Heart Defects in Connexin43-Deficient Mice


Abstract—Cardiac malformation in connexin43 (CX43)-disrupted mice is restricted to the junction between right ventricle and outflow tract, even though CX43 is also expressed abundantly elsewhere. We analyzed cardiac morphogenesis in immunohistochemically and hybridohistochemically stained and three-dimensionally reconstructed serial sections of CX43-deficient embryos between embryonic day (ED) 10 and birth. The establishment of the D configuration in the ascending loop of CX43-deficient hearts is markedly retarded, so that the right ventricle retains a craniomedial position and is connected with the outflow tract by a more acute bend in ED10 and ED11 embryos. Because of the subsequent growth of the right ventricle, this condition usually evolves into a D loop, but when it persists, a “crisscross” configuration develops, with the atrioventricular cushions rotated 90°, a horizontal muscular ventricular septum, and a parallel course of the endocardial ridges of the outflow tract. After ED12, large intertrabecular pouches develop at the ventricular side of both shell-like myocardial structures that support the endocardial ridges of the outflow tract, ie, at the location that was earlier characterized by the acute bend between the right ventricle and the outflow tract and that subsequently develops into the anterosuperior leaflet of the tricuspid valve. Retarded development of the D configuration in the ascending loop of the embryonic heart predisposes the myocardium at the junction of the right ventricle and outflow tract to excessive development of intertrabecular pouches during subsequent development. (Circ Res. 1998;82:360–366.)

Key Words: connexin  ■ cardiac malformation  ■ cardiac development

Congenital malformations of the heart are among the most common birth defects in humans. The systematic analysis of these defects almost entirely rests on findings in human neonates with these structural anomalies. As a consequence, little direct information is available about the development of these malformations. In particular, it is usually not known to what extent a particular malformation can be traced to a single morphogenetic event or process. A case in point is the development of the malformation of the subpulmonary outflow tract of the heart in mice that are deficient for gap junctions of the CX43 type that was reported by Reaume et al.1 The death of CX43-deficient mice has been associated homozgously for the CX43-null mutation is therefore remarkable. Thus, CX32 deficiency was shown to be the underlying cause of X-linked Charcot-Marie-Tooth neuropathy.3 However, congenital gene deficiencies usually remain undetected if the protein plays a crucial role during early embryonic development. CX43, for example, is already expressed in the eight-cell embryo.4 Furthermore, CX43 is abundantly expressed in the developing and adult heart,5–12 and gap junctions of this type are thought to be responsible for the behavior of the myocardium as an electrical syncytium.13 In this connection, it has recently been shown that the spread of the impulse in animals heterozygous for the CX43-null mutation is markedly impaired.14 The survival to birth of embryonic mice homozygous for the CX43-null mutation is therefore remarkable.1 The death of CX43-deficient mice has been associated with an obstruction of the subpulmonary outflow tract. However, the underlying cause of this abnormality and, in particular, why the malformation is restricted to the subpulmonary outflow tract, whereas other parts of the heart in which CX43 is also expressed abundantly are not affected, remain to be established. In the present study, we analyzed serially sectioned CX43 gene–disrupted mouse embryos between ED10 and birth. The sections were stained immunohistochemically or hybridohistochemically to aid in delineating the myocardial and fibrous structures and were subsequently reconstructed to...
visualize structural anomalies. Cardiac looping was found to be impaired during early organogenesis, resulting in a more acute bend between the embryonic right ventricle and outflow tract. Subsequently, an abnormal delamination of the anterosuperior leaflet of the tricuspid valve develops at this location.

