Targeted Disruption of hesr2 Results in Atrioventricular Valve Anomalies That Lead to Heart Dysfunction

Hiroki Kokubo, Sachiko Miyagawa-Tomita, Hirofumi Tomimatsu, Yasumi Nakashima, Makoto Nakazawa, Yumiko Saga, Randy L. Johnson

Abstract—Genes involved in the Notch signaling pathway have been shown to be critical regulators of cardiovascular development. In vitro studies have revealed that the Notch signaling pathway directly regulates transcription of hairy and enhancer of split-related (hesr) genes, encoding basic helix-loop-helix transcription factors. To assess the functional role of hesr genes in cardiovascular development, we generated mice with a targeted disruption of the hesr2 gene and used echocardiography to analyze heart function of the mutant mice. In the early postnatal period, a majority of hesr2 homozygous mice die as a result of congestive heart failure accompanied by pronounced heart enlargement. Transthoracic echocardiography on 5-day-old homozygous mice revealed tricuspid and mitral valve regurgitation and a dilated left ventricular chamber with markedly diminished fractional shortening of the left ventricle. The hemodynamic anomalies were accompanied by morphological changes, such as dysplastic atrioventricular (AV) valves, a perimembranous ventricular septal defect, and a secundum atrial septal defect. AV valve regurgitations attributable to dysplasia of the AV valves were most likely responsible for the heart dysfunction in hesr2 homozygous mice. These observations indicate that the Notch signaling target hesr2 plays an important role in the formation and function of the AV valves. In addition, hesr2 activity may be important for proper development of cardiomyocytes, thereby assuring normal left ventricular contractility. Because of the unique spectrum of cardiac anomalies expressed by hesr2-null mice, they represent a useful model system for elucidating the genetic basis of heart dysfunction. (Circ Res. 2004;95:540-547.)

Key Words: hesr2, notch signaling pathway ■ echocardiography ■ knockout mouse ■ heart anomaly

Notch signaling is an evolutionarily conserved mechanism for cell fate specification and embryonic development in organisms ranging from flies to humans.1 Notch encodes a transmembrane receptor with extracellular epithelial growth factor–like repeats and a short intracellular domain. After activation by its membrane-bound ligands, including Serrate/Jagged and Delta, the intracellular domain of the Notch receptor is released and enters the nucleus, where it regulates expression of downstream genes. Recently, several Notch signaling components have been reported to be responsible for cardiac development. For example, in humans, haploinsufficiency for Jagged 1 causes Alagille syndrome, in which development of the heart, liver, eyes, and limbs is affected. In addition, missense mutations in Jagged1 cause isolated congenital heart defects, such as pulmonic stenosis and tetralogy of Fallot.2–5 Mice that are double mutants of the Notch signaling components have been reported to as hairy, hrt, herp, chf, and gridlock, and their expression patterns have been described in the developing heart, somites, limb bud, and other tissues.9–13 In tissue culture assays, hesr gene products have been reported to be transcriptional repressors of the Notch signaling pathway.14–17 Gridlock, a zebrafish homologue of hesr2, has been shown to regulate arterial versus venous differentiation.18 However, hesr2-knockout mice do not exhibit a phenotype similar to that observed for zebrafish gridlock mutants.19–21 Instead, it was suggested that hesr2 plays a role in cardiac morphogenesis, especially in ventricular septum formation in mice.

Although previous characterization of hesr2 mutant mice revealed cardiac anomalies,19–21 the significant incidence of perinatal lethality suggested that additional cardiac defects might be present that were not revealed by histological and molecular marker analysis. To further assess the cardiac
phenotype of hesr2 null mutant mice, we used echocardiography to analyze the heart dysfunction of hesr2 homozygous neonates. Noninvasive assessment of cardiovascular physiology is necessary to better understand cardiac function in animal models. In particular, echocardiography is an advantageous and practical method to analyze cardiac function in mouse models of congenital cardiovascular anomalies. By using these methods, our study revealed that hesr2 mutant neonatal mice display tricuspid and mitral valve regurgitations together with a dilated left ventricular (LV) chamber accompanied by markedly reduced fractional shortening. Atrioventricular (AV) valve regurgitation, resulting from AV valve dysplasia, led to congestive heart failure in hesr2 mutant mice. In addition, our results suggest that lack of hesr2 induced the impairment of LV contractility. These data indicate that hesr2 plays an important role in the formation of the tricuspid and mitral valves and in cardiomyocyte development.

