Deficiency of Nectin-2 Leads to Cardiac Fibrosis and Dysfunction Under Chronic Pressure Overload


Abstract—The intercalated disc, a cell-cell contact site between neighboring cardiac myocytes, plays an important role in maintaining the homeostasis of the heart by transmitting electric and mechanical signals. Changes in the architecture of the intercalated disc have been observed in dilated cardiomyopathy. Among cell-cell junctions in the intercalated disc, adherens junctions are involved in anchoring myofibrils and transmitting force. Nectins are Ca²⁺-independent, immunoglobulin-like cell-cell adhesion molecules that exist in adherens junctions. However, the role of nectins in cardiac homeostasis and integrity of the intercalated disc are unknown. Among the isoforms of nectins, nectin-2 and -4 were expressed at the intercalated disc in the heart. Nectin-2–knockout mice showed normal cardiac structure and function under physiological conditions. Four weeks after banding of the ascending aorta, cardiac function was significantly impaired in nectin-2–knockout mice compared with wild-type mice, although both nectin-2–knockout and wild-type mice developed similar degrees of cardiac hypertrophy. Banded nectin-2–knockout mice displayed cardiac fibrosis more evidently than banded wild-type mice. The disruption of the intercalated discs and disorganized myofibrils were observed in banded nectin-2–knockout mice. Furthermore, the number of apoptotic cardiac myocytes was increased in banded nectin-2–knockout mice. In the hearts of banded nectin-2–knockout mice, Akt remained at lower phosphorylation levels until 2 weeks after banding, whereas e-Jun N-terminal kinase and p38 mitogen-activated protein kinase were highly phosphorylated compared with those of wild-type mice. These results indicate that nectin-2 is required to maintain structure and function of the intercalated disc and protects the heart from pressure-overload–induced cardiac dysfunction. (Hypertension. 2009;54:825-831.)

Key Words: nectin-2 ■ cell adhesion molecule ■ intercalated disc ■ heart failure ■ pressure overload

In the mature heart, the intercalated discs are located at the bipolar ends of the rod-shaped cardiac myocytes and mediate mechanical and electric coupling between adjacent cardiac myocytes. The intercalated disc mainly consists of 3 junctional complexes: adherens junctions (AJs), desmosomes, and gap junctions. The role of AJs in the intercalated disc is to anchor myofibrils and to transmit the force developed by the contracting myofibril to neighboring cardiac myocytes. Desmosomes provide structural support by anchoring the intermediate filament system, and gap junctions provide intercellular communication via ions and small molecules. Changes in the intercalated disc architecture have been reported in mouse models for dilated cardiomyopathy (DCM), such as muscle LIM protein knockout (KO) mice and tropomodulin-overexpressing mice. On the other hand, the targeted ablation of N-cadherin, which is predominantly localized at AJs in the intercalated disc, in the murine adult was shown to result in the disassembly of the intercalated disc structure and to exhibit a DCM-like phenotype. There are also several reports that the conditional KO mice of other AJ proteins in the heart, such as α-E-catenin, mXin-α, and vinculin, showed cardiac dysfunction with the disruption of the intercalated disc. These findings suggest that AJ proteins are indispensable for the homeostasis of the intercalated disc and cardiac function.

Nectins are Ca²⁺-independent, immunoglobulin-like cell-cell adhesion molecules found in AJs. Nectins are a family composed of 4 members, nectin-1, -2, -3, and -4. All of the

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nectins form homo-cis-dimers and then homo- or hetero-trans-dimers through the extracellular region in a Ca\(^{2+}\)-independent manner, causing cell-cell adhesion. The cytoplasmic region is associated with the actin cytoskeleton through afadin, a nectin and actin filament (F-actin) binding protein. Nectins first form cell-cell adhesion and recruit cadherins to the nectin-based cell-cell adhesion sites, and this leads to the formation of AJs. Thus, nectins are important for the formation of AJs.

Among the isoforms of nectins, nectin-2 is known to exist in the heart.\(^{10}\) However, the role of nectin-2 in the heart remains to be elucidated. The present study shows critical roles for nectin-2 in the heart under pressure overload.

