Human Connexin43E42K Mutation From a Sudden Infant Death Victim Leads to Impaired Ventricular Activation and Neonatal Death in Mice

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Background—Sudden infant death syndrome (SIDS) describes the sudden, unexplained death of a baby during its first year of age and is the third leading cause of infant mortality. It is assumed that ≤20% of all SIDS cases are because of cardiac arrhythmias resulting from mutations in ion channel proteins. Besides ion channels also cardiac gap junction channels are important for proper conduction of cardiac electric activation. In the mammalian heart Connexin43 (Cx43) is the major gap junction protein expressed in ventricular cardiomyocytes. Recently, a novel Connexin43 loss-of-function mutation (Cx43E42K) was identified in a 2-month-old SIDS victim.

Methods and Results—We have generated Cx43E42K-expressing mice as a model for SIDS. Heterozygous cardiac-restricted Cx43E42K-mutated mice die neonatally without major cardiac morphological defects. Electrocardiographic recordings of embryonic Cx43+/E42K mice reveal severely disturbed ventricular activation, whereas immunohistochemical analyses show normal localization and expression patterns of gap junctional Connexin43 protein in the Cx43E42K-mutated newborn mouse heart.

Conclusions—Because we did not find heterogeneous gap junction loss in Cx43E42K mouse hearts, we conclude that the Cx43E42K gap junction channel creates an arrhythmogenic substrate leading to lethal ventricular arrhythmias. The strong cardiac phenotype of Cx43E42K expressing mice supports the association between the human Cx43E42K mutation and SIDS and indicates that Connexin43 mutations should be considered in future studies when SIDS cases are to be molecularly explained. (Circ Cardiovasc Genet. 2015;8:21-29. DOI: 10.1161/CIRCGENETICS.114.000793.)

Key Words: connexin 43 ■ gap junctions ■ sudden infant death

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because of an obstruction of the right ventricular outflow tract. Cardiac-restricted Cx43 loss in young as well as induced cardiac-restricted deletion of Cx43 in adult mice results in impaired ventricular conduction velocities and leads to the death of mice. Different mutations in the human GJA1 gene (coding for the Cx43 protein) have been reported in oculodentodigital dysplasia patients, including 1 oculodentodigital dysplasia mutation causing cardiac arrhythmias in humans.

Recently, a novel Cx43 missense mutation was identified in a 2-month-old boy leading to the exchange of a glutamic acid with a lysine residue at position 42 of the Cx43 protein sequence (Cx43E42K mutation). Expression of the Cx43E42K mutation in transfected N2A cells resulted in normally localized but coupling-deficient Cx43 GJ channels whose function could not be rescued by the coexpression of Cx43 wild-type protein indicating a strong dominant negative effect of Cx43E42K. To date, this mutation is correlated but has not been proven to be the sole cause of the SIDS case as the impact of other mutations or the genetic context cannot be excluded in a single human case. Therefore, in this study, we describe the generation and characterization of a new transgenic mouse line carrying the human Cx43E42K mutation in cardiomyocytes as a model for SIDS.

