High Incidence of Cardiac Malformations in Connexin40-Deficient Mice

Hong Gu, Frank C. Smith, Steven M. Taffet, Mario Delmar

Abstract—Gap junctions are intercellular channels formed by oligomerization of a protein called connexin (Cx). The heart expresses at least three connexin isotypes: Cx40, Cx43, and Cx45. A possible role for Cx40 in cardiac morphogenesis remains to be determined. We have characterized the anatomy and histology of fetal and newborn hearts obtained from crossing Cx40-deficient mice of mixed genetic background (C57BL/6×129Sv). Hearts were serial-sectioned (5 μm) along the coronal plane, stained with hematoxylin-eosin, and visualized by conventional light microscopy. Cardiac malformations in mice lacking Cx40 in one allele (Cx40+/−) included bifid atrial appendage, ventricular septal defect, tetralogy of Fallot (TOF), and an aortic arch abnormality. In Cx40−/− mice resulting from crossing of Cx40+/− mice, the most common cardiac malformations were double-outlet right ventricle (DORV), TOF, and endocardial cushion defects. Overall incidence of cardiac malformations was 6/33 (18%) in Cx40+/− mice and 4/12 (33%) in Cx40−/− mice. No cardiac malformations were observed in 15 wild-type mice studied. In addition, we examined 39 hearts from offspring of Cx40−/− matings. Frequency of cardiac malformations was even higher in this group (44%). Over one third of the hearts (14 of 39) showed conotruncal malformations corresponding to either DORV or TOF. Endocardial cushion defects were found in 3 out of 39 hearts. Our results suggest that Cx40 participates in cardiac morphogenesis, likely in association with other (unknown) products whose expression may vary with the genetic background of the mice. (Circ Res. 2003;93:201-206.)

Key Words: connexin40 ▪ gap junction ▪ cardiac malformations ▪ cardiac morphogenesis ▪ mice

Gap junction channels are formed by oligomerization of a protein called connexin (Cx).1,2 These channels allow for the passage of ions and small molecules between adjacent cells.3 Twenty connexin isoforms have been identified in the human genome and nineteen in the mouse genome.4 Three isoforms are expressed in different regions of the cardiac muscle: Cx40, Cx43, and Cx45.5,6 Experiments in genetically modified mice have shown that Cx40 and Cx43 are necessary for synchronization of cardiac electrical activity,7,8 whereas Cx43 and Cx45 are essential for normal organogenesis.9–12 A possible role for Cx40 in cardiac morphogenesis has not been thoroughly studied, although a single report indicates that cardiac malformations may be found in Cx40-deficient mice.13

Cx40 gene expression in the developing heart is restricted in time and space. The studies of Delorme et al14 show the presence of low amounts of Cx40 mRNA as early as embryonic day (ED) 8.5, when the first rhythmic contractions appear in the tube heart. The presence of Cx40 extends to the primitive atrium and the common ventricular chamber by 9.5 ED, and to the right ventricle by 11.5 ED. Beyond this stage and until 14 to 14.5 ED (when the 4-chambered heart is formed), Cx40 is equally expressed in atria and ventricles.14 From 14.5 ED onward, the Cx40 distribution pattern in the atria is maintained, whereas ventricular expression fades. In the adult heart, Cx40 expression is restricted to the atria and to the His-Purkinje conduction system.6,15

The electrophysiological profile of the hearts of Cx40-deficient mice has been extensively studied.16–23 These mice show reduced atrial conduction velocity,17 atrioventricular (AV) nodal dysfunction,16–17,19–21 and impaired conduction in the bundle branches.16,22,23 However, the possible role of Cx40 in cardiogenesis remains unclear. In this study, we present a systematic study of the anatomy and histology of hearts of Cx40-deficient mice of mixed genetic background (C57BL/6×129Sv). Both Cx40 heterozygous (+/−) and homozygous-null (−/−) mice exhibited a variety of complex cardiac malformations including conotruncal defects and endocardial cushion defects. The results suggest that Cx40 expression is involved in cardiogenesis, but its potential impact may be dependent on genetic background. These studies open the possibility of Cx40 as a potential genetic modifier in humans.

