Mouse Model of Human Congenital Heart Disease
Progressive Atrioventricular Block Induced by a Heterozygous
Nkx2-5 Homeodomain Missense Mutation

Rajib Chowdhury, BS*; Hassan Ashraf, BS*; Michelle Melanson, BS*; Yohei Tanada, MD; Minh Nguyen; Michael Silberbach, MD; Hiroko Wakimoto, MD, PhD; D. Woodrow Benson, MD, PhD; Robert H. Anderson, MD; Hideko Kasahara, MD, PhD

Background—Heterozygous human Nkx2-5 homeodomain (DNA-binding domain) missense mutations are highly penetrant for varied congenital heart defects, including progressive atrioventricular (AV) block requiring pacemaker implantation. We recently replicated this genetic defect in a murine knockin model, in which we demonstrated highly penetrant, pleiotropic cardiac anomalies. In this study, we examined postnatal AV conduction in the knockin mice.

Methods and Results—A murine knockin model (Arg52Gly, Nkx2-5+/R52G) in a 129/Sv background was analyzed by histopathology, surface, and telemetry ECG, and in vivo electrophysiology studies, comparing with control Nkx2-5+/+ mice at diverse postnatal stages, ranging from postnatal day 1 (P1) to 17 months. PR prolongation (first degree AV block) was present at 4 weeks, 7 months, and 17 months of age, but not at P1 in the mutant mice. Advanced AV block was also occasionally demonstrated in the mutant mice. Electrophysiology studies showed that AV nodal function and right ventricular effective refractory period were impaired in the mutant mice, whereas sinus nodal function was not affected. AV nodal size was significantly smaller in the mutant mice than their controls at 4 weeks of age, corresponding to the presence of PR prolongation, but not P1, suggesting, at least in part, that the conduction abnormalities are the result of a morphologically atrophic AV node.

Conclusions—The highly penetrant and progressive AV block phenotype seen in human heterozygous missense mutations in Nkx2-5 homeodomain was replicated in mice by knocking in a comparable missense mutation. (Circ Arrhythm Electrophysiol. 2015;8:1255-1264. DOI: 10.1161/CIRCEP.115.002720.)

Key Words: animal models ▪ atrioventricular block ▪ congenital heart defects ▪ genetics ▪ human

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WHAT IS KNOWN

- Heterozygous missense mutations in the homeodomain of human NKX2-5 lead to near complete penetrance of AV conduction defects, along with high penetrance of diverse cardiac anomalies compared with mutations outside the homeodomain.
- We recently replicated a disease-causing missense mutation in the homeodomain in a knockin mouse model, Nkx2-5^{R52G}, which demonstrate a high incidence of diverse cardiac malformations.

WHAT THE STUDY ADDS

- This study showed that Nkx2-5^{R52G} mice also have developed PR prolongation sufficient to produce first degree AV conduction defects by 4 weeks of age, having by this age smaller AV nodes composed of smaller AV nodal cardiomyocytes.
- One substantial discordance between the phenotypes observed in Nkx2-5^{R52G} mouse model and human patients with NKX2-5 mutation is the prolongation of QRS, which was uniquely demonstrated in Nkx2-5^{R52G} mice from the neonatal stage onward.
- Our findings suggest that the mouse model is likely to provide important insights into the molecular mechanisms underlying conduction through the AV conduction axis.

In this study, we analyzed the signature phenotype of human patients having an NKX2-5 mutation, namely progressive AVB observed during postnatal life. We focused on the agreements and disagreements between mice and humans by using the combination of surface and telemetry ECG, in vivo electrophysiology studies (EPSs), and histopathology. As in human NKX2-5 mutations, we found a progressive and highly penetrant AVB phenotype in heterozygous Nkx2-5-KI mice.