Methods

Construction of the Conditional Cx43E42K Vector

The Cx43E42K mutation was generated by subsequent mutagenesis polymerase chain reactions (PCRs) using the KICx43ODDD targeting vector as a template that was shortened by Stul digestion for more efficient PCR reaction and temporary deletion of the Cx43 wild-type coding region. The first mutagenesis PCR converted the Cx43G138R-mutated sequence of KICx43ODDD into the wild-type Cx43 coding region (G138R → WT for: GAG GGT CAA GTA TGG GAT TGA AGA ACA CG and G138R → WT rev: GTG GGT CTT CAA TCC CAT ACT TGA ACT TC). The second mutagenesis PCR introduced the Cx43E42K mutation into the wild-type Cx43 sequence (E42K-for: CCA AGC TGA CTT AAC CGC TGT C and E42K-rev: GAC AGC GGT TAA TGC AGC TTG G; see below). During a third mutagenesis PCR a Clal restriction site was deleted to enable a CiaI cloning step (see below; Delete-CiaI for: GAG GTC GAC GGT AAC GAT AAG C and Delete-CiaI-rev: GCT TAT CGT TAC CGT CGA CCT C). Therefore, the Stul-fragment of the KICx43ODDD vector was reintroduced by Stul digestion into the newly generated Cx43E42K exchange vector. In addition, the 3′ untranslated region of the Cx43 wild-type sequence was enlarged by inserting a Clal restriction fragment of a vector containing the 3′ untranslated region of the mouse Cx43 gene followed by a neomycin resistance cDNA flanked by frt sites (constructed in our laboratory) and ligation of the resulting insert into the Clal-restricted Cx43E42K exchange vector. The final conditional Cx43E42K-vector contained the corresponding 5′ and 3′ homology regions, the wild-type Cx43 sequence flanked by loxp sites, neomycin resistance cDNA flanked by frt sites, and the mutated Cx43E42K sequence followed by an Internal Ribosomal Entry Site - enhanced Green Fluorescent (IRES-eGFP) reporter construct (Figure 1A). The targeting vector was analyzed by restriction mapping and partial sequencing (GATC, Konstanz, Germany).

Screening of ES Cell Clones

For transfection of HM1 embryonic stem (ES) cells via electroporation (0.8 kV, 3 μF), 300 μg DNA of the conditional Cx43E42K vector was linearized by NolI digestion. Screening of positively transfected ES cells was performed with 350 μg/μl G418-neomycin (Invitrogen, Darmstadt, Germany). Surviving ES cell clones were tested by 2 different PCR analyses. PCR1 amplifies the 3′ homology region (GFP-rev: CAT GGA CGA GCT GTA CAA GTA AAG CG and Cx43_3′HR: CAC TTT ATA GTA CAC TCT AAG CAA CC) and PCR2 the first loxp site (before_loxp: GCA CTT GGT AGG TAG AGC TTC TCA GGT C and in_Cx43: GCT GCC CAA AGG CGC TCC AGT CAC CC). ES cell clones positive in both PCRs were further characterized by Southern blot analyses for correct homologous recombination at the Cx43 locus (external probe) as well as for single integration of the vector construct (internal probe). DNA extracted from PCR positive clones was digested with HindIII (external probe) and BgIII or BamHI (internal probe). After electrophoresis in an agarose gel the digested DNA was transferred on Hybond-N+-membranes (Amersham Biosciences, Buck, UK) and fixed to the membrane via cross-linking by exposure to UV light. The external and internal probes were radioactively labeled with α-32P-dCTP (Amersham Biosciences, Buck, UK). Hybridization with the membranes was performed in Quick-Hyb solution (Stratagene, La Jolla, CA) at 68°C for 2 hours. The external probe consisted of a 550-bp AvfI fragment from the 3′ untranslated region of Cx43 and the internal probe of a 814-bp HindIII/Clal fragment from the coding region of Cx43.

Generation of Cx43E42K Mice

Homologously recombined ES cell clones were injected into C57BL/6 blastocysts. Resulting high-extent fur-colored chimeras were bred with C57BL/6 mice and agouti-colored offspring were genotyped by PCR analyses of isolated tail DNA. Heterozygous Cx43+/floxE42Kneo mice were backcrossed several times to increase the C57BL/6 genetic background to at least 87.5%. In addition, Cx43-/floxE42Kneo mice were mated to Fip recombinase-expressing mice to delete the neomycin resistance cDNA via the Fip/frt system. Therefore, Cx43+/floxE42K mice were interbred to generate homozygous Cx43floxE42K mice. Homozygous Cx43floxE42K mice were mated to αMyHC-Cre recombinase-expressing mice to delete the wild-type Cx43 cDNA sequence in cardiomyocytes using the Cre/frt system. After Cre-mediated deletion of the wild-type Cx43 region the mutated Cx43E42K sequence was expressed. Correct homologous recombination in floxed Cx43E42K mice was verified by Southern blot analyses. For this DNA was extracted from hearts of mice with different genotypes and digested with BgIII. Analyses with an internal probe were performed as described for screening of ES cell clones. For PCR genotyping, 2 different primers flanking the first loxp site (before_loxp: GCA CTT GGT AGG TAG AGC TTC TCA GGT C and in_Cx43: GCT GCC CAA AGG CGC TCC AGT CAC CC) resulted in a 350-bp fragment for the Cx43 wild-type allele and a 400-bp fragment for the Cx43E42K allele.

