Connexin31 cannot functionally replace connexin43 during cardiac morphogenesis in mice

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Accepted 18 November 2005
Journal of Cell Science 119, 693-701 Published by The Company of Biologists 2006
doi:10.1242/jcs.02800

Summary
In the gastrulating mouse embryo, the gap junction protein connexin43 is expressed exclusively in cells derived from the inner cell mass, whereas connexin31 is expressed in cells of the trophoblast lineage. Since connexin43 and connexin31 do not form heterotypic gap junction channels in exogenous expression systems, such as HeLa cells and Xenopus oocytes, previous studies have suggested that the incompatibility of these two connexins could contribute to the separation of connexin43-expressing and connexin31-expressing compartments between embryo and extraembryonic tissues at gastrulation, respectively. Thus, we have generated connexin43 knock-in connexin31 mice, in which the coding region of the connexin43 gene was replaced by that of connexin31. Interbreeding of heterozygous connexin43 knock-in connexin31 mice resulted in morphologically and functionally normal hearts. We conclude that connexin31 cannot functionally replace connexin43 during cardiac morphogenesis.

Key words: Gap junction, Connexin, Knock-in mice, Coupling compartment, Subpulmonary outlet, Low voltage QRS complex

Introduction
Connexin (Cx) proteins are subunits of gap junction channels that permit diffusional exchange of ions, secondary messenger molecules and metabolites up to 1000 Da and can serve for transmission of electrical signals between two contacting cells. Each gap junction channel consists of two hemichannels, the connexons, which are contributed by two neighboring cells. Each connexon is composed of six connexin protein subunits (Nicholson, 2003). In the mouse genome, 20 connexin genes (Cx) have been identified (Söhl and Willecke, 2003). They show cell type- and differentiation-dependent expression patterns, whereby most cell types express more than one connexin isoform. Connexins can assemble into homomeric or heteromeric hemichannels. The hemichannels can dock to hemichannels of the same or different connexin composition in the plasma membrane of apposed cells to form homotypic or heterotypic channels, respectively (Willecke et al., 2002). Gap junction channels composed of different connexin isoforms differ from each other in unitary conductance and permeability to small molecules (Harris, 2001). This raised the question ‘to what extent do the different connexin isoforms limit or support functional specialization of different cell types?’.

Of the 20 known mouse connexin genes, connexin43 (Cx43) is most abundantly expressed in different cell types. In mouse heart, Connexin43 protein (Cx43) is found in the working myocardium and Purkinje fibers (van Veen et al., 2001). Cx43-deficient mice died shortly after birth due to obstruction of the right ventricular outflow tract of the heart (Reaume et al., 1995). It has been described that the lethality of Cx43-deficient mice could be rescued by Cx32 or Cx40, indicating that Cx43, Cx40 and Cx32 share at least some common functions. However, Cx43 knock-in Cx32 (Cx43/Cx32) and Cx43 knock-in Cx40 (Cx43/Cx40) mice differed functionally and morphologically from each other and from wild-type mice (Plum et al., 2000). Cx31 is not expressed in the heart and Cx31 deficiency in mice caused transient placental dysmorphogenesis (Plum et al., 2001). In transfected HeLa cells or in RNA-injected Xenopus oocytes, Cx31 hemichannels did not form functional heterotypic channels with any other connexin, including Cx43 hemichannels, when analyzed by transfer of microinjected tracer molecules (Elfgang et al.,
1995) or by electrical conductance (Yeager and Nicholson, 2000). We refer to this as the incompatibility of Cx43 and Cx31 hemichannels. During embryonic development, both Cx43 and Cx31 are present in the compacted morula and throughout the blastocyst. After implantation, Cx43 protein is detected exclusively in cells derived from the inner cell mass, whereas Cx31 protein is localized in cells of the trophoectoderm lineage (Dahl et al., 1996; Reuss et al., 1996). It has been hypothesized that the incompatibility of these two connexins might contribute to the separation of Cx43-expressing and Cx31-expressing compartments between embryo and extraembryonic tissues at gastrulation (Dahl et al., 1996). Using Cx43 knock-in Cx31 mice (Cx4331/31), in which the coding region of the Cx43 gene was replaced by that of Cx31, we have now investigated whether the incompatibility between these two isoforms is necessary for embryonic development and whether the loss of Cx43 can be compensated for by Cx31.