Materials and Methods

Generation of Nkx2-5^{R52G} Mice

Nkx2-5^{R52G} knockin mice were generated as reported previously. In brief, a targeting vector was constructed by introduction of a CAG to GGT point mutation in exon 2, insertion of thymidine kinase (TK) gene for negative selection and floxed neomycin-resistant (NeoR) gene for positive selection in the intergenic regions. NeoR gene was deleted subsequently by crossing with Cre-deleter mice (ACTB-Cre). Cre-deleter transgenes were eliminated during backcrossing to 129/Sv mice purchased from Charles River (129/SvPasCrl) over 8 generations. All animal experiments were performed with approval from the University of Florida Institutional Animal Care and Use Committee.

Surface 6-Lead ECG and Telemetry ECG Recordings

Surface 6-lead ECG and telemetry ECG recordings were performed as previously described. Briefly, mice were anesthetized with isoflurane (1%–2%) and placed on a heated pad (using water circulation) at 37°C. ECG recordings were obtained from a 29 gauge subcutaneous electrode. For ambulatory ECG analysis in conscious unrestrained mice, 1.6-g transmitters (ETA-F10 DSI) were implanted. After 72 hours recovery time from surgical instrumentation, ECG recordings were performed in each mouse placed in a separate cage overlying a receiver (DSI) in daytime. Signal-averaged ECGs selected from ≈20 stable ECG beats from at least 3 different time points were analyzed by LabChart with the ECG analysis module (ADInstruments) to confirm the consistency of the data.

Electrophysiology Study

Simultaneous atrial and ventricular pacing and recording were performed via 1.1F octapolar catheter (EPR-800, Millar) inserted via a jugular vein in mice anesthetized with isoflurane (2%–3%). The standard pacing protocols were programmed in the multichannel stimulator STG 3008 (Multichannel systems), which was analyzed by LabChart (ADInstruments). Sinus nodal function was evaluated by measuring sinus node recovery time (SNRT) at 3 pacing drive rates (150, 120, and 100 ms) and corrected SNRT (SNRT minus sinus cycle length [CL]). AV conduction properties were assessed with rapid atrial pacing at rates with a minimum pacing cycle length of 50 ms (≤1200 beats per minute). The minimum cycle length maintaining 1:1 AV conduction, the Wenckebach paced cycle length, and the maximum paced cycle length causing 2:1 AV block were determined. AV nodal effective refractory periods were determined by the maximum coupling interval causing AV block at the 2 pacing drive rates (150 and 120 ms). Right ventricular effective refractory periods were determined by the maximum coupling interval that failed to stimulate ventricles at the 2 pacing drive rates (150 and 120 ms). Right ventricular burst pacing was performed at rates with a pacing cycle length of 150 to 50 ms (400–1200 beats per minute) to assess retrograde ventriculo-atrial conduction.

Histological Analysis

Serial paraffin-embedded tissue sectioning of 5-μm thickness was performed to reveal the location and dimensions of the AV conduction axis as described previously. For the purpose of this study, we discriminated the conduction axis as having a ventricular component (made up of the ventricular components of the axis and the bundle branches) and a penetrating component (made up of the bundle of His), from a compact AV nodal component and its inferior nodal extensions. We distinguished the penetrating component of the axis on the basis of its insulation from the atrial cardiomyocytes by the fibrous tissues of the central fibrous body. This definition of the node, as opposed to the penetrating bundle, was initially proposed by Tawara although subsequent investigators have described so-called lower nodal cells as being part of the AV node. We did not consider these cells as belonging to the node, although it was possible to trace the cells through the basal part of the node, where they became continuous with the inferior nodal extensions.