Treatment of Mice

All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals published by the US National Institutes of Health and were approved by the local veterinarians. Mice were kept under standard housing conditions with a 12-hour/12-hour dark/light cycle, with food and water ad libitum. Heterozygous Cx43+/floxE42K mice were used as controls in the following experiments.

Northern Blot Analyses

Hearts from newborn Cx43E42K and control mice were shock frozen in liquid nitrogen. Total RNA was extracted using TRIZOL reagent (Invitrogen, Darmstadt, Germany), following the instructions of the manufacturer. After electrophoresis of 10 μg RNA in an agarose gel, the RNA was transferred on Hybond-N membranes (Amersham Biosciences, Buck, UK) and fixed to the membrane via cross-linking by exposure to UV light. The internal probe was radioactively labeled with α-32P-dCTP (Amersham Biosciences, Buck, UK). Hybridization with the membranes was performed in Quick-Hyb.
solution (Stratagene, La Jolla, CA) at 68°C for 2 hours. The internal probe consisted of a 814-bp \textit{Hin}cII/\textit{Cla}I fragment from the coding region of Cx43.

**Histological Analyses**

For paraffin sections hearts from neonatal Cx43+/E42K:αMyHC-Cre and control mice were dissected from cervically dislocated animals and directly fixed in 4% paraformaldehyde. After dehydration in graded series of ethanol, hearts were embedded in Paraplast plus (Sherwood Medical Co., St Louis, MO) and sectioned (5 μm). Staining of the hearts was performed with hematoxylin and eosin.

**Surface ECG Recordings**

ECG recordings of Cx43+/E42K:αMyHC-Cre and control mice were performed at embryonic day 17.5. Embryos were removed from the uterus and kept in their yolk sack with placenta in oxygenated tyrode solution. Directly before measuring, the head was severed and embryos were placed in a recording chamber with the chest wall carefully removed and superfused with oxygenated tyrode solution at 33±2°C. Bipolar ECG was recorded by 2 silver chloride electrodes placed in the atrial region (negative pole) and the apex (positive pole) wall with a bioamplifier recording system (PowerLab 8/35, AD Instruments).

Analyses of RR, PQ, QRS, and QT duration as well as QRS amplitude were performed on averaged QRS complexes using the ECG analysis plugin of Chart (AD Instruments) with manual adjustments. Heart rate QTc were calculated according to the Mitchell’s formula (QTc=QT(RR/100)^0.5).\textsuperscript{27}

**Immunohistochemical Staining for Cx43, Plakophilin-2, N-Cadherin, Desmoplakin, and Zonula Occludens Protein-1**

Neonatal hearts from Cx43+/E42K:αMyHC-Cre and control mice were dissected from cervically dislocated animals, shock frozen in liquid nitrogen, and cryosectioned (12 μm). Cryosections were fixed in 4% paraformaldehyde for 5 minutes, washed 3× in PBS (133 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na$_2$HPO$_4$, 1.5 mmol/L KH$_2$PO$_4$, pH 7.2) with 0.1% Triton X-100 and blocked with 5% BSA, 0.1% Triton X-100 in PBS for 1 hour at room temperature. Rabbit anti-Cx43 (1:1000\textsuperscript{a}), rabbit anti-N-cadherin (N-cad; 1:500, Santa Cruz Biotechnoloy, CA), mouse anti–plakophilin-2 (PKP2; cell culture supernatant, 1:7, Meridian Life Science), mouse anti–desmoplakin 1 and 2 (DSP; cell culture supernatant; 1:7, Progen Biotechnik, Germany), and rat anti–zonula occludens protein-1 (1:100, Millipore, Temecula, CA) antibodies were diluted in blocking solution and incubated with the cryosections at 4°C overnight.