**Results**

**Generation of floxCx43KI31neo mice**

To circumvent possible embryonic lethality, we first generated a mouse line that carries a ‘floxed’ Cx43 coding region, i.e. flanked by loxP recognition sites for the Cre recombinase (Nagy, 2000). The Cx31 coding region was inserted behind the second loxP site (Fig. 1A). After crossing the floxed mice with PGK-Cre mice that expressed the Cre recombinase under control of the phosphoglycerate kinase (PGK) promoter, the Cx43 coding region was removed by Cre-mediated deletion and Cx31 was expressed under control of the Cx43 promoter. This approach allowed us not only to study the general replacement of Cx43 by Cx31, but will also be useful in the future for a cell-type-specific replacement by crossing with other mouse lines that express Cre under control of cell-type-specific promoters.

To check whether expression of Cx43 and Cx31 in the...
Cx43 knock-in Cx31 mice

floxCx43KI31neo mice (here abbreviated as Cx43floxflox) was influenced by the loxP sites, the PGK-neomycin cassette or the silent Cx31 gene, Cx43+/flox mice were crossed with each other. Cx43floxflox animals were born at the expected Mendelian frequency. We performed western blot analysis of heart and brain lysates using anti-Cx43 and anti-Cx31 antibodies. Compared to wild-type animals, the protein level of Cx43 in the heart was slightly increased in Cx43+/flox or Cx43floxflox mice (data not shown). By contrast, no significant change of Cx43 expression was found in the brain. As in wild-type mice, Cx31 was not expressed in brain or heart of Cx43+/flox and Cx43floxflox mice (data not shown). Therefore, we concluded that floxCx43KI31neo mice resemble wild-type mice with regard to Cx43 or Cx31 expression.

Generation of Cx4331/31 mice

To generate Cx4331/31 mice, in which the coding region of Cx43 was replaced by the coding region of Cx31, heterozygous male floxCx43KI31neo mice (Cx43+/flox) were mated with female PGK-Cre mice. Under these conditions, Cre activity commences in the diploid phase of oogenesis (Lallemand et al., 1998). The Cre-mediated recombination led to excision of the coding region of Cx43 and the PGK-neomycin cassette. The Cx31 gene was then expressed under control of endogenous Cx43 gene regulatory elements. Cx43311 mice were viable and fertile (Fig. 5). The offspring Cx43+/31 mice were intercrossed. Occurrence of three genotypes Cx433+/+, Cx433/31 and Cx4331/31 of mice was proved by PCR (Fig. 1E), Southern blot (Fig. 1B-D), western blot (Fig. 2) and immunofluorescence (Figs 3, 4) analyses.

A multiplex PCR (Fig. 1A) was established for genotyping using one common reverse primer from the 3’ untranslated region of Cx43 (primer 8), one forward primer from the coding region of Cx43 (primer 6) and the other from the coding region of Cx31 (primer 7). A 381 bp amplicon was derived from the wild-type allele and a 615 bp amplicon was delivered from the knock-in allele (Fig. 1E). In the Southern blot hybridization, the Cx43 external probe recognized a 8 kb wild-type fragment and a 2.9 kb knock-in fragment (Fig. 1B). The Cx43 internal probe detected the 8 kb wild-type fragment only in wild-type but not in homozygous mutant DNA (Fig. 1C). The Cx31 internal probe labeled the 12 kb wild-type fragment in all three genotypes and the additional 5.3 kb knock-in fragment in heterozygous and homozygous mutants (Fig. 1D). Western blot analyses of postnatal day 7 (P7) heart and brain lysates demonstrated that Cx43 protein was absent in the Cx4331/31 mice and Cx31 protein was translated in Cx43+/31 and Cx4331/31 mutants (Fig. 2). Immunofluorescence analysis of P0 ventricular myocardium confirmed the results of the western blot analysis: no gap junctional plaques due to Cx43 protein were detected in the Cx4331/31 mice, whereas signals of the Cx31 protein were found in Cx43+/31 and Cx4331/31 mutants (Fig. 3). In addition, we performed double immunofluorescence analysis of Cx43 and Cx31 on the Cx4331/31 heart tissue (Fig. 4). The working cardiomyocytes of the adult ventricle were extensively interconnected at the intercalated disk (Fig. 4D,E). Cx43- and Cx31-containing plaques were
predominantly co-localized (Fig. 4F). By contrast, P0 ventricular cardiomyocytes did not show intercalated disks (Fig. 4A,B) and the co-localization of Cx43 and Cx31 was low (Fig. 4C). Immunofluorescence analysis of Cx43+/31 heart showed regular expression of Cx40 in the atria and vessels. Upregulation of Cx40 was not found in Cx43+/31 mice (data not shown). Western blot analysis showed that the total amount of Cx40 was not changed in the P0 heart lysate of Cx43+/31 mice (data not shown). By immunofluorescence, Cx45 was detected in the conduction system, but not in the working cardiomyocytes of either Cx43+/+ or Cx43+31 mice (data not shown). In the western blot analyses, Cx45 was not found to be upregulated in the Cx43+/31 heart lysate (data not shown).