We then digitized the proximal part of the penetrating bundle immediately it had been insulated from the atrial cardiomyocytes, the
compact part of the node, and the inferior nodal extensions so as to measure their areas, nuclear numbers, and cell width using Image J as reported previously. The width of the cardiomyocytes within the compact node was measured in the troponin T–positive myocytes at the level of the nuclei as shown previously. AV nodal volume was calculated as \((\pi r^2t+\sqrt{2t}\pi r+1)\) \(r\), radius of maximum area size; \(l\), length, 5 \(\mu\)mnumber of tissue sections. Paraffin-embedded tissue sectioning or cryosectioning of paraformaldehyde-fixed hearts was
used for immunostaining. Immunostaining was performed with the following antibodies: actinin (Sigma A7811), connexin 40 (Alpha Diagnostic Inc cx40-A), connexin 43 (Zymed 71–700), hyperpolarization activated cyclic nucleotide-gated potassium channel 4 (Millipore, AB5808), Nav1.5 (α; Alomone Laboratories, ASC-005), troponin T (Sigma, T6277), and Nkx2-5.17 The presence of fibrosis was examined using picrosirius red–stained tissue sections. Briefly, the tissue sections were heated at 60°C for 45 minutes before deparaffinization and stained in 0.1% direct red 80, 0.1% fast green FCF in 1.2% picric acid for 60 minutes.

Acetylcholine esterase (AchE) staining using the serial frozen tissue sections (10-μm thickness) was performed as described previously.14

### Statistical Analysis

Data presented are expressed as mean values plus or minus the SE or SD of the mean. Results were analyzed by SPSS (version 22) using independent t test. Levene test was used for equality of variance, and P values were calculated depending on the assurance of equality.
Table 2. Summary of Telemetry ECG Analysis

<table>
<thead>
<tr>
<th>Age, mo</th>
<th>SCL, ms</th>
<th>HR, beats per minute</th>
<th>PR, ms</th>
<th>QRS, ms</th>
<th>QTc, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aged</td>
<td>W/W (n=10)</td>
<td>17.4±0.8</td>
<td>96±8</td>
<td>629±53</td>
<td>33.3±2.8</td>
</tr>
<tr>
<td></td>
<td>W/R52G (n=15)</td>
<td>17.5±1.2</td>
<td>96±9</td>
<td>628±55</td>
<td>38.0±3.6</td>
</tr>
</tbody>
</table>

Mean±SD. HR indicates heart rate; and SCL, sinus cycle length.

Table 3. Summary of EPSs and Simultaneous Surface ECG Analysis at 7 Months of Age

<table>
<thead>
<tr>
<th>WW (n=5)</th>
<th>W/R52G (n=6 or 7)</th>
<th>P Value</th>
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</thead>
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<tr>
<td>Body weight</td>
<td>23.1±0.6</td>
<td>24.2±1.5</td>
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<tr>
<td>SCL, ms</td>
<td>170±14</td>
<td>163±5</td>
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<tr>
<td>HR, beats per minute</td>
<td>352±29</td>
<td>368±12</td>
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<tr>
<td>PR, ms</td>
<td>40.9±1.6</td>
<td>53.8±6.2</td>
</tr>
<tr>
<td>QRS, ms</td>
<td>10.1±1.0</td>
<td>14.6±1.4</td>
</tr>
<tr>
<td>QT, ms</td>
<td>36.3±3.2</td>
<td>36.8±4.7</td>
</tr>
<tr>
<td>QTc, ms</td>
<td>27.8±3.1</td>
<td>31.5±4.2</td>
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<tr>
<td>SNRT 150, ms</td>
<td>212±45</td>
<td>204±23</td>
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<tr>
<td>SNRT 120, ms</td>
<td>243±56</td>
<td>277±58</td>
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<td>SNRT 100, ms</td>
<td>288±53</td>
<td>275±66</td>
</tr>
<tr>
<td>cSNRT 150, ms</td>
<td>48±42</td>
<td>40±21</td>
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<tr>
<td>cSNRT 120, ms</td>
<td>78±53</td>
<td>85±29</td>
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<tr>
<td>cSNRT 100, ms</td>
<td>123±51</td>
<td>82±44</td>
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<tr>
<td>AWBCL, ms</td>
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<td>115±15</td>
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<tr>
<td>AV2:1CL, ms</td>
<td>69±12</td>
<td>83±9</td>
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<tr>
<td>AVERP 150, ms</td>
<td>46±15</td>
<td>70±9</td>
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<tr>
<td>AVERP 120, ms</td>
<td>42±18</td>
<td>70±15</td>
</tr>
<tr>
<td>VERP 150, ms</td>
<td>37±10</td>
<td>60±14</td>
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<tr>
<td>VERP 120, ms</td>
<td>38±11</td>
<td>63±18</td>
</tr>
</tbody>
</table>

P values are 2-sided and those <0.05 were considered significant. There are no adjustments for multiple comparisons.