### Materials and Methods

#### Animals

A heterozygous pair of the Sv109 strain of CX43 gene–disrupted mice was purchased from the Jackson Laboratory, Bar Harbor, Me. Matings were overnight, and the day of finding a plug was designated day 0. The offspring were screened by PCR using primers specific for neo' and CX43. The sequence of both primers was as follows: neo', 5'-CAAGATGATTGCACCGAG-3' and minus 5'-TAT CACGGTTAGCCACGGC-3'; CX43, plus 5'-CCATCCAAA GACTTGGGATC-3' and minus 5'-TGATTACCCCTC CACGGGAAC-3'. A CX43-negative, neo'-positive PCR signal was used to identify embryos with both CX43 alleles disrupted. Embryos were collected every day between ED10 and ED18 and staged according to Theiler.15 Neonates were collected within 2 hours of birth. Embryos were fixed either in a mixture of ice-cold methanol/acetone/water (2:2:1) for immunohistochemical analysis or in 4% paraformaldehyde (wt/vol) in PBS (150 mmol/L NaCl and 10 mmol/L sodium phosphate, pH 7.4) for in situ hybridization studies. The embryos were fixed overnight, dehydrated in a graded series of ethanol, embedded in Paraplast Plus (Sherwood), serially sectioned at 7-μm thickness, and mounted on poly-L-lysine–coated slides for immunohistochemistry or in situ hybridization, respectively. The mounted sections were stored at 4°C until use.

#### Immunohistochemistry

After the paraffin was removed, the sections were pretreated, stained, and mounted as described.16 Serial sections were incubated overnight with alternately monoclonal antibodies against α-MHC (1:10),17 β-MHC (1:10),18 α-SMA (1:1000), Sigma IMM-H2), desmin (1:50), Monosan, Mon 3001, clone 33), and a polyclonal antibody against fibronectin (1:6000, AB 1942, Brunschwig Chemie). Sections incubated with monoclonal antibodies were stained with rabbit-antimouse IgG (1:7500, noncommercial) followed by incubation with goat anti-rabbit IgG (1:250, noncommercial); the sections incubated with the polyclonal anti-fibronectin antiserum were stained directly with goat anti-rabbit IgG. Antibody binding was demonstrated with rabbit peroxidase–anti-peroxidase complex (1:750, Nordic) and 3,3-diaminobenzidine tetrahydrochloride and H2O2 as substrates.

#### Immunofluorescence

Hearts were isolated and directly frozen in liquid nitrogen. Unfixed cryosections (10 μm) were collected on slides coated with aminopropyltriethoxysilane (Sigma) and stored at −80°C until used. Slides were equilibrated to room temperature and subsequently incubated with 0.2% Triton X-100 in PBS for 1 hour, followed by 30 minutes of blocking with 2% BSA. Sections were incubated overnight with appropriate dilutions of primary antibodies in PBS supplemented with 2% BSA and 10% normal goat serum. All incubation steps were performed at room temperature, and between all incubation steps the slides were thoroughly washed with PBS. The anti-CX40 and anti-CX37 antibodies were raised in rabbits against a synthetic peptide containing residues 335 to 356 of rat CX43 and residues 315 to 331 of mouse CX37, affinity-purified, and diluted to 5 to 15 μg/mL and 2 to 4 μg/mL, respectively. The specificity of the CX40 antibody was reported, whereas that of the CX37 antibody was demonstrated in communication-deficient cells transfected with mouse CX37.20 The next morning cells were preincubated with 2% BSA in PBS. Immunolabeling was carried out with fluorescein isothiocyanate–conjugated secondary antibodies (Jackson immunostaining) against rabbit immunoglobulins. Sections were mounted in Vectashield mounting medium (Vector Laboratories) and examined and photographed with an epifluorescence microscope equipped with the appropriate filter.