Materials and Methods

Identification of hesr2 Gene and Sequence Analysis

The expressed sequence tag clones (IMAGE 1261317 5' and 1331800 5'), named murine hesr2, were found by searching the GenBank Sequence Database with the entire coding region of hesr1. These sequences were cloned on both strands by automated DNA sequencing. Murine hesr2 appears in the DNA Data Bank of Japan, European Molecular Biology Laboratory, and GenBank with accession number AB093589.

Generating Mice That Have a Targeted Disruption of hesr2

The RPCI-22 mouse BAC library (BACPAC Resources) was screened with the hesr2 cDNA, and 4 specifically hybridizing BAC clones were identified. A targeting vector was designed and constructed that deletes the entire coding region (supplemental Figure 1 in the online data supplement available at http://circres.ahajournals.org). Two independent correctly targeted lines (lines 9 and 163) were identified from electroporated AB1 embryonic stem cells, and chimeras from both lines transmitted the targeted allele through the germ line. For genotyping, a polymerase chain reaction (PCR) method was established to detect the wild-type allele, using either 5' forward: 5'-CATTCCCCAGCCTCCTGGCAACGCCTCTCG-3'/5' reverse: 5'-GACCTCCTGGCAGATTGCCG-3' or 3' forward: 5'-CAATGTGTCAAGCTGCAAGATAAG-3'/5' reverse: 5'-GGCACAAGATTAGTGCTCTCC-3'; primer set. For the targeted allele using either 5' forward/Neo: 5'-CGACCCACCTCCGCTCAG-3' or 3' reverse/PGK: 5'-CCAGGAGGGCCACTTGTG-TAGCG-3' primer set. The neo cassette was removed by crossing hesr2 neo-positive mice with CAG-Cre mice, and the genotype was determined by using the 5'forward/3' reverse primer set.

Echocardiography

Transtracheal echocardiography was performed by using a PMS HDI-5000 SonoCT ultrasound imaging system with a 15-MHz linear-array transducer (PMS CL15-7; Philips Medical Systems). The pulsed Doppler operating frequency was set at 6 MHz to measure blood flow velocities. The pulse repetition frequency was set at 12 kHz to achieve a maximum measurable velocity of 180 cm/s. To assess LV function, echocardiograms of conscious 5-day-old mice were obtained to avoid any cardiodepression produced by anesthesia. The 5-day-old mice were restrained on a hand-made urethane foam board with rubber bands. ECG leads were attached using tungsten wires placed on the 4 limbs. Normal body temperature was maintained with a lamp and monitored by a digital thermometer (4000A; YSI Corp.) and disposable thermosensor (Sheridan Catheter Corp.). To ensure accurate and unbiased analysis, 2 independent investigators read the tracings. By using M-mode tracings, echocardiographic measurements of LV internal dimensions (LVID) were recorded at end-diastole (LVIDd) and end-systole (LVIDs) from 5 consecutive cardiac cycles by using the leading-edge method. Two-dimensional guided M-mode echocardiography of the LV at the papillary muscle level was obtained from the short-axis 2D view. An index of systolic function defined as the percentage of LV fractional shortening (LVFS) with the standard equation as follows: %LVFS = (LVIDd-LVIDs)/LVIDd × 100.