Results

Phenotypes Associated With Arg52(189)Gly, NKX2-5**R52G**, Homeodomain Missense Mutation

The pedigree of the family having Arg52(189)Gly mutation previously reported was recently found to include a new patient in the fifth generation who demonstrated AVB, as well as atrial and ventricular septal defects, resulting in 6 members of the family with a mutation (Figure 1). All 6 genotype-positive individuals demonstrate AVB (first, second, or third degree). Atrial septal defect was present in 5 patients, ventricular septal defect, or a small tricuspid valve was present in 1 patient each.

AVB in Heterozygous Knockin Nkx2-5 Mutant Mice, (Arg52(188)Gly, Nkx2-5**R52G**), Measured by Surface and Ambulant ECG Recordings

To confirm that the PR prolongation was not a side effect of anesthesia, and to examine the presence of advanced AVB, we used ambulatory ECG analysis in conscious unrestrained aged mice at 17 months of age (Figure 3A; Table 2). The PR interval was significantly longer in Nkx2-5-**KI** mice relative to the controls (38.0±0.9 versus 33.3±0.9 ms; P<0.01). One of the 16 Nkx2-5-**KI** mice also demonstrated advanced AVB (complete AVB; Figure 3B), whereas none of the control mice showed any degree of AVB.

In Vivo Endocardial EPSs

To determine the electrophysiological functions of conduction systems, in vivo EPS were performed in mice at 7 months of age, when AVB is well established, and mouse body size is large enough for EPS following the standard pacing protocol.
with the basal pacing rate of 150 ms. To be paced at 150 ms, endogenous sinus CL was kept longer than 150 ms by a higher dose of isoflurane (2%–3%). The sinus CL was maintained between 160 and 180 ms (heart rate, 350–370 beats per minute; Table 3), which was slower than those used for surface ECG recording at 7 months of age (Figure 2E; Table 1).

Under higher isoflurane anesthesia, the PR interval of mice at 7 months of age was prolonged similar to those at 4 weeks of age, even in the control mice (36.9±1.2 versus 40.9±0.7 ms), compared with those observed in the surface ECG recordings, but the effect was more profound in Nkx2-5-KI mice (42.5±2 versus 53.8±2.5 ms; Table 1 versus Table 3). EPS in Nkx2-5-KI mice consistently showed impaired AV nodal conduction properties, as assessed by AV nodal effective refractory period, and the longest pacing CL when 1:1 AV conduction failed to Wenckebach type (AVW/BCL) and 2:1 AV conduction (AV:2:1 CL; Table 3). In addition, Nkx2-5-KI mice demonstrated a prolonged ventricular effective refractory period, which reflects the wide QRS with an impaired ventricular peripheral conduction system. Sinus nodal conduction properties assessed by SNRT and rate-corrected SNRT (CSNRT) were unchanged in Nkx2-5-KI mice (Table 3).

One of Nkx2-5-KI mice from a total of 7 demonstrated advanced second-degree 3:1 AVB, shortly after induction of anesthesia (atrial sinus CL, 135 ms), which persisted (Figure 4A, atrial sinus CL 135 ms), whereas none of the control mice demonstrated advanced AVB. Because of lack of the 1:1 AV conduction, we could not assess PR and QRS interval from surface ECG, as well as AV nodal function and ventricular conduction from this Nkx2-5-KI mouse (Table 3).