### In Situ Hybridization

In situ hybridization was carried out on the sections of ED14, ED16, and ED18 embryos to visualize the expression of CX40, CX43, CX45, SERCA2, and ANF, as described in detail previously.21 Single-stranded antisense RNA probes were made by in vitro RNA transcription. All clones were inserted into pBluescript. The rat CX43 clone (1.5 kb)22 was linearized with Sal I and transcribed with T7 RNA polymerase, with both 3'S-UTP and 3'S-CTP as labeled substrates. The mouse CX40 (1.35 kb)23 and CX43 (1.68 kb)24 clones were linearized with Asp718 and transcribed with T3 RNA polymerase. The rat ANF clone (0.6 kb)25 was linearized with BanHI and transcribed with T7 RNA polymerase. The SERCA2 clone (1.6 kb)26,27 was linearized with Xho I and transcribed with T3 RNA polymerase. cRNA probes were degraded to fragments of ~100 bp by hydrolysis in 80 mmol/L NaHCO3 and 120 mmol/L Na2CO3 at 60°C for 10 to 20 minutes. The hybridization was performed with 30 pg of 32P-labeled antisense RNA (16 to 18 hours, 54°C) in 6 to 10 μL of hybridization mixture per section (50% formamide, 10% dextran sulfate, 300 mmol/L NaCl, 30 mmol/L sodium citrate, 2× Denhardt’s solution, 0.1% Triton X-100, 10 mmol/L dithiothreitol, and 200 ng/μL herring sperm DNA; pH 7.4). The specific activity of the cRNA was 1.5×106 cpm/μg for CX40, CX43, ANF, and SERCA2 cRNA and 3×106 cpm/μg for CX43 (double-labeled). After hybridization, the sections were washed and treated with RNase (30 minutes, 37°C) to reduce nonspecific binding. Under these conditions, the hybridization signal in sections is quantitative.

No CX43 protein is expressed in CX43-null mutants (not shown; see Reference 1). However, because the promoterless neo' gene was placed in frame after the second amino acid of CX43 and replaced the transmembrane regions of the CX43, but not its 3' tail in the CX43-targeting vector,7 the pattern of expression of the chimeric neo'-CX43 mRNA could be visualized with labeled antisense CX43 probe.

### Three-Dimensional Reconstruction

Selected hearts of CX43-mutant mice and their heterozygous littermates were reconstructed three-dimensionally as described28 and rendered with the help of a medical artist.

### Results

We have investigated the architecture and a number of gene expression patterns in the hearts of homozygous and heterozygous CX43–disrupted mice between ED10 and birth and compared them with wild-type littermates. A total of 44 homozygous mice, obtained from matings of heterozygotes, were available for study. These homozygotes represented 16.2% of the live animals, which is significantly below the expected 25% ($P<.01$) and confirms the earlier finding of Reamü et al.1 The reduced percentage of homozygotes was observed at all ages investigated. Apart from the malformations in CX43-deficient hearts described below, we observed a left
coronary artery that ended in the right ventricle in an ED14 heart (not shown). No abnormalities were found in the conduction system of affected hearts. The distribution of cardiac nerves was not studied. In addition to the homozygous CX43 knockout embryos and neonates, 20 heterozygotes and 20 wild-type littermates were analyzed. No abnormalities in architecture or gene expression pattern were observed in wild-type or heterozygous animals, in agreement with another recent study.14

**Development of the Heart Between ED10 and ED12**

In the mouse embryo, cardiac looping is initiated at ED8.5 (Theiler’s stage 13)15 and is virtually completed at ED9 (Theiler’s stage 14), i.e., before the respective segments of the embryonic heart have become identifiable by their lateral expansion. At this stage, the heart loop has a sagittally oriented component with inflow and outflow tract dorsal and the ventricle ventral and a transversely oriented component with the left ventricle left and the right ventricle right (the so-called dextro or D loop29,30). When we compared homozygous and heterozygous CX43-disrupted ED10 hearts (Fig 1A to 1F), we observed that in the homozygous animals, the transverse component of the loop had only incompletely developed. The right ventricle had retained a more cranial and medial position compared with that of control animals (so-called A loop)29,30 so that the developing interventricular septum occupied a transverse rather than a sagittal position and the connection of the outflow tract with the right ventricle was more acute than in control animals. This configuration was also seen in ED11 animals (Fig 1G to 1J). Subsequently, the rightward expansion of the right ventricle changed its distinct cranial position into a more lateral one, so that the developing abnormality became less obvious and most CX43 knockout hearts could no longer be identified externally at ED12. Although the pattern, as described, was seen in all homozygous embryos, some variation in the severity of the looping problems was observed.