Materials and Methods

Mice Breeding

Founder male mice of mixed genetic background (C57BL/6×129Sv) and deficient in Cx40 expression (Cx40−/−) were kindly provided by...
TABLE 1. Frequency of Cardiac Malformations in Cx40-Deficient Mice

<table>
<thead>
<tr>
<th>Malformation</th>
<th>+/+</th>
<th>+/-</th>
<th>-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total No. of animals studied</td>
<td>15</td>
<td>33</td>
<td>12</td>
</tr>
<tr>
<td>No. of animals with cardiac malformations</td>
<td>0</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Percentage</td>
<td>0</td>
<td>18</td>
<td>33</td>
</tr>
</tbody>
</table>

Drs Alex Simon (University of Arizona, Tucson, Ariz) and David Paul (Harvard University, Boston, Mass). The colony was initially expanded by crossings of Cx40+/− males with C57BL/6 females. After one generation, the colony was further expanded by breeding the heterozygous offspring and then maintained by crossing either Cx40+/− mice (to have all three genotypes in the same litter) or Cx40−/− mice (to produce all offspring homozygous null).

Surgical Procedures
Fetuses (18.5 ED; ie, 18 days after detection of the vaginal plug) were retrieved surgically from pregnant females anesthetized by exposure to CO2, followed by cervical dislocation, and then euthanized by removal of the heart. Fetal (18.5 ED) or newborn (6 hour) hearts and great vessels were exposed through a midsternal thoracic incision and separation of the thymus. The procedure was conducted under a dissecting microscope for careful gross anatomical inspection. The hearts and great vessels were then dissected, placed in sterile PBS containing 10 u/mL heparin, and photographed to document their morphology. All surgical procedures were consistent with established guidelines and approved by the "Committee for the Humane Use of Animals" at the SUNY Upstate Medical University.

Histological Analysis
Hearts and great vessels were embedded in Tissue-Tek OCT and frozen in liquid nitrogen. The specimens were serial-sectioned along the coronal plane (ventral to dorsal) in 5-μm slices; Cryosections were mounted on poly-L-lysine–coated glass slides. Sections were stained with hematoxylin and eosin and examined under bright-field illumination in an inverted microscope.

Genotyping
Mouse genomic DNA was prepared from tail biopsies. The genotypes were determined by PCR analysis using the following primers: primer 1, 5'-TGGAGCCACAGTTCAATGGT-3'; primer 2, 5'-GACCGAGACTAGTGAGACGGT-3'; and primer 3, 5'-TCTCTGACTCCGAAAGGCAAG-3'.

Results

Genotypic Distribution of Mice from Cx40+/− Crossings
A total of 15 litters (88 mice), obtained from crossings of Cx40−/− mice, were genotyped for Cx40. Twenty-five percent (22/88) of the mice presented the wild-type Cx40 genotype (Cx40+/+), 54% (47/88) of the mice were heterozygous (Cx40+/−), and 21% (19/88) were homozygous-null (Cx40−/−). Lack of Cx40 expression in Cx40−/− mice and reduction in Cx40+/− mice were confirmed by immunohistochemistry (data not shown).

Cardiac Malformations in Offspring of Cx40+/− Mice
Sixty hearts were sectioned for anatomical and histological analysis as described in Materials and Methods. As shown in Table 1, no malformations were detected in any of the 15 wild-type (Cx40+/+) animals studied. Yet, 6 of the 33 hearts (18%) from Cx40−/− mice and 4 of the 12 hearts (33%) from Cx40+/− mice were morphologically abnormal. A summary of these malformations is presented in Table 2. The following descriptions of four characteristic lesions correspond with illustrations in Figures 1 through 4.

Bifid Atrial Appendage in Cx40+/− Mice
The hearts of three heterozygous mice demonstrated a bifid atrial appendage (one in the right atrium, two in the left atrium). An example of bifid left atrial appendage is shown in Figure 1A. Figure 1A depicts the heart of a Cx40+/− fetus at 18.5 ED, and Figure 1B is a 5-μm section of the heart along the coronal plane. The right atrial appendage anatomy (RA) is normal. The left atrial appendage (LA) was cleaved, forming two separate "pouches" connected by a thin strand of muscular tissue. All other cardiac structures were normal. This abnormality was not observed in any of the Cx40−/− mice.