Materials and Methods

Animal Experiments

Animal care and experiments were conducted according to guidelines for animal experimentation at Kobe University. Nectin-2–KO mice were generated as described previously, but the cardiac phenotype of the mice was not assessed in detail.\(^{11}\) Ascending aortic constriction was performed in male wild-type (WT) or nectin-2–KO mice at the age of 11 weeks, as described previously,\(^{12}\) except that the aorta was ligated with a 26-gauge needle. The mice were euthanized at the indicated time points.

The following materials and methods used for this study are described in detail in the online Data Supplement (please see http://hyper.ahajournals.org): echocardiography and electrocardiography, antibodies, histological analysis, Western blotting, real-time PCR, and statistics.

Results

Expression and Localization of Nectins and Other Proteins in the Intercalated Disc

To examine the expression and localization of nectins in the murine heart, immunostaining of nectin-1, -2, -3, and -4 was performed. As shown in Figure 1A, nectin-2 and -4 were specifically expressed in the intercalated disc. Bar, 50 μm. B, Nectin-2 was coexpressed with N-cadherin, which localized to the AJ, in the WT mouse hearts but not in the nectin-2–KO mouse hearts. Bar, 10 μm.

Cardiac Hypertrophy and Dysfunction Induced by Pressure Overload in Nectin-2–KO Mice

The functional phenotypes of the nectin-2–KO mouse heart were investigated under physiological conditions. The heart weights of nectin-2–KO mice were similar to those of the WT mice (Figure S3, see sham-operated groups), and normal systolic function assessed by echocardiography was observed in nectin-2–KO mice (Table and Figure 2A, see sham-operated groups), suggesting that nectin-2–KO mice exhibit normal cardiac function under physiological conditions. The function of nectin-2 was then explored in the heart under pathological conditions. A left ventricular (LV) pressure-overload model was used in nectin-2–KO and WT mice to assess cardiac dimensions and function. The heart weights of WT and nectin-2–KO mice were

<table>
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<th>KO</th>
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<td>BW, g</td>
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<td>LVE SD, mm</td>
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<td>1.60 ± 0.09*</td>
</tr>
<tr>
<td>FS, %</td>
<td>51 ± 1</td>
<td>43 ± 1*</td>
</tr>
<tr>
<td>AoPg, mm Hg</td>
<td>5.2 ± 1.0</td>
<td>28.1 ± 2.5*</td>
</tr>
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</table>

BW indicates body weight; IVS, interventricular septum thickness; LVPW, LV diastolic posterior wall thickness; LVEDD, LV end-diastolic dimension; LVE SD, LV end-systolic dimension; FS, fractional shortening; AoPg, aortic pressure gradient. Values are expressed as mean ± SE.

*P < 0.05 vs sham.

†P < 0.05 vs banded WT mice.
increased significantly, and the degrees of hypertrophy were similar between WT and nectin-2–KO mice after aortic banding (Figure S3). Histological analysis showed that the increase in the cross-sectional areas of cardiac myocytes in nectin-2–KO mice under chronic pressure overload was similar to that in WT mice (Figure 2B). Echocardiographic measurements revealed similar extents of increases in LV wall thickness in nectin-2–KO mice with aortic banding compared with corresponding WT mice (Table and Figure 2A). However, LV end-diastolic dimension tended to be larger, and LV end-systolic dimension was significantly greater in banded nectin-2–KO mice than in those in banded WT mice (LV end-diastolic dimension: 2.03±0.10 versus 1.60±0.09 mm; banded nectin-2–KO mice versus banded WT mice: P<0.05). Systolic function was significantly depressed in banded nectin-2–KO mice compared with banded WT mice (percentage of fractional shortening: 33±2 versus 43±1; banded nectin-2–KO mice versus banded WT mice: P<0.05; Table and Figure 2A). Furthermore, a significant increase in lung weight was observed in banded nectin-2–KO mice, whereas the lung weight in WT mice was not changed after banding, indicating that pulmonary congestion was induced by pressure overload in nectin-2–KO mice but not in WT mice (Figure 2C). The mRNA expression of atrial natriuretic factor (ANF), a marker of cardiac stress that is elevated in the heart in pathological hypertrophy or heart failure,12,13 was significantly increased in the myocardium of hypertrophic cardiomyopathy, DCM, and myocardial infarction and is considered to induce cardiac fibrosis.14,15 The levels of transforming growth factor-β1 mRNA in the banded nectin-2–KO mouse hearts were increased twice as much as those in the banded WT mouse hearts (Figure 2C).