Histological examination of ED10, ED11 (Fig 1A, 1D, 1I, and 1J), and ED12 hearts (not shown) of CX43 knockout animals did not reveal structural abnormalities. The sinus venosus, left and right atria, and atrial septum were positioned normally. These structures showed high α-MHC expression, whereas in the outflow tract and ventricles, α-MHC expression was low (not shown). β-MHC expression in the hearts between ED10 and ED12 was mainly restricted to the outflow tract and ventricles (Fig 1). A strong expression of α-SMA and desmin was detected throughout the heart between ED10 and ED12, especially in the outflow tract, where fibronectin delineated the endocardial cushions/ridges (Fig 2A to 2D). Apart from the looping abnormality and the cases to be described in the next paragraph, no structural abnormalities were observed in the ventricles and outflow tract, in particular not at the junction of the right ventricle and the outflow tract. Nevertheless, it was observed that the embryos with the more severe looping problems showed some developmental retardation.

Especially interesting cases that illustrate the looping abnormalities observed in CX43 knockout mice were provided by the ED11 and the ED12 embryo illustrated in Figs 1H to 1J and 2, respectively. The ED11 embryo shown in Fig 1G to 1J is considered to be the most seriously affected of 4 CX43-deficient ED11 embryos studied because of its pronounced cranial position of the right ventricle and its leftward convex bend midway in the outflow tract and is thought to be a likely precursor of the condition seen in the ED12 embryo shown in Fig 2. In this latter embryo, which is one of four knockouts of this age group studied, the inflow tract and the atrium appeared normal. The systemic veins drained into the right atrium, which was substantially larger than the left. The developing
sinoatrial node could be identified at the entrance of the superior cardinal vein into the right atrium. The atrioventricular canal was still located over the embryonic left ventricle. However, the fusion line of both atrioventricular cushions was oriented craniocaudally instead of laterolaterally, with the tricuspid opening positioned cranially and the mitral opening positioned caudally. The right ventricle had remained positioned cranially to the left ventricle, so that the interventricular septum retained a transverse orientation and so that a pronounced leftward convex bend developed midway in the outflow tract (Fig 2A and 2B). The parietal and septal endocardial ridges of the outflow tract followed a parallel rather than the usual spiral course, with the parietal ridge located cranially and the septal ridge caudally. In addition, both ridges had developed asymmetrically and were apposed in the inner curvature of the outflow tract. Since the outflow tract had not (yet) septated, the single outlet channel split, on its emergence from the myocardial sleeve, into bilateral sixth arches (the left being approximately twice the size of the right one) and an initially single, cranially running aortic channel. This ED12 embryo had, therefore, retained the A-loop configuration that is normally only found in CX43-deficient ED10
Figure 4. Development of intertrabecular pouches in the right ventricle (RV) at its junction with the outflow tract (OFT). AO indicates aorta; P, pulmonary trunk. Panels A and B show the expression of the chimeric neo-CX43 mRNA; panels C and D show the expression of SERCA2 mRNA in hearts of CX43-deficient mice at ED14 (panels A and C) and ED16 (panels B and D). Bar=200 μm.

Figure 5. The trabecular pouches of CX43-deficient hearts develop in the right ventricle (RV) at its junction with the outflow tract (OFT). AO indicates aorta; P, pulmonary trunk. Panels A and B show the expression of the chimeric neo-CX43 mRNA; panels C and D show the expression of SERCA2 mRNA in hearts of CX43-deficient mice at ED14 (panels A and C) and ED16 (panels B and D). Bar=200 μm.

and ED11 embryos. In summary, this ED12 heart presented as situs solitus, with the tricuspid orifice and right ventricle cranial instead of lateral and a leftward-bent outflow tract.