Cx43+/31 mice die postnatally because of a dysmorphogenesis of the right ventricular outflow tract

To obtain Cx43+/31 mice, Cx43+/+ mice were intercrossed. At birth, we found the expected ratio of homozygous, heterozygous and wild-type mice (Fig. 5). This indicated that the embryos developed to term, although Cx31 was presumably expressed both in the extraembryonic tissue and the embryo proper (instead of Cx43). However, no Cx43+31 mice survived to adulthood. 50% of the Cx43+31 animals survived at P0, 30% at P1, none of them lived longer than 10 days (Fig. 5). At P0, the average weight of the wild-type mice was 1.4±0.15 g and the weight of Cx43+31 mice was 1.2±0.14 g. Several days later, the living Cx43+/31 animals could be identified by their lower weight. They did not grow as fast as their Cx43+31 and Cx43+/31 littermates. There was no obvious difference in the phenotype between Cx43+31 and Cx43+/31 mice.

Since Cx43-deficient mice died shortly after birth due to a dysmorphogenesis of the right ventricular outflow tract (Reaume et al., 1995), we investigated neonatal Cx43+/31 hearts histologically (Fig. 6). We examined six Cx43+/+ (three P0, two P4 and one P5), six Cx43+/31 (two P0, two P4 and two P5) and ten Cx43+31 (eight P0, one P4 and one P5) hearts. The pulmonary outlet of all ten Cx43+31 heart was prominent with irregular hypertrophic trabeculation forming abnormal pouch-like spaces, surrounding the narrow but still open substantial lumen. The morphology of Cx43+/31 hearts was basically normal. We conclude that the morphological defect of Cx43+31 hearts was similar to Cx43-deficient hearts and Cx31 could not replace the functions of Cx43 during cardiac morphogenesis.

Neonatal Cx43+31 mice show low voltage of the QRS complex in the electrocardiogram

A total of 81 ECG recordings of P0 mice were performed (Fig. 7), 23 Cx43+/+, 43 Cx43+/31 and 15 Cx43+31 mice. Mean heart rate was 362±65 beats per minute (bpm; asterisk denotes statistical significance, see Materials and Methods), 325±35 bpm and 295±25 bpm, respectively. Thus, it was significantly lower in Cx43+31 mice than in Cx43+/+ mice (ANOVA, P<0.05). Results of signal-averaged ECG analyses are summarized in Table 1. Significant prolongation of P wave (P<0.01) and QRS duration (P<0.001) were detected in Cx43+31 mice. The QRS amplitudes of Cx43+31 mice were
significantly lower than those of Cx43+/+ and Cx43+3/31 mice (P<0.001). Owing to severely decreased QRS amplitude, QT and QTc quantification could not be performed reliably in Cx4331/31 mice. One Cx4331/31 mouse demonstrated a spontaneous AV-block III°. No further arrhythmias were documented during the 5 minute period of ECG recording.