Aortic Arch Branch Vessel Abnormality in a Cx40−/− Mouse
One Cx40−/− mouse demonstrated a peculiar aortic arch abnormality. Figure 2A depicts a normal heart of a wild-type 6-hour-old mouse for comparison. The pulmonary artery (PA) is anterior to the ascending aorta (AAo) and the aorta gives rise to a left aortic arch with branch vessels that course upward and anterior to the trachea (tr) and esophagus (es). Figure 2B reveals the heart of the affected Cx40−/− mouse. The take-off of two branch vessels from the aorta (arrows) is more proximal than normal, and the left vessel courses more proximal than normal. Yet, 6 of the 33 hearts (18%) from Cx40−/− mice were abnormal. A summary of these malformations is presented in Table 2. The following descriptions of four characteristic lesions correspond with illustrations in Figures 1 through 4.

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leftward and lateral to the trachea and esophagus. Right (RA) and left (LA) atrial appendages appear normal in both cases.

Ventricular Septal Defect, Tetralogy of Fallot, and Double-Outlet Right Ventricle in Both Cx40/H11545 and Cx40/H11546 Mice

Two heterozygous mice had isolated ventricular septal defects and one had tetralogy of Fallot (TOF). Two homozygous-null mice had variants of double-outlet right ventricle (DORV). An example of DORV in a 6-hour-old Cx40/−/− mouse is shown in Figure 3. Figures 3A through 3C reveal hematoxylin-eosin–stained serial coronal sections of the heart. Figures 3D through 3F demonstrate similar sections from a normal Cx40/−/− littermate for comparison. In Figure 3A, there is significant muscular obstruction (open arrow) proximal to the pulmonary valve (PV). Figure 3B depicts a section posterior to the section in Figure 3A in which the aortic valve (Ao) overrides a ventricular septal defect (asterisk) and arises nearly completely from the right ventricle. The diameter of the pulmonary artery (PA) seems narrower when compared with that observed in the normal littermate (Figure 3E). In Figure 3C, the most posterior section shows the ventricular septal defect (VSD, asterisk) with aortic override (Ao) and mild fibrous discontinuity between the anterior mitral valve leaflet (open arrow) and the aortic valve. These findings are consistent with double-outlet right ventricle. Figures 3D and 3E demonstrate a normal pulmonary valve (PV) arising from right ventricle (RV) without subvalvar obstruction and a normal aorta (Ao) arising from the left ventricle (LV). Figure 3F is a tangential section of the aortic valve leaflets, and there is continuity with the mitral valve leaflet (open arrow).

Partial Endocardial Cushion Defects in Cx40/−/− Mice

Two Cx40/−/− newborn mice had partial endocardial cushion defects. Figures 4A through 4C depict serial coronal hematoxylin-eosin–stained sections from one of the two mice. Figure 4A demonstrates the four cardiac chambers: right atrium (RA), left atrium (LA), right ventricle (RV), and left atrium (LA) for comparison. Right (RA) and left (LA) atrial appendages appear normal in both cases.
ventricle (LV). In Figure 4B, there is a common atrioventricular valve (arrow) that has papillary muscle attachments within both ventricles. The “mitral” portion of the valve that is over the LV appeared closed on all other sections, suggesting the presence of severe mitral stenosis. The section in Figure 4C demonstrates a very large ostium primum atrial septal defect (double-headed arrow). Because there is no significant ventricular septal defect, the anomaly is best described as a partial endocardial cushion defect. Because the right ventricle is larger and receives most of the common AV valve inflow, the defect is also unbalanced.