Development of the Heart Between ED13 and Birth

Three-dimensional reconstruction of ED13 hearts showed that, externally, homozgyous CX43 knockout animals could be distinguished from their heterozygous littermates by a slight dilatation of the right ventricle at its junction with the outflow tract (compare Fig 3C with 3F). In sections, the structural abnormality that is characteristic for CX43-disrupted hearts, namely, the excessive development of intertrabecular pouches from the lumen of distal portion of the right ventricle, had become identifiable. These pouches develop at the ventricular side of two shelflike myocardial structures, which support the base of both endocardial ridges of the outflow tract (Fig 3D and 3E). Therefore, the pouches develop at the location that in previous stages was characterized by an acute bend between the right ventricle and the outflow tract as a result of the persisting A-loop configuration. The medial shelf is located on the right ventromedial wall of the interventricular septum, and the lateral shelf is located on the lateral free wall of the right ventricle (Fig 3G and 3H, respectively). These shelves, which we observed in both normal and abnormal embryos, were not yet seen in ED12 hearts and arise as a result of a local ingrowth of myocytes into the base of the endocardial ridges. In the CX43 knockout embryos, the extended intertrabecular pouches that develop underneath the shelves emphasized the presence and location of these shelves.

The endocardial and myocardial components of these shelves will evolve into the smooth atrial and rough ventricular surface of the anterosuperior leaflet of the tricuspid valve, respectively.31 The development of the intertrabecular pouches at the site where, in normal development, the anterosuperior valve leaflet delaminates explains the involvement of this valve leaflet in CX43 deficiency.

External inspection of ED14 hearts revealed that the myocardium at the junction of right ventricle and outflow tract continued to dilate symmetrically (not shown). In the embryonic sections, we observed that the intertrabecular spaces near the junction of the right ventricle with the outflow tract that we first observed at ED13 had already become so enlarged at ED14 that sizable cavities had developed (Fig 4A and 4B). On the basis of the staining pattern of SERCA2 and CX43 (Fig 5), which are lower and absent, respectively, in the outflow tract,8,21 we concluded that the cavities developed within the confines of the right ventricle but at its junction with the outflow tract. In older animals, the pouches continued to increase in diameter and to expand dorsolaterally, thereby surrounding the pulmonary semilunar valves (Fig 4C and 4D). As a result, the bulging wall of the right ventricle gradually came to surround and enclose the root of the pulmonary trunk (compare the normal heart in Fig 4G with the CX43-deficient one in Fig 4H and 4I). No such process was seen to occur near the aortic outlet. The trabeculae traversing the pouches clearly had a hypertrophic appearance. In particular, the developing medial (septal) papillary muscle, which attaches the anterosuperior leaflet of the tricuspid valve to the interventricular septum and which can be recognized as medially flanking the central channel near its transition into the subpulmonary portion of the outflow tract, was markedly hypertrophied (Fig 4B). As a consequence of the strong development of the trabeculae, the lumen of the original endocardial tube remained an easily identifiable, centrally located, tortuous, and
The intensity of expression of a
However, the expression of these connexins was similar in control
(CX37 and CX40) or in situ hybridization (CX40 and CX45).
CX45, these connexins were studied by immunofluorescence
accompanied by changes in the expression of CX37, CX40, or
mRNA in a control and a CX43-deficient ED18 heart, respectively.
Bar=200 μm.

Figure 6. CX43-deficient hearts express ANF. AO indicates aorta; LA, left atrium; P, pulmonary trunk; RA, right atrium; and RV, right ventricle. Panels A and B show ANF mRNA in control and a CX43-deficient ED18 heart, respectively.

narrow channel (asterisk in Fig 4B and 4E), to which the
intertrabecular pouches were connected by sievelike openings.
The compact myocardium that borders the pouches externally
remained relatively thin. The region involved in this pathological remodeling of the right ventricle remained confined to its distal portion, ie, the myocardium supporting the developing subpulmonary outlet portion of the outflow tract. In a caudal direction, the pouches rapidly decreased in size and did not extend beyond (caudal to) the myocardial component of the developing anterosuperior leaflet and the anterior papillary muscle; ie, the caudal part of the right ventricle and the entire left ventricle were not visibly affected (Fig 4F).