Echocardiographical analyses
The ejection fraction was 87±7%* in Cx43+/+, 78±10% in Cx43+/31 and 65±10% in Cx4331/31 mice on postnatal day 5, indicating significantly reduced systolic function in neonatal Cx4331/31 mice (ANOVA P<0.05). Mean Doppler-derived systolic velocities in the aorta (Cx43+/+: 98±21 cm/second; Cx43+/31: 90±12 cm/second; Cx4331/31: 93±16 cm/second) and the pulmonary trunk (Cx43+/+: 55±15 cm/second; Cx43+/31: 52±16 cm/second; Cx4331/31: 42±8 cm/second) were not significantly different. However, P5 Cx4331/31 mice had slightly but not significantly lower flow velocities in the pulmonary trunk. Left-ventricular mass (LVM) was not significantly different between the three groups. However, LVM normalized to body weight (BW) revealed significantly higher values in Cx4331/31 mice (LVM/BW Cx43+/+: 4.0±0.3 mg/g*; LVM/BW Cx43+/31: 5.1±1.0 mg/g*; LVM/BW Cx4331/31: 6.0±0.9 mg/g; ANOVA P<0.001). These results are summarized in Table 2.

Fig. 6. Transverse sections through P4 hearts of Cx43+/+ (A), Cx43+/31 (B) and Cx4331/31 mice (C). The heart of the Cx4331/31 mouse showed irregular hypertrophic trabeculae with abnormal pouches (asterisks) in the subpulmonary outlet of the right ventricle. The heart of the Cx43+/+ mouse was almost normal. aa: ascending aorta; la, left atrium; ra, right atrium; rv, right ventricle; pv, pulmonary valve. Bar, 500 μm.

Fig. 7. Representative signal-averaged ECG recordings performed at P0. Cx43+/+ (A) and Cx43+/31 mice (B) show normal ECG patterns. Note significant low voltage and prolonged P wave as well as QRS duration in Cx4331/31 mice (C).

No differences in development and placental architecture were found in Cx4331/31 conceptuses
Cx43 is expressed in spongiotrophoblast and giant cells in the mature placenta. In order to test whether or not replacement of Cx43 by Cx31 leads to impairment of trophoblast cell lineage differentiation, we examined placentas for phenotypic changes. Placental morphology was investigated at embryonic day (E) 10.5 and E17.5. No obvious differences in placental architecture, correlated to the developmental stage, were observed between Cx43+/+, Cx43+/31 and Cx4331/31 mice from the same litter (Fig. 8). In mature placentas, all different trophoblast cell types analyzed were normally developed, for example the labyrinthine trophoblast, the spongiotrophoblast, interspersed by nests of glycogen cells, and trophoblast giant cells located at the feto-maternal border.

Discussion
We prepared the floxed Cx43KI31neo construct, since we anticipated that Cx4331/31 mice might die during development at the gastrulation stage. At this time, Cx43 is expressed in the embryonic compartment, whereas Cx31 was found exclusively in the surrounding extraembryonic compartment (Dahl et al., 1996). Since Cx31 and Cx43 did not form heterotypic gap junction channels in transfected HeLa cells (Elfgang et al., 1995) and in RNA-injected Xenopus oocytes (Yeager and Nicholson, 2000), we hypothesized that this incompatibility might be important for the further development of the gastrulating mouse embryo. Our results presented here clearly show that the incompatibility of Cx43 and Cx31 hemichannels cannot be essential for mouse embryonic development, because Cx43+/31 and Cx4331/31 mice were born at the expected Mendelian frequencies after interbreeding of Cx43+/+ mice.
There might be several reasons why the separation between embryonic and placental lineage might not have any effect in our experimental system. For example, the separation between the embryo and the surrounding extraembryonic tissues at gastrulation might have other explanations, such as discrete tissue masses that do not establish gap-junctional communication across an extracellular matrix barrier or, alternatively, the abolition of the separation does not impair the development of the two cell lineages. newborns showed a significantly reduced weight compared to their Cx43+/+ and Cx43+/31 littermates. The weight of embryos or newborns has not been analyzed so far for Cx43−/− mice (Reaume et al., 1995), but recently Kibschull et al. (Kibschull et al., 2005) showed for the Cx31/Cx43 double knockout mouse that at 17.5 dpc the Cx31+/−/Cx43−/− embryos were also significantly smaller compared to their Cx31+/−/Cx43+/− and Cx31+/−/Cx43+/+ littermates. Neither the weight nor the morphology of the Cx31/Cx43 double knockout placentas appeared to be affected by Cx43 expression. In accordance to this data, the Cx43+/31 placentas also did not show any morphological changes compared to wild-type littermates. Therefore, we assume that the weight of the Cx43+/31 embryos is unlikely to have been influenced by placental development or function. The reduced weight of the Cx43+/31 newborns is probably the result of the loss of Cx43 in the embryo itself, as has been discussed for the Cx31/Cx43 double knockout mouse (Kibschull et al., 2005). Since the replacement of Cx43 by Cx31 did not lead to any obvious impairment in embryogenesis and placental development, we focused our characterization of Cx43+/31 mice on the cardiac phenotype.