Cardiac Defects in the Offspring of Cx40⁻/⁻ Mice

The frequency of cardiac malformations was higher in the offspring of Cx40⁻/⁻ mice. Seven litters generated 50 offspring. Three mice, later found to have severe cardiac malformations (DORV, dilated and hypertrophic cardiomyopathy), died shortly after birth. We performed a histological examination of 39 hearts and found cardiac malformations in 17 of them (44%). As shown in Table 3, over one third of the hearts (14 of 39) showed conotruncal malformations (DORV or TOF). Endocardial cushion defects were found in 3 out of 39 hearts, and in one case, there was DORV as well as a mitral valve cleft (the simplest endocardial cushion defect in humans).

Tetralogy of Fallot With Pulmonary Atresia (Pulmonary Atresia With VSD) in a Homozygous-Null Mouse Offspring of Cx40⁻/⁻ Mice

Figure 5 demonstrates the heart of a Cx40⁻/⁻ mouse offspring of Cx40⁻/⁻ mice. A through C, Serial coronal hematoxylin-eosin-stained sections from a 6-hour-old Cx40⁻/⁻ mouse. B, Common atrioventricular valve (arrow). C, Large atrial septal defect (double-headed arrow). Bar=1 mm.

There is concentric hypertrophy of both ventricles, and there is an interventricular cleft (open arrow) as well. Figures 5B and 5C are hematoxylin-eosin–stained sections confirming severe ventricular hypertrophy and hypoplasia of the PA with atresia of its origins from the right ventricle. Figure 5C demonstrates a ventricular septal defect (open arrow) with override of the aorta (AO) over the ventricular septum. Because there was no definite fibrous discontinuity between the aortic and mitral valves the lesion could be described as tetralogy of Fallot or pulmonary atresia with ventricular septal defect.

Discussion

Connexin40 is necessary for normal action potential propagation in the heart. However, the role of this protein on the formation of the cardiac structures is unclear. In the present study, we report a careful anatomical analysis of hearts obtained from Cx40-deficient mice and demonstrate that cardiac malformations are prevalent in these animals. This is not the first report of cardiac malformations in a Cx40⁻/⁻ mouse. In a previous study, Kirchhoff et al reported a relatively high incidence of newborn and young adult death in the Cx40-null population. These authors analyzed the hearts of three newborn and two young adult mice and found a small septum primum defect in three of these hearts, a persisting interventricular foramen in one case, a persistent foramen ovale in two cases, and one case with a ventricular septal defect. Four hearts showed myocardial hypertrophy. The present study significantly expands on these observations. We have sampled 99 hearts and included crossings from heterozygous as well as from homozygous null. Our analysis allowed us to quantify the frequency of malformations and identify the presence of various defects of the heart and great vessels. Our data show the following: (1) in Cx40⁻/⁻ mice, there is a high likelihood of cardiac defects; (2) malformations can be found in Cx40⁻/⁻ animals but the incidence is higher, and the malformations more severe, in Cx40⁻/⁻; (3) the highest incidence is found in the offspring of Cx40⁻/⁻ mice; and (4) although the most common malformations are of conotruncal origin, endocardial cushion defects and other malformations can also be found. Overall, the data suggest a role for Cx40 as a factor in cardiac development.

A number of investigators have characterized the Cx40⁻/⁻ animals from a physiological standpoint. None of those publications report the presence of malformations, and yet, we found that the overall incidence of DORV/TOF in the offspring of Cx40⁻/⁻ mice was rather high (33%). A number of factors may explain this discrepancy. First, electrophysio-

![Figure 4. Unbalanced partial endocardial cushion defect in Cx40⁻/⁻ mouse. A through C, Serial coronal hematoxylin-eosin-stained sections from a 6-hour-old Cx40⁻/⁻ mouse. B, Common atrioventricular valve (arrow). C, Large atrial septal defect (double-headed arrow). Bar=1 mm.](https://example.com/image.png)
logical studies do not require a detailed characterization of the anatomical structures. Without careful histological analysis, some of the malformations could have gone undetected.