Reduced AV Nodal Size Composed of Smaller Cardiomyocytes in Nkx2-5+/R52G in 4 Weeks Old but Not in P1 Nkx2-5+/R52G Mice

Using the combination of immunostaining with hyperpolarization activated cyclic nucleotide-gated potassium channel 4 and hematoxylin and eosin staining, we defined the AV nodal area as being hyperpolarization activated cyclic nucleotide-gated potassium channel 4 positive, containing less-organized sarcomere structure (reduced hematoxylin and eosin staining), and having cardiomyocytes in direct contact with atrial muscle.16,20 We excluded from consideration the area of the conduction axis insulated from the atrial muscle by connective tissue, defining this component as the penetrating AV bundle (Figure 4B and 4C, Histological analysis section of this article).

Atrophy of AV conduction axis has been postulated as an underlying mechanism of the PR prolongation observed in multiple conditional homozygous Nkx2-5 knockout mice.9,10,19

In keeping with this notion, we found that, in Nkx2-5-KI mice at 4 weeks of age, when the PR prolongation was first displayed, the area made up of the compact node was markedly reduced, analyzed by AV nodal maximum area size, and estimated AV nodal volume modeled as a three-dimensional cylindrical structure (see Methods; Figure 5A and 5B). The areas of the penetrating bundle and nodal extensions were also smaller in Nkx2-5-KI mice. Nuclear density, in contrast, calculated as the number of nuclei per area size in the maximum AV node, was increased. The width of the cardiomyocytes visualized by sarcomeric troponin staining revealed that the AV nodal cardiomyocytes were smaller in Nkx2-5-KI hearts (Figure 5B). The ratio of the number of Nkx2-5 positive (indicating cardiomyocytes) versus Nkx2-5-negative (nonmyocytes) nuclei was unchanged (Figure 5C and 5D), suggesting that the cellular component of myocytes and nonmyocytes is most likely unchanged between the Nkx2-5-KI mice and the controls.

Massive AV nodal fibrosis has been demonstrated in conditional Nkx2-5 knockout mice using a myosin-light chain 2v-Cre.19 As judged by the extent of picrosirius red staining
in our material, we did not observe any apparent increase of fibrosis, either at 4 weeks (data not shown) or at 7 months of age (Figure 5E).

In contrast, in P1 mice, when PR prolongation of Nkx2-5-KI mice was not evident, AV nodal maximum area size and nuclear density were unchanged between the Nkx2-5-KI mice and the controls (Figure 6A–6D). In addition, immunostaining of positive markers or negative markers for AV node, penetrating AV node and left bundle branch, including hyperpolarization activated cyclic nucleotide-gated potassium channel 4, connexin 40, connexin 43, and Na_1.5 was not different between the 2 groups neither at P1 (Figure 7A) nor at 4 weeks of age, except that the left bundle branch was underdeveloped in Nkx2-5-KI mice (Figure II in the Data Supplement).

Notably, to detect P1 AV nodes, we need to use picrosirius red staining instead of hematoxylin and eosin staining, because of less organized sarcomeric structure in the surrounding atrial myocytes at this age.

In summary, therefore, Nkx2-5-KI mice were found to have smaller AV nodes at 4 weeks but not at P1. The
cardiomyocytes within the compact AV node at 4 weeks were smaller than those of the wild-type animals, but without any noted difference in the ratio of myocyte to nonmyocyte nuclei, and without any noted increase in fibrosis.

Reduced AchE Activity in Ventricular Trabeculations

Wide QRS, indicative of prolonged ventricular conduction times, was demonstrated in Nkx2-5-KI mice at all ages from the neonatal stage onward. This finding was consistent with P1 hearts demonstrating the underdeveloped left bundle branch in Nkx2-5-KI mice compared with control mice (Figure 7A).

Furthermore, histopathology of the ventricular conduction system in E18.5 hearts was examined by AchE activity, which is known to coincide with parts of the developing ventricular conduction system and probably controls conduction of electric impulses during embryonic stages. AchE staining in ventricular trabeculations and left bundle branch was slightly weaker in Nkx2-5-R52G hearts. Areas a–d selected on the left are shown enlarged on the right.
weaker in E18.5 Nkx2-5-KI hearts than in control hearts under the same experimental conditions (Figure 7B, n=4 each). An underdeveloped ventricular conduction system during the embryonic stage, therefore, could be causative, at least in part, of the wide QRS.

