., Iruela-Arispe, M.L., Weinmaster, G., 2001. Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. Mech. Dev. 108, 161–164.
Wang, H.U., Chen, Z.F., Anderson, D.J., 1998. Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell 93, 741–753.
Xue, Y., Gao, X., Lindsell, C.E., Norton, C.R., Chang, B., Hicks, C., Gendron-Maguire, M., Rand, E.B., Weinmaster, G., Gridley, T., 1999. Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. Hum. Mol. Genet. 8, 723–730.
Yamagishi, H., Olson, E.N., Srivastava, D., 2000. The basic helix–loop-helix transcription factor, dHAND, is required for vascular development. J. Clin. Invest. 105, 261–270.
Zhong, T.P., Rosenberg, M., Mohideen, M.A., Weinstein, B., Fishman, M.C., 2000. Gridlock, an HLH gene required for assembly of the aorta in zebrafish. Science 287, 1820–1824.
Zhong, T.P., Childs, S., Leu, J.P., Fishman, M.C., 2001. Gridlock signalling pathway fashions the first embryonic artery. Nature 414, 216–220.