Furthermore, to analyze the pathological features of diffuse and multifocal fibrosis in banded nectin-2–KO mice, cell death was assessed by TUNEL staining of cardiac sections. As shown in Figure 3D, the number of TUNEL-positive nuclei was more prominently increased in the banded nectin-2–KO mouse hearts than in the banded WT mouse hearts. These results suggest that nectin-2 has a protective role in cardiac myocytes against chronic pressure overload.

**Disruption of Intercalated Discs in Nectin-2–KO Mice by Pressure Overload**

Immunoblotting and immunofluorescence confocal microscopy were performed to examine the expression of component proteins and structural changes of the intercalated disc after banding. N-cadherin is well known to be present in the transmembrane part of AJs. On the cytoplasmic side of AJs, β-catenin binds to N-cadherin via the cytoplasmic domain,
whereas it interacts with α-catenin at the other end. Then, α-catenin contacts the actin cytoskeleton. Deficiency of nectin-2 did not affect N-cadherin expression or distribution, even after banding (Figures 4A and S4). The notable change observed in the banded nectin-2–KO mouse hearts was the broader fluorescent line of the intercalated disc (Figure 4B). Such phenomena were reported in the hearts of DCM mice because of the higher degree of convolution of the plasma membrane in the intercalated disc.3 For other AJ proteins, the expression and distribution of afadin, β1-integrin, and α-catenin in the heart did not differ between WT mice and nectin-2–KO mice after banding, whereas broad intercalated discs were observed similarly (Figure 4A and 4B). On the other hand, a gap junction protein (Cx43) expression in the banded hearts was reduced in both WT and nectin-2–KO mice (Figures S4 and 4A), whereas the distribution of Cx43 was not changed (Figure 4B). The expression of Cx43 in banded nectin-2–KO mouse hearts was significantly lower than that in banded WT mouse hearts (fold difference of sham-operated WT mice: 0.760±0.045 versus 0.438±0.040; banded WT mice versus banded nectin-2–KO mice after 4 weeks: P<0.05). To elucidate whether such a reduction in Cx43 causes electrocardiographic abnormality in nectin-2–KO mouse hearts, we performed electrocardiography (Figure S5). After banding, both WT and nectin-2–KO mice showed a prolonged QT interval to the same extent (Table S1), but no arrhythmia was observed in both WT and nectin-2–KO mice.

Transmission electron microscopy was performed to explore changes in the structure of the intercalated disc. As shown in Figure 4C, no significant change was observed in the structure of the intercalated disc in both sham-operated and banded WT mice. In sham-operated nectin-2–KO mice, the intercalated discs appeared intact. In banded nectin-2–KO mice, the intercalated discs were severely disrupted at only 2 weeks after the operation by the higher degree of membrane convolution that resembled the features of DCM, disarray of myofibrils, and disconnection from adjacent intercalated discs. These results suggest that nectin-2 is indispensable for maintaining the intercalated disc structure under chronic pressure overload.

Impaired Cellular Signalings in Nectin-2–KO Mice

Cell-cell junctions have important roles not only in cell adhesion but also in cell polarization, survival, movement, and proliferation via regulating signal transduction.16 Because the activation of signaling molecules, such as extracellular signal–regulated kinase, c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), and Akt, has been reported to be associated with the development of the pressure-overload–induced hypertrophy and heart failure,13,17 the activity of these molecules was examined after banding using Western blotting. The activation of extracellular signal–regulated kinase did not differ between WT and nectin-2–KO mice (Figure 5A). On the other hand, activation of JNK and
p38 MAPK was more significant in nectin-2–KO mice than in WT mice after banding (Figure 5B and 5C). It is noteworthy that the activation of Akt was reduced more significantly in sham-operated nectin-2–KO mice than in sham-operated WT mice, and at 2 weeks after banding it decreased further in the nectin-2–KO mouse hearts (Figure 5D). However, Akt activation rose again and was increased further at 4 weeks after banding, although the significance of this finding was not further examined. These results indicate that nectin-2 is involved in intracellular signaling in the heart in response to chronic pressure overload.