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**Figure 1.** Generation of conditional Cx43E42K mice. **A**, Homologous recombination of the conditional Cx43E42K-vector with the genomic locus of the Cx43 gene leads to the recombined allele mCx43floxE42Kneo. Besides the 5′ and 3′ homologous regions the vector consists of a \textit{loxP} (locus of crossing over P1) site flanked region including the Cx43WT sequence, its 3′-untranslated region and an inverted neomycin resistance cDNA driven by a PGK (phosphoglycerate kinase) promoter and flanked by \textit{frt} (Flp recognition target) sites. Furthermore, the vector contains the mutated Cx43E42K sequence followed by an Internal Ribosomal Entry Site - enhanced Green Fluorescent (IRES-eGFP) construct. Flp-mediated deletion of the neomycin resistance cDNA results in the genotype mCx43floxE42K. Cre-mediated recombination causes the deletion of the Cx43WT sequence together with its 3′-untranslated region and leads to the genotype mCx43E42K. **B**, Southern blot analysis with an internal probe on \textit{Bgl}II-digested liver DNA from different genotypes confirmed homologous recombination in Cx43floxE42K mice. Detection of a 7.5-kb fragment indicates the Cx43 wild-type allele and a 12.4-kb fragment the Cx43floxE42K allele. **C**, Northern blot analysis with an internal probe on total RNA from neonatal hearts of different genotypes verifies the transcripitional expression of the Cx43E42K mutation after Cre-mediated recombination. Detection of a 3.1-kb fragment indicates the Cx43 wild-type mRNA and a 4.2-kb fragment the Cx43E42K-IRES-eGFP bicistronic mRNA. **D**, Polymerase chain reaction genotyping of Cx43E42K mice results in a 350-bp fragment for the Cx43 wild-type allele and a 400-bp fragment for the Cx43floxE42K allele.
On the next day, the sections were washed 3x in PBS with 0.1% Triton X-100 and incubated with secondary donkey antitoxin or goat antitoxin antibodies both conjugated with Alexa 488 (1:1000; Invitrogen) and goat antitoxin antibodies conjugated with Alexa 564 (1:1000; Invitrogen) for 1 hour at room temperature, diluted in blocking solution. After washing the sections twice in PBS with 0.1% Triton X-100, nuclei were stained by incubating sections in PBS with 0.1% Triton X-100 and 0.5 mg/mL bisbenzimide (1:1000; Hoechst 33258 stain; Sigma, Germany) for 10 minutes at room temperature. After brief washing in double-distilled water, the cryosections were mounted with Glycergel mounting medium (Dako, Glostrup, Denmark) and viewed with a Laser Scanning Microscope (Zeiss, Jena, Germany).

**Immunoblot Analyses for Cx43, PKP2, and N-cad**

Total proteins were extracted from hearts of newborn Cx43+/E42K;αMyHC-Cre and control mice by homogenization using a Precellys tissue homogenizer (Peqlab, Erlangen, Germany) in lysis buffer (2×complete [Roche, Mannheim, Germany], 1% Triton X-100, 0.5% Nonidet P40, 50 mmol/L NaCl, 30 mmol/L Na_P2O5×10 H2O, 1 mmol/L Na_2VO5, 50 mmol/L NaF, 1 mmol/L PMSF, 20 mmol/L HEPES), 3× for 25 s and 5000 rpm. Laemmli buffer was added to all samples and proteins (50 μg) were separated by electrophoresis on 10% sodium dodecyl sulfate containing polyacrylamide gels. Proteins were transferred to a Hybond ECL membrane, and membranes were incubated for 1 hour at room temperature. The membranes were incubated overnight at 4°C with 5% milk powder in Tris-buffered saline with Tween-20 (TBST) (10 mmol/L HEPES), 3× for 25 s and 5000 rpm. Laemmli buffer was added to all samples and proteins (50 μg) were separated by electrophoresis on 10% sodium dodecyl sulfate containing polyacrylamide gels. Afterward, proteins were transferred to a Hybond ECL membrane, and the membranes were incubated overnight at 4°C with 5% milk powder in Tris-buffered saline with Tween-20 (TBST) (10 mmol/L Tris, 150 mmol/L NaCl, 0.1% Tween-20, pH 7.5) for 1 hour at room temperature. The membranes were incubated with goat antirabbit or goat antimouse horse-radish peroxidase–conjugated antibodies (diluted 1:10000, Dianova, Hamburg, Germany). Finally, the membranes were incubated with reagents for enhanced chemiluminescence (Pierce, Rockford, IL) and developed on X-ray films (Super RX, FUJIFILM Europe, Düsseldorf, Germany). Loading controls were performed with mouse anti-GAP-DH (diluted 1:10000, Millipore, Temecula, CA) antibodies.