### Table 1. ECG parameters of 23 Cx43+/+, 43 Cx43+/31 and 15 Cx4331/31 mice at P0

| Genotype          | Heart rate (1/min) | P (ms) | PQ (ms) | QRS (ms) | QT (ms) | QTc (ms) |
|-------------------|--------------------|--------|---------|----------|---------|----------|
| Cx43+/+          | 362±65             | 11±2   | 54±7    | 11±2     | 1300±418| 25±5     |
| Cx43+/31         | 325±35             | 12±2   | 59±11   | 12±2     | 1096±390| 29±6     |
| Cx4331/31        | 295±25*            | 19±2** | 63±10   | 28±7***  | 188±77***| n.d.     |

Table 2. Echocardiographic data from five Cx43+/+, sixteen Cx43+/31 and five Cx4331/31 mice at P5

| Genotype          | Ejection fraction (%) | Vmean Aorta (cm/second) | Vmean Pulmonary artery (cm/second) | LVM/BW (mg/g) |
|-------------------|-----------------------|-------------------------|-----------------------------------|---------------|
| Cx43+/+          | 87±7*                 | 78±10                   | 65±10                             | 4.0±0.3***    |
| Cx43+/31         | 98±21                 | 90±12                   | 93±16                             | 5.1±1.0***    |
| Cx4331/31        | 55±15                 | 52±16                   | 42±8                              | 6.0±0.9       |

Vmean, mean systolic velocity; LVM, left ventricular mass; BW, body weight; n.s., not significant (P>0.05).

Cx4331/31 newborns showed a significantly reduced weight compared to their Cx43+/+ and Cx43+/31 littermates. The weight of embryos or newborns has not been analyzed so far for Cx43−/− mice (Reaume et al., 1995), but recently Kibschull et al. (Kibschull et al., 2005) showed for the Cx31/Cx43 double knockout mouse that at 17.5 dpc the Cx31+/−/Cx43−/− embryos were also significantly smaller compared to their Cx31+/−/Cx43+/− and Cx31+/−/Cx43+/+ littermates. Neither the weight nor the morphology of the Cx31/Cx43 double knockout placentas appeared to be affected by Cx43 expression. In accordance to this data, the Cx4331/31 placentas also did not show any morphological changes compared to wild-type littermates. Therefore, we assume that the weight of the Cx4331/31 embryos is unlikely to have been influenced by placental development or function. The reduced weight of the Cx4331/31 newborns is probably the result of the loss of Cx43 in the embryo itself, as has been discussed for the Cx31/Cx43 double knockout mouse (Kibschull et al., 2005). Since the replacement of Cx43 by Cx31 did not lead to any obvious impairment in embryogenesis and placental development, we focused our characterization of Cx4331/31 mice on the cardiac phenotype.

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Our results show that Cx31 cannot replace the essential role of Cx43 during cardiac morphogenesis. Mice lacking Cx43 die shortly after birth from an obstruction of the right ventricular outflow tract (Reaume et al., 1995). In Cx43<sup>31/31</sup> mice, the right ventricular outflow tract is still open, but its lumen is so narrow that the supply of blood is likely to be limited. As a consequence, all of them died within ten days (Fig. 5). By contrast, Cx43<sup>40/40</sup> and Cx43<sup>32/32</sup> mice can survive to adults, although 40% of them died during the first 3 weeks after birth and most of them were infertile (Plum et al., 2000). Thus, Cx40 and Cx32 can replace Cx43 during cardiac development, but Cx31 cannot. Our results fit into the general scheme, that the different connexin isoforms can replace each other in certain tissues but fulfill unique essential functions in other tissues (Plum et al., 2000; White et al., 2002; Alcolea et al., 2004).