**Discussion**

In the nectin-2–KO mouse hearts, the distribution and expression of the proteins localized in AJs, such as N-cadherin, α-catenin, β1-integrin, afadin, and nectin-4, were unaffected. Because nectins have important roles in the recruitment of cadherins to the nectin-based cell-to-cell adhesion sites and the formation of AJs in epithelial cells,8,9 nectin-4 in the nectin-2–KO mouse hearts may contribute to the recruitment and expression of AJ proteins. However, nectin-2–KO mice showed the disruption of the intercalated discs and the myofibril disarray after pressure overload, suggesting that nectin-4 alone does not compensate for the function of nectin-2 in the pressure-overloaded hearts. The digitation and convolution of the intercalated disc membrane in banded nectin-2–KO mice resembled the morphology observed in the DCM models.2,3,18 Such changes in the intercalated-disc membrane between neighboring cardiac myocytes lead to a decrease in flexibility of the contractile tissue and an increased stiffness.1 Therefore, these changes are in part attributable to cardiac dysfunction in banded nectin-2–KO mice. Expression of connexins in the failing myocardium is reduced in both animal models and human diseases.19 We showed the reduction in Cx43 expression in the banded hearts of both WT and nectin-2–KO mice. The expression of Cx43 was significantly reduced in banded nectin-2–KO mouse hearts compared with banded WT mouse hearts. However, no arrhythmia was observed in nectin-2–KO mice even after banding. In selectively bred, cardiac-restricted Cx43-KO mice, reduction of Cx43 expression to 59% of matched controls does not induce susceptibility to arrhythmia, but
when the Cx43 reduction reaches 18% of control levels, 80% of the mice are inducible into lethal ventricular arrhythmias, implying that the reduction in Cx43 expression in banded nectin-2–KO mice might not be sufficient to induce arrhythmia.

Increased myocardial apoptosis, the resultant loss of cardiac myocytes, and consequential fibrosis are other mechanisms of cardiac dysfunction in banded nectin-2–KO mice. We considered that signaling impairment in nectin-2–KO mice heart provides some explanation for the distinguishing cell death and fibrosis. Extracellular signal–regulated kinase, JNK, p38 MAPK, and Akt have been reported to be activated under pressure overload. The phosphorylation levels of JNK and p38 MAPK in the nectin-2–KO mouse hearts were notably increased after banding and were much higher compared with those in the WT mouse hearts at each corresponding time point. Recent in vivo experiments using genetically modified animals have provided insights into important aspects of MAPK activity induced by the platelet-derived growth factor. We showed that the phosphorylation level of Akt was significantly lower in the nectin-2–KO mouse hearts than in the WT mouse hearts even before the operation, which continued until 2 weeks after the operation (Figure 5D), suggesting that nectin-2 in the heart regulates Akt signaling. Akt is involved in the survival of various types of cells and Akt1-KO mice and transgenic mice expressing dominant-negative phosphatidylinositol 3-kinase with the reduced Akt activity under basal conditions display an accelerated heart failure phenotype to pressure overload. Impaired activation of Akt in the nectin-2–KO mouse hearts may be involved in the increased susceptibility of cardiac myocytes to apoptosis after banding. We also showed that the phosphorylation level of Akt at 4 weeks after banding was even greater in the nectin-2–KO mouse hearts than in the WT mouse hearts. A possible explanation is provided with the evidence that Akt is usually activated in a failing myocardium, which is regarded as a compensatory reaction. Because Akt is regulated by various upstream molecules because of its critical role in cell survival, signaling molecules regulating Akt activation, conceivably from the costameres, are likely to be upregulated at the stage of heart failure.