**Statistical Analysis**

Statistical analyses of ECG data were performed using Graph Pad Prism (GraphPad Software). Normality was assessed with the D’Agostino and Pearson omnibus normality test. Data sets that followed a normal distribution were statistically compared with unpaired Student t test, otherwise unpaired t test with Welch’s correction was applied. A P value of <0.05 was considered significant. All data are expressed as mean±SEM.

**Results**

**Generation of Cx43E42K-Expressing Mice**

For targeted mutation of the mouse Cx43 gene, we used the conditional Cx43E42K vector shown in Figure 1A. The vector was transfected into HM1 embryonic stem cells for homologous recombination with the mouse Cx43 locus. Blastocyst injections of correctly recombined ES cell clones yielded germ-line transmission chimeras. Breeding with C57BL/6 mice yielded heterozygous Cx43+/floxE42K mice. However, we observed dead pups on the first day after birth, which expressed the mutant Cx43 protein in the heart revealing that all heterozygous Cx43+/E42K mice die shortly after birth (Table 1). This is in contrast to their heterozygous Cx43+/floxE42K control vector.

**Table 1. Percentage of Living Offspring From Breeding αMyHC-Cre-Recombinase With Homozygous Cx43floxE42K-Expressing Mice at the Day of Birth (PD 0) and PD**

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Cx43+/E42K</th>
</tr>
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<tbody>
<tr>
<td>PD 0</td>
<td>45.2% (50%)</td>
<td>54.8% (50%)</td>
</tr>
<tr>
<td>PD 1</td>
<td>52.6% (50%)</td>
<td>0% (50%)</td>
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The number in parentheses represents the expected Mendelian ratio for each age. n=5 litters for each age (each containing 6–8 pups). PD indicates postnatal day.

**Figure 2. Morphology of neonatal heterozygous Cx43E42K hearts.** Newborn Cx43+/E42K;αMyHC-Cre (left) display irregularly thickened myocardial trabeculae with abnormal pouch formation (*) at the subpulmonary outflow tract, which was absent in controls (right). No other morphological alterations in heterozygous Cx43E42K hearts were detected. n=5 per group. Bar, 500 μm. LA indicates left atrium; LV, left ventricle; MV, mitral valve; PV, pulmonary vein; RA, right atrium; RV, right ventricle; and TV, tricuspid valve.
littermates, which showed a normal survival frequency reaching adulthood.

**Newborn Heterozygous Cx43E42K Hearts Show No Severe Morphological Defects**

In contrast to controls, morphological analyses of neonatal Cx43+/E42K hearts revealed abnormal thickening and irregularity of the right ventricular trabeculae with abnormal pouch formation at the subpulmonary outflow tract (Figure 2). Yet, in contrast to Cx43-deficient neonates that die from a closed right ventricular outflow tract, the pulmonary outflow tract was still open in Cx43+/E42K hearts. No further morphological alterations of heterozygous Cx43E42K hearts were detected when compared with control littermates.

**Electrocardiographic Recordings in Cx43+/E42K Embryos**

Because no major structural cardiac abnormalities were found in heterozygous Cx43E42K newborn mice, we investigated whether neonatal lethality was associated with impaired cardiac function. Therefore, we performed surface-ECG recordings of heterozygous Cx43E42KωMyHC-Cre (Cx43+/E42K) embryos at embryonic day 17.5. We found in mutant embryos compared with controls highly significant longer QRS durations (1.7-fold) and significant reduced QRS amplitudes (3.7-fold) suggesting impaired ventricular activation (Figure 3; Table 2). Also, the RR intervals were slightly but significant prolonged (1.3-fold), indicating slower spontaneous beating rates (Table 2). Furthermore, the QT duration was slightly prolonged but rate QTc and PQ durations were not significantly different.