Histological Analysis of hesr2 Mutants

Mice with a mixed B6/129Sv genetic background were analyzed. The animals were genotyped by Southern hybridization, PCR, or both. In situ hybridization was performed as described previously. For observation under light microscopy, the hearts, liver, and lung were dissected and fixed in 4% paraformaldehyde in PBS. Fixed tissues were embedded in paraffin, sectioned at 7 μm, and stained with hematoxylin-eosin. Preparation, sectioning, and analysis of samples for transmission electron microscopic (TEM) were performed using standard methods.

Reverse Transcription–Polymerase Chain Reaction

Total RNA was isolated from the hearts of 5-day-old hesr2 homozygous and heterozygous mice using a RNAeasy mini kit (Qiagen). The reverse transcription reaction was performed with SuperScriptII (Invitrogen), and the cDNA products were amplified at 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s for 20 to 25 cycles using RTaq (Takara). Sequences of primers were as follows: myosin light chain (MLC); 5'-GGCTGATCCTGGAAAGACCAT-3', 5'-TTATTGTGGCACACGCCCTGG-3', myosin heavy chain (α-MHC); 5'-GAAGACGCTGGGAAGGGGGA-3', 5'-GTGAGCTGAGATTCCCTCAGG-3', β-MHC; 5'-CCCTGCGGGAAGCA-TCCAT-3', 5'-CAGACTCTGGAGGCTTCTC-3', cardiac actin; 5'-CCCTGTTATGGCCGATGTGTAAC-3', 5'-CCAAGAACACAATACGTTATCC-3', skeletal actin; 5'-CCCTGGTATCGCTGACCATGCAG-3', atrial natriuretic factor (ANF); 5'-TGGTGTCGTCCTC-3', 5'-TTTATTT-3'; cardiac MHC; 5'-GGTGA- CACATGAGTAACCTGCTTCTC-3', primer set. For the targeted allele using either 5' forward/Neo: 5'-CGACCCACCTCCGCTCAG-3' or 3' reverse/PGK: 5'-CCAGGAGGGCCACTTGTGGTAGCG-3' primer set. The neo cassette was removed by crossing hesr2 neo-positive mice with CAG-Cre mice, and the genotype was determined by using the 5'forward/3' reverse primer set.

Results

Growth Retardation and Heart Defects in hesr2 Homozygous Mice

From intercrosses of heterozygous mice, homozygous hesr2 progeny were recovered at numbers lower than those predicted by Mendelian ratios at 10 days after birth (supplemental Table S1). However, homozygous mutant embryos were isolated at the expected frequency at 10.5 days postcoitum. Hence, homozygosity at the hesr2 locus leads to lethality with incomplete penetrance. Some homozygotes were viable and fertile. However, most homozygotes exhibited growth retardation and died within the first 10 days after birth (Figure 1A). In the affected homozygotes, the atria and ventricles were markedly enlarged, and the heart occupied almost the entire thoracic cavity (Figure 1B and 1C). The ratios of heart weight to body weight and lung weight to body weight were significantly increased in 5-day-old mice (Table 1). Hearts of hesr2 heterozygotes and homozygotes had the same d-ven-
tricuspid loop as a normal heart. Although the tricuspid and mitral valves had similar dimensions in both heterozygous and wild-type mice (Figure 1D), the tricuspid valve of hesr2 homozygous mice was significantly smaller (Figure 1E, arrowhead) than that of the mitral valve. The number of valvular leaflets in homozygous mice was normal (Figure 1E). These observations suggest that heart malformation was one of the main causes of growth retardation and early mortality in hesr2 homozygous mice.

Figure 1. Growth retardation and heart anomalies in 5-day-old hesr2 homozygous mice. A, The homozygote exhibits growth retardation. Bar=1 cm. B and C, Frontal views of the hearts. Homozygous heart exhibits enlargement and occupies entire thoracic cavity (C). Bar=1 mm. D and E, Comparison of the AV valves from the cranial side after removal of most of the atrial and vessel components. Dimension of the tricuspid valve (TV) of the homozygous heart is smaller (E, arrowhead). AO indicates aorta; LA, left atrium; MV, mitral valve; PT, pulmonary trunk; RA, right atrium.