The expressions of α- and β-MHC, α-SMA, desmin, SERCA2, and fibronectin, which highlight the structural and functional development of the embryonic myocardium and the endocardial cushions/ridges, were found to be comparable in homozygous and heterozygous animals (not shown). The staining intensity of α-MHC in the ventricles and outflow tract remained relatively low or absent. Elsewhere in the heart, CX45 expression was detected more prominently in the affected portion of the heart in CX43-deficient hearts, clearly expressed CX45 in both control and affected embryos (not shown). In this respect, mouse neonates are apparently more vulnerable than human neonates. However, both malformations are structurally different. The hearts of neonatal and mutant embryos. The vessel walls were positive for CX37 and CX40, with CX40 also being abundantly expressed in the atrial and the ventricular trabeculae (not shown). The marked difference in the developmental timing of the disappearance of CX40 mRNA from the trabeculae of left and right ventricles that we previously noted in rat was also seen in control and mutant mice (not shown). The myocardium of the shelves at the base of the endocardial ridges of the outflow tract, ie, the structures below which the intertrabecular pouches develop in the CX43-deficient hearts, clearly expressed CX45 in both control and affected embryos (not shown). Elsewhere in the heart, CX45 expression was very low or absent.

Discussion

The major abnormalities of cardiac development in CX43-deficient embryos are a delay in the normal looping of the ascending limb of the heart tube and, subsequently, the progressive development of intertrabecular pouches at the junction of the right ventricle and the outflow tract, resulting in an abnormal delamination of the anterosuperior leaflet of the tricuspid valve. In normal mouse embryos, the ascending limb of the heart tube, which includes the right ventricle and the outflow tract, will adopt a rightward position (D loop) during ED9, but in CX43-deficient embryos, it temporarily retains a more symmetric middle position (A loop). This A-loop configuration is metastable and usually changes into a D loop in CX43 knockout embryos after ED11. However, if the A-loop configuration persists, a heart develops in which both atria retain their respective left- and right-sided position but in which the position of the more distal parts of the heart has rotated by 90°. Thus, the atrioventricular cushions occupy lateral positions in the atrioventricular canal, while the right ventricle occupies a cranial position relative to the left ventricle. Accordingly, the left atrioventricular connection is caudal and the right atrioventricular connection is cranial, while the ventricular septum occupies a transverse plane. This configuration is reminiscent of that found in “crisscross” hearts, although the development of such malformed hearts as a result of a persisting A loop remains to be shown.

The increasingly overt structural malformation that is typical for CX43-deficient hearts is confined to the myocardium supporting the ventricular base of the endocardial outflow tract ridges. The markers CX43 and SERCA2 show that these shelf-shaped myocardial structures, which are involved in the formation of the anterosuperior leaflet of the tricuspid valve, develop at the boundary of the right ventricle with the outflow tract. Topographically, therefore, the malformation develops where, in preceding stages, the junction of the embryonic right ventricle and the outflow tract made a more acute bend in CX43-deficient embryos than in morphologically normal embryos, but we do not know at present how such a temporary geometric distortion contributes to the subsequent developmental abnormalities at this location.

Both CX43-deficient mice and mice in which local overexpression of CX43 is driven by the CMV promoter/enhancer suffer from an obstruction of the subpulmonary outlet and neonatal death. In this respect, mouse neonates are apparently more vulnerable than human neonates. However, both malformations are structurally different. The hearts of neonatal
CMV-CX43 transgenes are characterized by a narrowing of the subpulmonary outlet as a result of hypertrophy of the compact myocardium of the right ventricle and the interventricular septum. In CX43-deficient hearts, on the other hand, it seems likely that contraction of the markedly hypertrophied trabeculae, including the papillary muscles of the tricuspid valve, squeezes the narrow and tortuous outlet channel and prevents the ejection of the blood. Similarly, the bilateral dilatation of the right ventricular conus probably results from the squeezing of the narrow muscular openings of the pouches into the outlet channel of the right ventricle during contraction. In accordance with this scenario, we observed a locally increased expression of ANF, a sensitive marker for cardiac dilation of the right ventricular conus probably results from hypertrophy of the compact myocardium of the right ventricle and the interventricular septum.

Acknowledgments

The drawings of the three-dimensional reconstructions were skillfully prepared by I.E.M. Oosterling; C. Graveniezer is credited for the photographs.

References

Heart Defects in Connexin43-Deficient Mice


Circ Res. 1998;82:360-366
doi: 10.1161/01.RES.82.3.360

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1998 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/82/3/360

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/