**Localization and Expression of Cx43 and Mechanical Junction Proteins in Ventricular Myocardium of Heterozygous Cx43E42K Hearts**

To determine the expression and localization of ventricular proteins, we performed immunostaining of cryosectioned newborn hearts. No difference in the expression and distribution of Cx43 between control and heterozygous Cx43+/E42K hearts could be detected (Figure 4). In both genotypes Cx43 immunoreactive signals were present at the cell surface between ventricular cardiomyocytes with an equal punctate pattern consistent throughout control and mutated ventricles. Besides gap junctional Cx43, desmosomal and adherens junctional proteins are expressed in ventricular cardiomyocytes providing mechanical stability. We determined the expression and abundance of desmosomal PKP2, DSP, and zonula occludens protein-1 as well as the adherens junctional protein N-cad in heterozygous Cx43E42K-mutated hearts. PKP2 and N-cad immunoreactivity were unaltered between control and mutated hearts (Figure 5). Both proteins localized to the contact sites of neighboring ventricular cardiomyocytes with distinct areas of coloculation. Furthermore, immunoreactive signals indicating DSP and zonula occludens protein-1 in Cx43+/E42K hearts were indistinguishable from controls (Figure 1 in the Data Supplement).

In line with the corresponding immunohistochemical stainings, immunoblot analyses showed no significant difference in Cx43 protein levels in neonatal Cx43+/E42K hearts compared with controls (Figure 6, left), revealing that Cx43 protein expression was not affected in the mutated hearts. Moreover, no changes in the abundance of desmosomal PKP2 or the adherens junctional protein N-cad were detected in newborn heterozygous Cx43E42K hearts (Figure 6, middle and right).

**Discussion**

In this study, we have investigated the effects of the human Cx43E42K mutation identified in a SIDS victim on cardiac morphology and function in a new transgenic mouse model.
In this mouse line the Cx43 coding region is replaced by the Cx43E42K mutated sequence in cardiomyocytes. This mutation leads to complete absence of junctional conductance of Cx43 GJ channels in transfected N2A cells. Furthermore, coexpression of Cx43 wild-type with Cx43E42K in transfected N2A cells did not rescue gap junctional conductance suggesting a dominant-negative effect of the E42K mutation on Cx43 GJ channel function. This is in line with the fact that heterozygous cardiac-restricted Cx43E42K-expressing mice die on the first day after birth, which is similar to unrestricted Cx43-deficient mice, which die neonatally because of a malformation of the pulmonary outflow tract. However, detailed morphological analyses of newborn Cx43+/E42K hearts revealed no severe morphological defects. We observed only a minor thickening of the right ventricular trabeculae with abnormal pouch formation at the subpulmonary outflow tract. In contrast to Cx43KO mice, the right ventricular outflow tract of cardiomyocyte-restricted Cx43E42K-expressing mice was still open. From this we conclude that the minor cardiac malformations are unlikely to cause the lethal phenotype of Cx43+/E42K mice. Furthermore, the minor morphological phenotype of Cx43E42K hearts is comparable with another transgenic mouse line with a cardiac-restricted expression of a truncated Cx43 protein. Taken together, our observations are in line with previous publications describing that Cx43-deficient neural crest cells and noncrest neuroepithelial cells but not cardiomyocytes are responsible for the aberrant right ventricular outflow tract formation.

To explain the neonatal death of heterozygous Cx43E42K mice, we investigated their cardiac function performing surface-ECG measurements with embryos at embryonic day 17.5. Heterozygous Cx43E42K embryos revealed highly significant longer QRS durations and significant decreased QRS amplitudes indicating impaired activation of the ventricles. These results are similar to ECG analyses of embryonic Cx43KO mice. Rate QTc intervals were not different between heterozygous Cx43E42K and control embryos indicating no major changes in repolarization. We conclude that the expression of the mutated Cx43E42K protein leads to a severe impairment of ventricular activation, which most likely results from coupling impaired Cx43 gap junction channels containing Cx43E42K protein subunits. Because Cx43 is mostly expressed in ventricular cardiomyocytes and only to a smaller extent in the Purkinje fiber endings, we speculate that these altered ECG parameters occur because of impaired electric conduction between ventricular cardiomyocytes. However, because of the dominant negative nature of Cx43E42K, malfunctioning conduction system as the cause for QRS broadening cannot be excluded.