**Perspectives**

The present results indicate that nectin-2 plays a protective role in the heart against cardiac dysfunction induced by

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**Figure 5.** Impaired signal transduction in nectin-2–KO mice. Representative Western blots of LV lysates from different groups after banding. The graphs under each blot represent the fold increases in band intensities of phosphorylated extracellular signal–regulated kinase (ERK), JNK, p38 MAPK, and Akt normalized to the total amount of each protein as compared with the sham-operated WT mouse heart. White bars, Sham-operated mice; gray bars, banding-operated mice after 2 weeks; black bars, after 4 weeks. *P<0.05, #P<0.01. n=6 in each group. The panel was made by cutting each lane from the same Western blotting data and posted.
pressure overload. The effect of nectin-2 is considered to be unique and independent from other AJ proteins. Additional analysis of the roles of nectin-2 and other proteins in the intercalated disc may contribute to deepen our understanding of the complicated mechanisms of heart failure.

Acknowledgments
We thank Toshiko Kojima for technical assistance.

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Disclosures
None.

References
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1. On page 830, Figure 5 legend, the following text was added, “The panel was made by cutting each lane from the same Western blotting data and posted.”

2. In supplemental data, Figure S4, the third band from left in the Western blotting probed for the β1-integrin antibody was corrected, and in the legend the following text was added, “The panel was made by cutting each lane from the same Western blotting data and posted.” The corrected figure is shown below.

The authors apologize for these errors.

These corrections have been made to the current online version of the article, which is available at http://hyper.ahajournals.org/content/54/4/825.full.
Deficiency of Nectin-2 Leads to Cardiac Fibrosis and Dysfunction under Chronic Pressure Overload

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Short title: The Protective Role of Nectin-2 in the Heart

Abstract, 243 words. Manuscript, 4784 words and 5 figures and 1 table.

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**Materials and Methods**

**Echocardiography and Electrocardiography**

Four weeks after aortic banding, echocardiography and electrocardiography were performed as described previously.\(^1,2\) Mice anesthetized with 2,2,2-tribromoethanol (0.20 mg/g) by intraperitoneal injection were analyzed by echocardiogram and multilead-surface electrocardiogram. We used Envision C (PHILIPS) and JB-101J (Nihon Kohden, Tokyo, Japan) for experiments. M-mode recordings of the left ventricle were obtained at the level of the papillary muscles from a parasternal window.

**Antibodies**

Rat monoclonal anti–nectin-1, -2, and -3 antibodies (Abs), rabbit polyclonal anti–nectin-1, -3 Abs, and rabbit anti-l-afadin Abs were produced as described previously.\(^3,4\) Rabbit polyclonal anti–nectin-4 Ab was produced using C-terminus (1111-1530) of nectin-4. Abs for phospho-Akt (Ser473), Akt, phospho-p44/42 MAP kinase (ERK1/2), p44/42 MAP kinase (ERK1/2), phospho-SAPK/JNK, SAPK/JNK, phospho-p38 MAPK, p38 MAPK (Cell Signaling Technology), N-cadherin, α-catenin, β-catenin (Zymed), α-actinin, connexin43 (BD Biosciences), and β1-integrin (SIGMA) were purchased.

**Histological Analysis**

Histological analysis of the hearts was performed as described previously.\(^2\) Immunofluorescence microscopy was performed using a LSM5 Pascal microscope (Carl Zeiss). Nuclei were visualized using TO-PRO-3 (Molecular Probes) as described previously.\(^5\) The area of cardiac myocytes was calculated by measuring at least 500 cells per mouse using ImageJ® software. Masson’s trichrome staining was used to quantify fibrosis in the left ventricle (LV). The area of fibrosis was quantified using ImageJ® software and the fibrosis ratio was calculated by dividing the area of fibrosis by the total myocardial area. TUNEL reaction was performed on heart sections with an Apoptosis Detection Kit (Takara Bio). At least 10\(^4\) nuclei from one heart section were counted to assess the ratio of TUNEL-positive nuclei to myocardial nuclei. For transmission electron microscopy, LV walls were fixed in Karnovsky’s fixative (pH 7.4) (2% paraformaldehyde, 2.5% glutaraldehyde, 0.2 M phosphate buffered saline) for 2 h, postfixed with 1.5% OsO\(_4\), dehydrated in graded ethanol, and embedded in Quetol 812. Thin sections were examined using a HITACHI H-600A electron microscope.