Table 1. Analysis of Body and Heart Weight in hesr2 Mice at 1 and 5 Days After Birth

<table>
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<tr>
<th>Age (dpp)/Genotype</th>
<th>Body Weight (g)</th>
<th>Heart Weight (mg)</th>
<th>Ratio (HW/BW %)</th>
<th>Lung Weight (mg)</th>
<th>Ratio (LW/BW %)</th>
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<tr>
<td>+/− (n=4)</td>
<td>1.47±0.1</td>
<td>17.5±4.2</td>
<td>1.17±0.22</td>
<td>ND</td>
<td>ND</td>
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<tr>
<td>−/− (n=7)</td>
<td>1.38±0.1</td>
<td>16.4±3.9</td>
<td>1.18±0.25</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td></td>
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<tr>
<td>+/− (n=9)</td>
<td>2.32±0.37*</td>
<td>10.6±9.8*</td>
<td>1.22±0.27*</td>
<td>96.62±11.3*</td>
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<td>−/− (n=7)</td>
<td>1.65±0.17*</td>
<td>46.1±7.4*</td>
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<td>61.7±10.8*</td>
<td>4.13±0.85*</td>
</tr>
</tbody>
</table>

Values are means±SE.
Along each column, the asterisk indicates a significant difference (P<0.05) among the values of the same parameter.
dpp indicates days postpartum; BW, body weight; HW, heart weight; LW, lung weight.

Regurgitation of the AV Valves in hesr2 Mutant Mice

We used echocardiography to analyze the heart function in hesr2 heterozygous and homozygous mice. Typical 2D and M-mode echocardiograms of the LV were captured (Figure 2A and 2B) in the parasternal short-axis view (Figure 2C). The LVIDd and LVIDs in homozygous mice were larger than those in heterozygous mice. LVFS, an index of systolic function, was decreased in homozygous mice compared with
that in heterozygous mice (Figure 2D), indicating that LV systolic function was impaired in homozygous mice.

Pulsed-wave Doppler recordings at the RV and LV were performed at sampling points indicated in the 2D images from the apical 4-chamber views (Figure 3A and 3E). The pulsed Doppler waveforms at the RV and LV inflow tracts in heterozygous mice showed a biphasic pattern, an early diastolic (Ed) velocity, and an atrial systolic (As) velocity during diastole (Figure 3B and 3F). Tricuspid flow waveforms in homozygous mice were similar to those of heterozygous mice during diastole, whereas Ed and As waveforms were sometimes fused (Figure 3C). During systole, abnormal downward waveforms were observed at the tricuspid orifice, which were similar to the regurgitation pattern in human patients (Figure 3D). Such a wave pattern was not observed in heterozygous mice. In flow recordings in the LV, the mitral flow waveforms were normal during diastole, although Ed and As waves were sometimes fused (Figure 3G). The abnormal downward waveforms observed at the LV inflow tract during systole were attributed to mitral regurgitation (Figure 3H). To observe flow through the ventricular septal defect (VSD) clearly, the pulsed-wave Doppler flow was recorded at sampling points indicated in the 2D images from the long-axis view (Figure 3I). Abnormal upward waveforms observed during systole (Figure 3J) were attributed to a VSD. To evaluate LV function, we measured diastolic inflow LV parameters (Table 2). Peak velocities of the early ventricular filling wave (E wave) and the late ventricular filling wave attributable to atrial contraction (A wave) in homozygous mice were significantly (13%) increased compared with those of heterozygous mice ($P<0.01$). However, the E wave/A wave ratio was the same in heterozygous and homozygous mice, indicating that LV diastolic function was not impaired.

Aortic and pulmonary outflow tracts in heterozygous and homozygous mice also showed normal waveform patterns (data not shown).