In embryonic and newborn mouse hearts Cx43 protein is uniformly dispersed on the entire surface of ventricular cardiomyocytes, whereas in adult mammalian hearts Cx43 is localized at the end-to-end contact sites of ventricular cardiomyocytes, at the intercalated disc. During postnatal development (20–90 postnatal days) Cx43 is incorporated progressively into the cell termini of ventricular cardiomyocytes. This is similar to human hearts where redistribution of Cx43 from the entire cell surface to the intercalated disc takes also place during postnatal development and continues to ≈7 years of age. Consistent with this we observed punctate staining of Cx43 on the ventricular cardiomyocyte surface in newborn control and Cx43+/E42K hearts with no obvious differences in expression or localization between the genotypes. In contrast to Cx43E42K-mutated mouse hearts, in the human Cx43E42K victim’s heart a patchy Cx43 protein expression pattern was reported suggesting...
somatic mosaicism. However because of poor tissue quality in case of the Cx43E42K victim, the authors were not able to confirm mosaicism.\textsuperscript{20} It is possible that the patchy Cx43 staining pattern observed in the myocardial tissue from the Cx43E42K victim results from poor quality of necropsy tissue. This would also be in line with the normal Cx43E42K protein distribution observed in transiently expressing N2A cells cotransfected with Cx43 wild-type protein.\textsuperscript{20} From their findings the authors suggested that heterogeneous Cx43E42K protein expression could create an arrhythmogenic substrate leading to lethal ventricular dysfunction in the SIDS victim. It was shown before that heterogeneous Cx43 protein expression can affect ventricular function\textsuperscript{21,42} and ventricular arrhythmias were reported in patients with heart failure with heterogeneous connexin expression.\textsuperscript{33}

Yet, Cx43E42K-expressing mice show a strong phenotype, despite an evenly Cx43 distribution pattern in the heart (Figures 4 and 6). Thus, a patchy distribution of Cx43 protein is not necessary and does not account for the severe cardiac consequences in heterozygous Cx43E42K-expressing mouse hearts. Moreover, neonatal lethality of heterozygous Cx43E42K mice supports the described dominant negative effect of the Cx43E42K protein over the Cx43 wild-type protein\textsuperscript{20} and suggests that the Cx43E42K mutation has the potential to be the sole cause of death in the human SIDS victim. Because the presence of Cx43E42K-mutated protein in a homogeneously Cx43-expressing heart is sufficient to cause significant impaired ventricular activation, we conclude that the Cx43E42K mouse line might represent the better mouse model for the clinical phenotype of the human SIDS victim than the previously proposed chimeric Cx43 mice.\textsuperscript{20,42}

It is important to note that the Cx43 antibodies used cannot differentiate between the Cx43 wild-type and the Cx43E42K protein. However, Northern blot analyses verified the expression of mutated Cx43E42K transcript in neonatal Cx43+/E42K hearts (Figure 1C) and quantitative immunoblot analyses demonstrated equal Cx43 protein expression levels between control and Cx43+/E42K cardiac tissue (Figure 6). From this we conclude that ≤50% of the Cx43E42K protein is expressed in heterozygous newborn mutated hearts.