Second, those studies have been conducted in young animals. Although other studies suggest a reduction in life expectancy in these animals, we found major malformations in some pups (such as the ones shown in Figure 5), most of the cases were compatible with life extra uterus. These animals may be able to compensate their hemodynamic function given the very low demand that is required for their lifestyle. Third, although some authors have looked at the cardiac histology of Cx40−/− mice, the incidence of malformations is low enough to suggest that maybe malformed hearts were coincidentally missed from being sampled. Fourth, it is possible that the incidence of malformations increased in our colony due to the fact that we backcrossed the C57BL/6 mice into the Cx40−/− line. Yet, it is worth noting that two independent Cx40 KO lines have been generated, one by Kirchhoff et al.17 and the other one by Simon et al.16

The previous report of cardiac defects in Cx40-deficient mice used the mice generated by Kirchhoff et al.13 whereas we used the other line. Thus, although it is clear that genetic background may play a major role in determining whether or not a Cx40-deficient mouse develops cardiac malformations, the presence of these defects is not exclusive to the mice in our colony.

The molecular mechanisms by which a Cx40-deficiency could lead to cardiac malformations are unknown. Conotruncal defects represented the majority of malformations observed in Cx40−/− mice (Table 3). These defects have been associated with deficiencies in the cardiac neural crest as well as abnormalities in cardiac looping.24,25 Given that, in mice, heart looping is completed at 9 ED and Cx40 gene expression in heart begins at 9.5 ED, we favor the possibility that Cx40 alters conotruncal formation by interfering with the interaction of the cardiac neural crest with the developing heart. Further studies should be directed at determining the potential role of Cx40 in cardiac neural crest function. Lack of Cx43, a different connexin isotope, is known to disrupt neural crest function and cause cardiac malformations of conotruncal origin.26,27

No expression of Cx40 has been detected in the neural crest, although a detailed analysis of its expression throughout development has not been performed. Whether the defects reported in this study are directly consequent to deficient intercellular communication in embryonic tissues remains to be determined. Yet, given that not all hearts showed malformations, an indirect role for Cx40 is suggested.

Although the incidence of cardiac malformations in the Cx40-deficient mice studied is much higher than in wild-type, malformations are present only in a fraction of the animals. Given that these mice are of mixed genetic background, our data suggest that genetic modifiers exist that influence cardiogenesis and either compensate for the absence of Cx40 or are modulated by the Cx40 protein. The fact that genetic modifiers may be responsible for variations in the phenotype of connexin-deficient animals has been reported before.28

Absence of Cx46 (a connexin isotype expressed in lens) causes cataracts in mice. A large variance in the cataracts was observed in mice of mixed 129SvJae X C57BL/6J genetic background. When the mice were backcrossed into a single background, the severity of the cataracts segregated according to the strain. It is therefore possible that backcrossing our Cx40-deficient mice into a single background will allow us to segregate the presence of cardiac malformation to a single strain. The latter could lead us to the identification of the specific gene that acts as a modifier of Cx40 during cardiac development.

The results in this study also open the possibility that variations in the function or regulation of the Cx40 gene may be part of the genetic profile leading to cardiac malformations in humans. A recent study reported that a Cx40 polymorphism concurrent with a cardiac sodium channel mutation were responsible for a rare arrhythmogenic disorder called “atrial standstill,” found in a large Dutch family.29 Other connexin polymorphisms have been associated with prevalent human diseases, such as myocardial infarction30 and atherosclerosis.31 In this context, it will be interesting to determine whether Cx40 polymorphisms may be associated with cardiac congenital malformations, particularly those segregated under altered genotypes such as Down syndrome.

In summary, we have described the frequency and the morphological characteristics of cardiac malformations present in Cx40-deficient mice. Our results show that Cx40 is not essential for cardiogenesis; yet, its absence or limited expression increases the probability of cardiac malformations. In addition to the relevance of these data to the understanding of the role of Cx40 in normal physiology, our results are relevant to those that use this animal model for functional studies. Given the mixed genetic background of the mice, it is likely that genetic modifiers may cosegregate with those mice where malformations are present. Future studies will be aimed at identifying those modifiers. Our data further open
the possibility that Cx40 polymorphisms may cosegregate with groups of patients where congenital cardiac malformations are commonly found.

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References
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