Discussion

Heterozygous missense mutations in the homeodomain of human NKX2-5 lead to near complete penetrance of AV conduction defects, along with high penetrance of diverse cardiac anomalies compared with mutations outside the homeodomain.7 We recently replicated a disease-causing missense mutation in the homeodomain in a knockin mouse model, Nkx2-5-KI R52G, which is currently the most clinically relevant mouse model.8 These mice demonstrate a high incidence of diverse cardiac malformations.9 Our current study showed that Nkx2-5-KI mice also have developed PR prolongation sufficient to produce first degree AVB by 4 weeks of age, having by this age smaller AV nodes composed of smaller AV nodal cardiomyocytes. In addition, 2 Nkx2-5-KI mice demonstrated advanced AVB: 3:1 AVB, and complete AVB, at 7 and 18 months of age, respectively.

As yet, the steps involved in the formation of the AV node during development are not fully understood, but it is known that the atrial components of the AV conduction axis, including parts of the AV node, are derived from the embryonic AV canal, whereas the penetrating AV bundle is developed from the interventricular ring.24 It has been postulated that the rate of proliferation of whole AV nodal cells originating from the engaged myocytes from the embryonic stage could be insufficient, and recruitment from adjacent working myocardium is required.25 A recent study, in contrast, argued that Tbx3-positive AV nodal cells engaged from the embryonic stage would be sufficient, and would proliferate from embryonic day E14.5 to postnatal day 14, increasing nearly 5-fold.24 To our knowledge, however, maintenance of the cellular architecture of the AV node has not been investigated. Additional studies, possibly using laser capture to compare the content of mRNA and proteins in the AV node between control and Nkx2-5-KI mice, could cast further light on this possibility. We are unable to explain, however, why the Nkx2-5-R52G mutant protein should produce the situation of the compact node being composed of smaller cardiomyocytes.

The population of the Nkx2-5-positive cardiomyocytes as opposed to Nkx2-5-negative nonmyocytes was unchanged in the AV nodes of Nkx2-5-KI mice compared with theiragematched controls. Thus, the previously hypothesized mechanisms, including failure of development of the AV nodal cardiomyocytes, or recruitment of cardiomyocytes from the adjacent working myocardium demonstrated in Nkx2-5 knockout mice,18,19 would not be conserved in Nkx2-5-KI mice.

One substantial discordance between the phenotypes observed in Nkx2-5-KI mouse model and human patients with NKX2-5 mutation is the prolongation of QRS, which was uniquely demonstrated in Nkx2-5-KI mice from the neonatal stage onward. AchE-positive ventricular trabeculations, which likely represent components of the developing ventricular conduction system, are morphologically changed and poorly developed in E18.5 Nkx2-5-KI mice shortly before birth. Ventricular noncompaction demonstrated in Nkx2-5-KI mouse could be attributed to abnormal development of the ventricular conduction system. Prolongation of QRS complex has been also demonstrated in multiple heterozygous and conditional homozygous Nkx2-5 knockout mice, and related to poor development of the ventricular conduction system, with reduced expression of selected gap junction protein, such as connexin40 and transcription factor Id2.9,10,14,18,19,26 This suggests that, in mice, Nkx2-5 plays a more substantial role in the formation and function of the ventricular conduction system than it does in humans. Reduction of connexin40, however, was not evident in Nkx2-5-KI mouse when using neither immunostaining (Figure 7A) nor expression profile from whole hearts (data not shown).