Color flow Doppler echocardiography, in which flow movement toward the transducer is shown in red and that away from the transducer is shown in blue, was performed. Normal color flow patterns were seen in the heterozygous mice (Figure 4A and 4B; supplemental Movie I). However, in homozygous mice, mitral valve regurgitation was captured as a red, blue, and yellow mosaic pattern (Figure 4C and 4D; supplemental Movie II). We observed blood flow from the LV to the RV through VSD at a semisubcostal position in the semilong-axis view (Figure 4E and 4F, supplemental Movie III). These data suggest that mitral and tricuspid valve regurgitation and VSD were present in hesr2 homozygous mice.

**Morphological and Histological Analyses**

The defects detected by echocardiography in hesr2 homozygotes were analyzed further by gross morphology and histological methods. These studies revealed that the orifice of the tricuspid valve was small compared with that of the mitral valve. The defects in homozygous mice were analyzed further by gross morphology and histological methods. These studies revealed that the orifice of the tricuspid valve was small compared with that of the mitral valve.

**TABLE 2. Comparison of LV Diastolic Inflow Parameters**

<table>
<thead>
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<th>Genotype</th>
<th>+/− (n=6)</th>
<th>−/− (n=4)</th>
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<tr>
<td>Heart rate (bpm)</td>
<td>368±12*</td>
<td>312±31*</td>
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<tr>
<td>Peak E in LV (cm/s)</td>
<td>39.8±4.76</td>
<td>51.1±4.49</td>
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<tr>
<td>Peak A in LV (cm/s)</td>
<td>40.5±6.12</td>
<td>50.3±5.93</td>
</tr>
<tr>
<td>Peak E/A ratio</td>
<td>1.00±0.11</td>
<td>1.11±0.22</td>
</tr>
</tbody>
</table>

Values are means±SE.

Peak E indicates peak velocity of the early ventricular filling wave (E wave); Peak A, peak velocity of the late ventricular filling wave attributable to atrial contraction; Peak E/A ratio, the ratio of peak E to peak A.

Along each column, the asterisk indicates a significant difference ($P<0.05$) among the values of the same parameter.
valve in the homozygous hesr2 mutant mice (Figure 5B, arrow; compare with Figure 5A). In addition, the left and right atria were enlarged. The mitral and tricuspid valves in the hearts of homozygous mice were dysplastic compared with those of heterozygous mice (Figure 5E; compare with Figure 5C and 5D), suggesting that the AV valve regurgitation found by echocardiography was caused by dysplastic AV valves. The endocardium of the left atrium and left ventricle in homozygous mice was thickened compared with the monolayer in heterozygous mice and contained elastic and collagen fibers (supplemental Figure S2), indicating severe mitral regurgitation. Obvious secondum atrial septal defect (ASDII) and perimembranous VSD (Figure 5E and 5F) were observed in homozygous mice, supporting the echocardiographic findings. In contrast, the semilunar valves had normal 3 leaflets, although these were relatively dysplastic (Figure 5I and J; compare with Figure 5G and 5H). Histological analysis of the lung and liver in hesr2 homozygous mice revealed congestion; however, their bile ducts were normal (data not shown).

Because the impaired LV systolic function that was revealed by reduced fractional shortening could have been

Figure 4. AV valve regurgitation and VSD revealed by color flow Doppler recording in 5-day-old hesr2 homozygous mice. A, C, and E, Typical color flow images in heterozygous (A) and homozygous (C and E) mice. B, D, and F, Schematic drawings of the blood flow pattern at the LV and right ventricular (RV) inflow tracts or VSD are shown in A, C, and E, respectively. A and B, Inflow tracts are recorded as red during diastole in heterozygous mice. C and D, Turbulent color flow at the mitral orifice during systole demonstrates mitral regurgitation. E and F, Turbulent color flow from the LV to the RV during systole indicates VSD. LA indicates left atrium; RA, right atrium.