The reasons, at the molecular level, for the malformation of hearts during development of general Cx43-deficient mice are still a matter of debate. The hearts of mice with cardiac-restricted inactivation of Cx43 by use of the Cre/loxP system are structurally normal (Gutstein et al., 2001). Studies of transgenic mice exhibiting gain or loss of Cx43 suggest that the migration rate of cardiac neural crest cells is increased with overexpression of Cx43 and decreased with deficiency of Cx43 (Lo et al., 1999). Additional studies have shown involvement of another extracardiac cell population, the proepicardial cells in these cardiac anomalies (Li et al., 2002; Wei et al., 2004). Cx43<sup>31/31</sup> mice show similar cardiac malformations as the general Cx43-deficient mice. The hearts of Cx43<sup>32/32</sup> mice also suggest a similar morphologic defect, but to a much lesser extent. This underlines that the special properties of Cx43 lead to normal morphogenesis of the heart.

Functionally, replacement of Cx43 by Cx31 in the heart leads to low voltage of the QRS complex, and prolonged P and QRS duration in the electrocardiogram. Recently, Danik et al. (Danik et al., 2004) generated a heart-specific Cx43-deficient subline (O-CKO mice) in which cardiac Cx43 expression decreased progressively throughout the ventricular myocardium. From 25 to 45 days, the number of immunofluorescent clusters in the O-CKO hearts decreased from 67% of control to 34% and the percentage area from 59% to 30%. The QRS amplitude of O-CKO mice showed an age-dependent decrease, which closely paralleled the loss of Cx43 expression. In order to determine the mechanism of diminished QRS amplitude, the authors later used voltage-sensitive dyes and high resolution optical mapping techniques and found that reduced intercellular coupling leads to paradoxical propagation across Purkinje-ventricular junctions and aberrant myocardial activation (Morley et al., 2005). The diminution of the QRS amplitude seems to be related to the pattern of ventricular excitation. Compared with O-CKO mice, in Cx43<sup>31/31</sup> and Cx43<sup>32/32</sup> hearts Cx43 is replaced by Cx31. Double immunofluorescence using anti-Cx43 and anti-Cx31 showed in the ventricle of Cx43<sup>42/42</sup> mice at P0 that Cx43 and Cx31-containing plaques were not co-localized, whereas in the adult Cx43<sup>42/42</sup> ventricle, Cx43 and Cx31 were predominantly co-localized in intercalated disks (Fig. 4). Since the ECG of Cx43<sup>42/42</sup> mice was normal, we assume that the presence of Cx31 plaques does not lead to an increase in impulse propagation across the Purkinje-ventricular junction. The reduction of the QRS amplitude in the Cx43<sup>31/31</sup> heart is possibly due to deletion of Cx43 and the inability of Cx31 to function like Cx43 in the myocardium. Although Cx43 is the predominant connexin expressed by ventricular and atrial cardiomyocytes in wild-type mice, Cx45 is also expressed at very low levels (Severs et al., 2004). The total amount of Cx45 in the Cx43<sup>31/31</sup> heart was not upregulated, as determined by western blot analysis (data not shown).

The relationship between QRS duration and uncoupling due to Cx43-deletion is controversial in the literature. A modest increase in QRS duration was detected only at 25 days in the O-CKO mice. At 35 and 45 days of age, however, there was no significant difference in the QRS duration between the CKO and control mice (Danik et al., 2004). Our data are consistent with the results of Eckardt et al. (Eckardt et al., 2004) who showed in inducible Cx43<sup>Cre-ER(T)fl</sup> mice that ventricular activation was significantly delayed after deletion of Cx43, as indicated by significant prolongation of the QRS complex. Prolongation of the P wave was found in Cx40-deficient mice (Kirschhoff et al., 1998; Simon et al., 1998). Gap junction channels among atrial myocytes contain abundant Cx40, co-localized with Cx43 (Vozzi et al., 1999; Severs et al., 2001). An increase in P wave has not been shown in conditional Cx43-deficient mice (Danik et al., 2004; Eckardt et al., 2004). The Cx43<sup>32/32</sup> mice exhibited significant prolongation of the P wave. This might be due to the different level of atrial Cx43 in different mouse lines. Atrial expression of Cx43 in the O-CKO hearts at 45 days of age was not changed in immunoblot analyses (Danik et al., 2004). By contrast, no Cx43 protein was detected in the Cx43<sup>31/31</sup> heart, either by western blot (Fig. 2) or by immunofluorescence (Fig. 3) analyses. Direct compensation of Cx43 deletion by upregulation of Cx40 was not found in Cx43<sup>31/31</sup> heart, similar to O-CKO mice (Danik et al., 2004) and inducible Cx43<sup>Cre-ER(T)fl</sup> mice (Eckhardt et al., 2004).