Nkx2-5 is also expressed in the sinus node, and has been implicated in playing a role in nodal formation under the control of Shox2 homeodomain transcription factor.27–31 Some heterozygous Nkx2-5 knockout mice, furthermore, demonstrated sinus bradycardia,32 whereas other heterozygous and conditional homozygous Nkx2-5 knockout mice did not.9,10,18,19,33 We also failed to observe any sinus arrhythmias, including sinus bradycardia, nor did we observe sinus nodal dysfunction in our EPS, consistent with the findings in human patients with NKX2-5 mutations.5

In summary, we report the presence of progressive AVB in our recently developed Nkx2-5-KI mouse model having a heterozygous Nkx2-5 missense mutation in the homeodomain. We have focused on the phenotypic similarities and differences between our mice and humans. Our findings suggest that the mouse model is likely to provide important insights into the molecular mechanisms underlying conduction through the AV conduction axis.

Acknowledgments

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Disclosures

None.

References


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http://circep.ahajournals.org//subscriptions/
Supplemental Table 1: Summary of surface ECG analysis 4 weeks of age with 1 or 3% isoflurane anesthesia.

<table>
<thead>
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<th>genotype</th>
<th>Isoflurane</th>
<th>SCL (ms)</th>
<th>HR (bpm)</th>
<th>PR (ms)</th>
<th>QRS (ms)</th>
<th>Qtc (ms)</th>
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</thead>
<tbody>
<tr>
<td>W/W (n=5)</td>
<td>1%</td>
<td>146 ± 8</td>
<td>416 ± 23</td>
<td>54.2 ± 2.2**</td>
<td>12.2 ± 0.4**</td>
<td>29.0 ± 1.2</td>
</tr>
<tr>
<td>W/ R52G (n=6)</td>
<td>1%</td>
<td>146 ± 8</td>
<td>416 ± 23</td>
<td>54.2 ± 2.2**</td>
<td>12.2 ± 0.4**</td>
<td>29.0 ± 1.2</td>
</tr>
<tr>
<td>W/W (n=5)</td>
<td>3%</td>
<td>139 ± 7</td>
<td>434 ± 23</td>
<td>41.5 ± 1.5</td>
<td>10.3 ± 0.3</td>
<td>26.0 ± 1.0</td>
</tr>
<tr>
<td>W/R52G (n=6)</td>
<td>3%</td>
<td>141 ± 4</td>
<td>428 ± 12</td>
<td>36.5 ± 0.6*</td>
<td>11.8 ± 0.4**</td>
<td>26.0 ± 0.6</td>
</tr>
</tbody>
</table>

SCL, sinus cycle length; HR, heart rate; bpm, beats per minute.
Mean ± SE. *P, P value between two different mouse genotypes. **P < 0.01
Figure S1. Profound effects of higher dose of isoflurane (3%) anesthesia on prolongation of PR interval and QRS duration in Nkx2-5+/R52G mice. Representative surface ECG recordings obtained from 4 weeks old control Nkx2-5+/+ (top) and Nkx2-5+/R52G (bottom) mice either with 1% or 3% isoflurane anesthesia. Signal-averaged ECG waves were utilized for analysis shown in Supplemental Table S1. HR, heart rate.

<table>
<thead>
<tr>
<th>1% isoflurane</th>
<th>3% isoflurane</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>+/+</strong></td>
<td><strong>+/R52G</strong></td>
</tr>
<tr>
<td>HR=470 bpm, PR=35.7 ms, QRS=8.2 ms</td>
<td>HR=420 bpm, PR=39.1 ms, QRS=9.6 ms</td>
</tr>
<tr>
<td>HR=509 bpm, PR=32 ms, QRS=11 ms</td>
<td>HR=458 bpm, PR=54 ms, QRS=13.5 ms</td>
</tr>
</tbody>
</table>

100 ms
**Figure S2:** Representative images of serial tissue sections positively (+) or negatively (-) stained with HCN4, connexin 40, connexin 43, and Na_{1.5} (green) in AV node, penetrating AV bundle (traced by white dots) and left bundle branch (arrowheads) from 4 week-old mice. Anti-actinin antibody was used for co-immunostaining (red). Of note, connexin40 staining was not evident in penetrating AV bundle and left bundle branch differing from P1 hearts demonstrated in Figure 6E.