Figure 5. Histological analysis of heart anomalies in hesr2 homozygous mice. Hematoxylin-eosin staining (A–J) and TEM pictures (K and L) of the 5-day-old heterozygous (A, C, D, G, H, and K) and homozygous (B, E, F, I, J, and L) heart. A and B, The homozygous heart (B) shows enlargement of both atria and ventricles compared with that of heterozygous heart (A). The arrow in B indicates tricuspid valve stenosis. C–F, Tricuspid valves (TVs) and mitral valves (MVs) are dysplastic in the homozygote (E) compared with that of heterozygote (C and D). An ASDII and a perimembranous VSD are observed (arrows in E and F, respectively). Bar=100 μm. G–J, Aortic (Ao) and pulmonary (PulV) valves have 3 leaflets (arrows) in both heterozygote (G and H) and homozygote (I and J). Bar=100 μm. K, Normal cytoarchitecture of cardiomyocyte with aligned Z-bands, well-organized myofibrils (Mf), and ordered many mitochondria (Mt) are observed in the heterozygous heart. L, Cardiomyocytes of homozygote contains fewer, disorganized myofibrils that are replaced with a decreased number of irregular-shaped and vacuolized mitochondria and numerous glycogen particles (Gly). The nucleus (Nu) had a nucleolus with relatively low electron density and many heterochromatin. Bar=1.0 μm. IVS indicates interventricular septum; LA, left atrium; RA, right atrium; LV, left ventricle; RV, right ventricle; PT, pulmonary trunk.
caused by abnormal cardiomyocytes, the ultrastructure of cardiomyocytes was analyzed with TEM. Well-organized myofibrils and many round mitochondria were observed in cardiomyocytes from heterozygous mice (Figure 5K). In contrast, cardiomyocytes from homozygous mice contained many disorganized myofibrils, vacuolized and irregular-shaped mitochondria, and numerous glycogen particles (Figure 5L), although hypertrophy was not observed. These observations suggested that abnormal cardiomyocytes could be a significant cause of impaired LV systolic function in the hearts of hesr2 homozygote mice.

Changes of Cardiac Gene Expression in the Hearts of hesr2 Homozygous Mutant Mice

The morphological finding of an enlarged heart and reduced (15%) heart rate (P<0.05) in homozygous mice (Table 2) led us to investigate changes in cardiac gene expression that accompany cardiac hypertrophy and cardiomyopathy.24–28 Generally, embryonic β-MHC is replaced by α-MHC after birth. However, the cardiac expression level of β-MHC in 5-day-old homozygous mice was 1.7× greater than that of heterozygous mice (Figure 6). Reduced contractile velocity of the heart has been correlated with increased β-MHC expression,29,30 suggesting that this may contribute to the decreased heart rate that we observed in hesr2 homozygotes. Expression levels of ANF are affected by changes in the tension sensed by blood vessels.31 In the hearts of homozygous mice, ANF expression was 2.4× greater than in heterozygous mice.

However, the expression level of SERCA, which regulates Ca\(^{2+}\) for muscle relaxation, was not changed, indicating that the diastolic heart function in homozygous mice is unaffected. There was no significant change in the expression level of C-actin or S-actin, which encode structural proteins of the heart. Our findings support the idea that anomalies of hemodynamics may be responsible in part for the observed changes in gene expression in hesr2 homozygous hearts.