Quantitative echocardiography of neonatal mice on P0 could not be performed in this study because of the small size of the hearts. On P5, echocardiography revealed an impaired systolic function in surviving Cx43<sup>31/31</sup>. In these animals, mean Doppler-derived systolic velocities in aorta and pulmonary trunk showed no significant differences between the genotypes. Assessment of Doppler-derived flow velocities within the stenotic outflow tract was not possible at this age. Slightly lower velocities measured in the putative post-stenotic pulmonary trunk of Cx43<sup>31/31</sup> mice could be the consequence of right-ventricular outflow tract stenosis.

In recent years, several heart-specific conditional Cx43-deficient mice have been generated (Gutstein et al., 2001; Eckardt et al., 2004; van Rijen et al., 2004; Danik et al., 2004; Gros et al., 2004). In summary, 80-95% decrease of Cx43 expression resulted in delayed ventricular activation, arrhythmia and sudden death. Cx43<sup>31/31</sup> mice died before P10. At this young age, telemetric ECGs could not be measured. Thus, we do not know the ECG shortly before death. Since we can induce cardiac-specific replacement of Cx43 with Cx31 in adult mice by breeding floxCx43KI31neo mice with Cx43<sup>Cre-ER(T)fl</sup> mice, we can study the consequence of this replacement also in adult hearts in the future. Furthermore, the floxCx43KI31 mice that were generated in this work could be bred with mice that express the Cre recombinase cell type specifically, in order to study the functional consequence of replacement of Cx43 by Cx31 in other cell types. We assume that the functional diversity of different connexin isoforms in
AGC CTG TCA GGT C-3
PGK-neo cassette was analyzed by using primer 3 (5'-GCC ACC TCA CCC ATG GTC TGG GCA CCT C-3').

CAG TCA CCC ATG GTC TGG GCA CCT C-3

The mutated allele. The mutated allele gave a 375 bp fragment because

326 bp DNA fragment, whereas the mutated allele gave a 375 bp fragment because

/ H11032
PGK-neo cassette was analyzed by using primer 3 (5'-GCC ACC TCA CCC ATG GTC TGG GCA CCT C-3').

CAG TCA CCC ATG GTC TGG GCA CCT C-3

/ H11032
PGK-neo cassette was analyzed by using primer 3 (5'-GCC ACC TCA CCC ATG GTC TGG GCA CCT C-3').

CAG TCA CCC ATG GTC TGG GCA CCT C-3

/ H11032
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Echocardiography of neonatal mice

High resolution mouse echocardiography was performed in 26 mice (five Cx43<sup>+/−</sup>x Cx43<sup>−/−</sup> and five Cx43<sup>1/2</sup>/<sup>1/2</sup> on C57 using a commercially available ultrasound system (HDI 5000, Philips-Ultrasound, Bothell, WA) equipped with a 15 MHz linear array transducer. Volumetric analyses of left ventricular (LV) structures was performed to assess mass and function (Tiemann et al., 2003; Ghanem et al., 2005). Structures that are known to be prone to congenital defects in Cx43-deficient mice, in particular, the right-ventricular outflow tract, were investigated carefully by B-mode and pw-Doppler. Additionally, indirect signs of congestive heart failure or pulmonary hypertension (Doppler-derived velocities of the aorta and pulmonary trunk) were assessed.

Statistical analysis

Parameters were compared between the three genotypes by means of one-way ANOVA along with post-hoc Tukey-Kramer Multiple Comparisons Test. P values <0.05 for ANOVA were considered as significant. Statistical significance of post-hoc analyses of Cx43<sup>+/−</sup> versus Cx43<sup>−/−</sup> versus Cx43<sup>+/+</sup> mice are defined as values of P<0.05 (**), P<0.01 (***) and P<0.001 (****). For comparisons of two groups, 2-sided unpaired Student’s t-test was performed. P values <0.05 were considered as significant.

We gratefully acknowledge the technical assistance of Ina Fiedler. We thank Joachim Degen and Dominik Eckardt as well as Maria Kreuzberg for helpful discussions. Work in the Bonn laboratory was supported by the German Research Association (Wa270/2-2) and through the research group on keratinocytes at Bonn University) to K.W.

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