Together with gap junctional Cx43, adherens junctional and desmosomal proteins are expressed in ventricular cardiomyocytes providing mechanical stability of the heart. It has been published before that defects in cardiac mechanical junction proteins can lead to severe cardiac consequences in mice and humans including (dilated) cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy as well as impaired conduction velocities.\textsuperscript{44–48} To investigate whether a mechanical dysfunction might contribute to the observed cardiac defect in Cx43E42K mice, we performed immunostainings and immunoblots for the adherens junctional protein N-cad and desmosomal PKP2 protein. Neither N-cad nor PKP2 abundance or their distribution patterns were affected in newborn heterozygous Cx43E42K hearts. In addition, we analyzed desmosomal DSP and zonula occludens protein-1, which also did not show any differences in their localization pattern (Figure 1 in the Data Supplement). These results suggest that mechanical dysfunction is unlikely to contribute to the observed impaired ventricular activation in Cx43E42K mice. Similarly, the Cx43E42K victim’s case report showed unaltered staining for N-cad and DSP.\textsuperscript{20}

Overall our data demonstrate that the presence of non-functional Cx43E42K protein in the heart leads to severe delayed ventricular activation and neonatal lethality of mice without structural malformations. We conclude that ventricular dysfunction because of functional impaired heteromeric Cx43/Cx43E42K GJ channels and not a patchy Cx43 protein expression pattern creates an arrhythmogenic substrate resulting in ventricular arrhythmias and cardiac arrest of newborn Cx43E42K mutated hearts. The strong cardiac phenotype of Cx43E42K-expressing mice supports the Cx43E42K human case report linking the Cx43E42K mutation to SIDS. Therefore, genetic screening of newborns for the Cx43E42K mutation followed by a constant electrocardiographically monitoring of mutation carriers might contribute to prevent certain cases of sudden infant death. Yet, based on current knowledge Cx43 mutations linked to SIDS seem to occur only infrequently in contrast to mutations in ion channel genes, which have been reported for up to 10% to 20% of SIDS cases.\textsuperscript{31} Nevertheless, the identification of the Cx43E42K mutation in a human SIDS victim is the first example for a Cx43 gene variant associated with SIDS. Future screening for Cx43 variants in SIDS victims might reveal further cases.

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Disclosures
None.

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CLINICAL PERSPECTIVE

Sudden infant death syndrome (SIDS) is the leading cause of postneonatal mortality and describes the unexpected death of an infant aged <1 year. In 2010, >200 babies died of SIDS in the United States. To date, it is suggested that ≤20% of all SIDS cases are caused by mutations in ion channel or ion-channel–associated proteins. In the mammalian heart, electric activation and propagation are mediated by cardiac ion channels and gap junction channels, respectively. Recently, a novel point mutation in the gap junction protein Connexin43 (Cx43) leading to coupling-deficient gap junction channels (Cx43E42K mutation) was identified in a 2-month-old SIDS case. Because additional unknown mutations or the genetic context might influence the impact of this mutation, we have generated transgenic Cx43E42K mice as a genuine model for SIDS. We show that heterozygous Cx43E42K-expressing mice die on the first day after birth without major cardiac morphological defects. Electrocardiographic recordings from embryonic mice on day 17.5 revealed severely impaired ventricular activation in heterozygous Cx43E42K hearts. Immunohistochemical analyses showed a normal localization and distribution pattern of the Cx43 protein in Cx43E42K ventricles. Our data demonstrate that the presence of nonfunctional Cx43E42K protein in the heart is sufficient to cause severe delayed ventricular activation and neonatal lethality of mice. The strong phenotype of heterozygous Cx43E42K mice supports that the Cx43E42K mutation is the cause for the previously reported human SIDS victim. Thus, a possible impact of Cx43 mutations should be considered when SIDS cases are to be molecularly explained. Genetic screening of newborns followed by constant electrocardiographical monitoring of mutation carriers might prevent certain cases of SIDS.
Human Connexin43E42K Mutation From a Sudden Infant Death Victim Leads to Impaired Ventricular Activation and Neonatal Death in Mice
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Supplemental Material:

Supplemental Figure Legends:

Supplemental Figure 1. Localization of desmoplakin and zonula occludens protein-1 in newborn heterozygous Cx43E42K hearts. Immunostaining of desmoplakin (green), zonula occludens protein-1 (red) and nuclei (blue) in sections of control (A) and Cx43+/E42K:αMyHC-Cre (B) ventricles. Neither DSP (desmoplakin) nor ZO-1 (zonula occludens protein-1) immunoreactivity was altered in heterozygous Cx43E42K hearts compared to controls. n=3 for both groups. Bar: 25 µm.
Supplemental Figure 1