Discussion

In our study, echocardiographic assessment of hesr2 mutant neonatal mice revealed dysfunction of the AV valves and reduced contractility of the LV with decreased LVFS. Anatomical observations of malformed AV valves, VSD, and ASDII confirmed the echocardiographic findings. Although previous reports described cardiac anomalies of hesr2 knockout mice,19–21 they did not describe the cardiac hemodynamics of the mutant mice. Previous reports suggested that abnormalities in cardiac formation are the direct consequence of lack of hesr2 function, although these abnormalities might indirectly result from alterations in hemodynamics. Moreover, the cause of death in hesr2 mutant mice has been attributed solely to abnormalities of cardiac morphology. In this study, we examined hesr2 mutant mice by echocardiography, which clearly revealed abnormal cardiac hemodynamics. Our findings suggest that the early mortality in hesr2-null mice was initiated by mitral regurgitation, resulting in thickening of the endocardium of the left atrium and ventricle. We further suggest that the valve defects led to LV failure and pulmonary congestion in hesr2 homozygous mice. Moreover, we observed congestion, edema, and collapse of some alveoli in the lungs of homozygous mice and believe that these defects eventually caused congestive heart failure and dyspnea leading to death. Although Sakata et al have pointed out that isolated VSD is the main cardiac defect in hesr2 mutant mice,21 it would be difficult to attribute the high rate of perinatal lethality of hesr2 homozygotes to this single defect.

Function of hesr2 in Heart Development

Notch signaling is required for cardiovascular development. Jagged1 is a gene that causes Alagille syndrome.4 The phenotype of Jagged1/Notch2 double heterozygous mutant mice mimics the phenotype of human Alagille syndrome.7 Because hesr2 has been implicated as a target gene of the Notch signaling cascade,15 it might function downstream of the Jagged1/Notch2 signaling pathway in cardiac development. Donovan et al pointed out this possibility on the basis of their observation of stenosis of the pulmonary trunk in hesr2-null mice that is similar to that of Jagged1/Notch2 double heterozygous mutant mice.19 However, pulmonary stenosis was not present in our hesr2 mutant mice. Jagged1 is expressed only in the pulmonary trunk and aorta, whereas hesr2 is expressed in the ventricular myocardium. Well-formed bile ducts were observed in hesr2 homozygous mice, although Jagged1/Notch2 double heterozygous mutant mice lack bile ducts.7 These observations indicate that hesr2 may not work downstream of the Jagged1/Notch2 signaling pathway. Because we have shown previously that hesr1 is a target...
of DLL1 signaling.8 hesr2 might be downstream of Delta/Notch but not the Jagged (Serrate)/Notch signaling pathway.

The major defect we found in our hesr2 mutant mice is dysfunction and deformation of the AV valves, which are derived from cushion tissues.32 Interaction with the myocardium and endocardium is essential for formation of endocardial mesenchyme within the cushion matrix. These mesenchymal cells subsequently proliferate and differentiate to form valvular leaflets. In the heart, hesr2 expression is detected in whole myocardium in 8.5 days postcoitum and later is restricted to ventricular myocardium.12,15 In hesr2 mutant mice, important signals between endocardial and myocardial cells may be lacking, leading to incomplete AV valve formation. Further research is needed to elucidate the mechanism by which hesr2 contributes to AV valve formation.

The gridlock gene, a zebrafish homologue of hesr2,18 plays a role in control of arterial differentiation.13 However, no corresponding phenotype was observed in hesr2 mutant mice in our study or other previous studies.20 Because the expression of hesr1 and hesr2 overlaps in the great arteries, we speculate that some hesr family members have redundant functions with regard to blood vessel development.