**Western Blotting**

Heart lysates were obtained by homogenization in ice-cold buffer (10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA (pH 8.0), 150 mM NaCl, 0.5% Triton X-100, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride). The lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore). The bands were quantified
by densitometry analysis using ImageJ® software ($n=6$–$8$). L fibroblasts stably expressing nectin-1, nectin-2, and nectin-3 were used as the positive controls of each nectin.\textsuperscript{6} Human umbilical vein endothelial cells were used as a positive control of nectin-4.

**Real-Time PCR**

Total RNA was isolated from left ventricular wall samples and real-time PCR was performed using an Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems) with Takara One Step SYBR\textsuperscript{®} RT-PCR Kit (Takara Bio). The following primers were designed by Takara Bio for murine atrial natriuretic factor (ANF) (forward, 5’-GGACTAGGCTGCAACAGCTTC-3’; reverse, 5’-GTGACACACCACAAGGGCTTA-3’), murine type I collagen (forward, 5’-ATGCCGCGACCTCAAGATG-3; reverse, 5’-TGAGGCACAGACGGCTGAGTA-3’), murine TGF-$\beta_1$ (forward, 5’-GTGTGGAGCAACATGTGGAACTCTA-3’; reverse, 5’-TGAGGCACAGACGGCTGAGTA-3’), murine TGF-$\beta_1$ (forward, 5’-GTGTGGAGCAACATGTGGAACTCTA-3’; reverse, 5’-TGAGGCACAGACGGCTGAGTA-3’), and murine GAPDH (forward, 5’-TTGCTGGGCTGCTGGAAGTCGAGGAG-3’). GAPDH was used for normalization, and the comparative threshold method was used to assess the relative abundance of the targets.

**Statistics**

All experiments were performed at least three times. Results are expressed as mean ± SEM. Differences between groups were compared using one-way analysis of variance, followed by Fisher’s protected least significant difference post hoc test or Scheffé’s post hoc test. A value of $p<0.05$ was considered significant.
References


Table S1. Electrocardiographic Assessment

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Values are expressed as means ± standard errors. *p <0.05 compared to sham.
Figure S1. Expression of nectin-1 and -3 in nectin-2-KO mice. Representative Western blots of nectins using the heart lysates from nectin-2\textsuperscript{+/+} (WT), nectin-2\textsuperscript{+/−} (HET), and nectin-2\textsuperscript{−/−} (KO) mice. The extreme left lane showed positive control of each nectin protein. Ten μg of protein was applied to each lane.
Figure S2. Distribution of intercalated disc protein in nectin-2-KO mice heart. Immunofluorescence of heart sections from WT and nectin-2-KO mice. AJ proteins, such as α-catenin, β-catenin, and afadin, and GJ protein, connexin43, were expressed normally in the intercalated discs of both WT and nectin-2-KO mice. Bar indicates 10 μm.
Figure S3. Cardiac hypertrophy induced by aortic banding at 4 weeks after the operation. A, HW/body weight (BW) (mg/g). B, HW/tibial length (TL) (mg/mm). White bars and black bars indicate sham-operated and aortic banding-operated mice, respectively. The degree of cardiac hypertrophy induced by aortic banding was similar in WT and nectin-2-KO mice. Numbers for each group in A and B are shown in Table 1.
Figure S4. Changes in intercalated disc proteins under pressure overload. Representative Western blots of the molecules existing in the intercalated disc. There was no significant change in the expression of AJ proteins in both genotypes after banding. Connexin43 was significantly decreased in nectin-2-KO mice after banding. Ten μg of protein was applied to each lane. The panel was made by cutting each lane from the same Western blotting data and posted.
Figure S5. Echocardiogram (lead I) from WT and nectin-2-KO mice at 4 weeks after operation. No arrhythmia was detected through whole recording in both genotypes even after aortic banding.