Cardiomyocyte Defects in hesr2-Null Mice

An enlarged heart accompanied by reduced LV contractile function was observed in hesr2 homozygous mice. Humans with mitral regurgitation usually exhibit enlarged heart size and normal-to-increased LV systolic function.53 Therefore, the cause of decreased LVFS in hesr2 mutant mice is probably not limited to AV valve regurgitation. We speculate that a main cause of decreased LV systolic function in hesr2 mutant mice is the presence of abnormal cardiomyocytes, which must occur independently from AV valve regurgitation. In fact, TEM analysis of cardiomyocytes from hesr2 homozygous mice revealed abnormal mitochondria, abnormal accumulation of glycogen particles, and disorganized myofibrils (Figure 5L). These abnormalities might induce metabolic defects that cause a lack of energy for muscle contraction, which would lead to decreased LVFS. These alterations likely impact cardiac gene expression, including increased expression of β-MHC and ANF (Figure 6). It is known that increased production of ANF is caused by cardiac hypertrophy,34,35 as seen in our hesr2 mutant mice, but that increased expression of β-MHC and ANF genes is observed with cardiomyopathy.18 Gessler et al speculated that myofibrillar disorganization leads to heart enlargement and diagnosed hesr2 mutant mice as having hypertrophic cardiomyopathy.20 Although our TEM analysis revealed abnormalities in cardiomyocytes from homozygous mice, we found no histological characteristics that suggested hypertrophic cardiomyopathy such as hypertrophy or disarray of cardiomyocytes or fibrosis. Hence, we could reach the conclusion that hesr2 mutant hearts were not diagnosed as cardiomyopathy. Rather, we speculate on the basis of the data presented herein that the hemodynamic abnormalities in hesr2 homozygotes were caused by congenital heart defects, which resulted in heart enlargement after birth, and by intrinsic myocardial anomalies, which, in turn, induced LV contractile dysfunction.

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References

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Supplemental data

Supplemental table S1.

Number of progeny obtained by intercrossing heterozygous hesr2 mice.

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<td>19 (17)</td>
<td>35 (34)</td>
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Two independent lines, 9 and 163 were examined at 10 day after postcoietum (dpc) and 10 day after postpartum (dpp). Expected numbers are parenthesized in the panel.

**Supplemental Figure S1.** Target strategy of hesr2 gene.

To analyze the function of hesr2 gene in mouse development, a targeting vector was constructed. Almost entire coding region was replaced to the lox neo cassette. Southern blot analysis using 5' probe showed that the locus was targeted. Null-locus was created by intercross with CAG-Cre mouse and the complete deletion of the normal allele confirmed by PCR analysis.
Supplemental Figure S2. Fibrosis in thickened endocardium of the left atrium and ventricle in *hesr2* homozygote.

The 5-day-old heterozygous (A, C-H) and homozygous (B, I-N) hearts were sectioned transversely and stained with hematoxylin-eosin (A-C, F, I, L), Victoria blue-van Gieson (D, G, J, M), and Masson-Trichrome (E, H, K N). (A, B) Approximate enlarged points of the left atrium (LA) and left ventricle (LV) are indicated with rectangles. (C-H) The LA (C-E) and LV (F-H) of the heterozygous heart show monolayer of the endocardium (arrowheads in C, F). (I-N) Endocardium of the LA (arrowheads in I) and LV (arrowheads in L) in the homozygous heart are thickened, and contain more elastic fibers (J, M) and collagen fibers (K, N) showing in blue color, compare with heterozygote (D, E, G, H). A thrombus is also observed (arrow in L). RA, right atrium; RV, right ventricle.

Supplemental movie 1

Color Doppler echocardiography recording from the apical 4-chamber view in the subcostal position of the transducer in the heart of 5-day-old heterozygous mouse. The
apical 4-chamber view is characterized by visualizing the left atrium on the right lower side, and the left ventricle on the right upper side. On the left lower side, the right atrium is seen and the right ventricle is seen on the left upper side (Figure 4 B).

**Supplemental movie 2**

Color flow Doppler echocardiography recording from the apical 4-chamber view in the subcostal position of the transducer in the heart of 5-day-old homozygous mouse. View of the heart is illustrated in Figure 4D. Mitral valve regurgitant flow is recorded during systole.

**Supplemental movie 3**

Color flow Doppler echocardiography recording from the semi-long axis view in the semi-subcostal position of the transducer in the heart of 5-day-old homozygous mouse. View of the heart is illustrated in Figure 4F. The turbulent flow through VSD from the LV to the RV is recorded during systole.
Wild-type locus

Targeting vector

Targeted locus

Null locus

Diagram showing the structure of genetic loci with annotations for probe binding and genetic markers.