Fate of connexin43 in cardiac tissue harbouring a disease-linked connexin43 mutant

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Aims More than 40 mutations in the GJA1 gene encoding connexin43 (Cx43) have been linked to oculo-dentodigital dysplasia (ODDD), a pleiotropic, autosomal dominant disorder. We hypothesized that even with a significant reduction in the levels of Cx43 in a mutant mouse model of ODDD (Gja1Jrt/þ) harbouring a G60S mutation (Cx43G60S), cardiomyocyte function may only be moderately compromised given that a majority of mutant mice typically survive.

Methods and results Western blotting and quantitative reverse transcriptase-polymerase chain reaction in conjunction with immunofluorescence were used to assess the expression and localization of Cx43 in hearts and cultured cardiomyocytes from wild-type and Gja1Jrt/þ mice. Dye-coupling and dual whole cell patch-clamp recordings were also used to assess the gap junction channel status in cultured cardiomyocytes from wild-type and mutant mice. Cardiac tissue from adult Gja1Jrt/þ mice revealed a 60–80% reduction in Cx43 protein with a preferential loss of the highly phosphorylated forms of Cx43. Compensation via the up-regulation of Cx40 or Cx45 was not observed. Immunofluorescent analysis of cultured cardiomyocytes revealed a trafficking defect, with a decrease in Cx43 plaques and a large population of Cx43 being retained in the Golgi apparatus. However, cultured cardiomyocytes from mutant mice remained beating with a 50% decrease in coupling conductance.

Conclusion These results suggest that the Cx43G60S mutant impairs normal trafficking and function of co-expressed Cx43 with no dramatic effect on cardiomyocyte function, suggesting that Cx43 is biosynthesized in excess of an essential need.

1. Introduction

Gap junctions allow direct communication through the exchange of ions and small molecules from the cytoplasm of one cell to another,1 a critical mechanism necessary to maintain normal organ function. Gap junctions are comprised of connexin subunits,2 which form hexamers called connexons. Connexons typically dock with connexons from opposing cells to form intercellular channels,3 which mediate gap-junctional intercellular communication (GJIC).

In the heart, GJIC contributes to the normal rhythmic contraction of cardiomyocytes as a synchronous unit.4 Cx43 is found in almost all ventricular and atrial working cardiomyocytes of the heart, and is localized mainly at the intercalated discs.5 Along with Cx40, Cx43 forms channels of high unitary conductance in the atria6 and are thought to be responsible for the rapid spread of excitation waves throughout the heart. Cx43 is the prominent connexin in the ventricular myocardium, although Cx45 and more recently, Cx30.2 have been localized to the rodent ventricular conduction system.7 Cx45 can also be up-regulated in human heart failure conditions when Cx43-mediated coupling is reduced8 suggesting that, at least in some circumstances, Cx45 may compensate for the reduction in Cx43.

Cx43 and GJIC play essential roles in the development and function of the cardiac system, as Cx43 knockout mice (Gja1−/−) die perinatally because of ventricular outflow tract defects.9 Isolated cardiomyocytes from mice heterozygous for the Cx43 knockout (Gja1+/−) demonstrated similar coupling and cell beating frequencies as cardiomyocytes from wild-type mice,10 suggesting that mice can tolerate a substantial reduction in Cx43 levels without establishing an obvious pathology. However, when Cx43 was specifically ablated from the heart, mice develop morphologically normal hearts with proper contractile function but die suddenly owing to spontaneous ventricular arrhythmias.11

In 2003, mutations in the GJA1 gene encoding Cx43 were genetically linked with the human disease oculo-dentodigital dysplasia (ODDD).12 This rare, autosomal dominant disease...
displays a plethora of symptoms affecting face, eyes, teeth and digits. Some cases of ODDD have been associated with cardiac disturbances, while surprisingly, the majority of ODDD patients display little evidence of cardiac malfunction. Several ODDD-linked Cx43 mutants exhibit loss of gap junction channel function and act as dominant negatives when co-expressed with wild-type Cx43.

ODDD can now be further studied in a mouse model (Gja1<sup>−/−</sup>), which was generated through an N-ethyl-N-nitrosourea (ENU) screen. This mutant mouse harbours a G605 mutation in Cx43 and displays a phenotype similar to human ODDD. In a previous paper, we reported that Cx43 levels were severely reduced in the heart of 11–14-week-old Gja1<sup>−/−</sup> mice resulting in a prolonged PQ interval and P wave width, a decrease in heart rate, and some spontaneous cardiac events, including bradycardia and atrioventricular block. In 2007, two new mouse models of ODDD were generated. The first one was engineered to harbour one allelic copy of an I130T Cx43 mutant (Cx43<sup>I130T/I</sup>), while the second expresses the G138R mutant, in addition to exhibiting hallmark characteristics of ODDD, mice often exhibited a slowing of action potential conduction velocity in the myocardium and an increase in spontaneous and inducible ventricular tachyarrhythmias.

Likewise, the Cx43<sup>G138R/+</sup> mice displayed increased spontaneous arrhythmias and high mortality likely owing to heart defects. In all three ODDD mouse models, while the generic features of ODDD were retained, each mouse model exhibited some unique features that may be linked to the molecular motif of Cx43 that contained the missense mutation. Intriguingly, although it is known that a complement of Cx43 is critical for cardiac function, very few patients with ODDD exhibit cardiac symptoms. Thus, we hypothesized that normal cardiac function can tolerate substantial reductions in Cx43 levels.

2. Methods

2.1 Animals and tissue collection

Animal studies were carried out in accordance with the Animal Care Committee of the University of Western Ontario and with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Gja1<sup>−/−</sup> mice were obtained from Dr Janet Rossant (Centre for Modeling Human Disease, Toronto, ON, Canada). These mice were originally generated from ENU-mutagenized C57BL/6J males crossed with C3H/HeJ females and the resultant Gja1<sup>−/−</sup> mouse line survived, but approximately 27% of all mutant mice died prematurely. Original Gja1<sup>−/−</sup> mice were back-crossed with C57BL/6J mice to produce generations 2, 3, and 4, which were found to exhibit similar birth ratios of wild-type and Gja1<sup>−/−</sup> mice as predicted by Mendelian genetics (data not shown) while spontaneous premature death of mutant mice was negligible.

2.2 Western blot analysis

Hearts were removed from neonatal or 3-week-old Gja1<sup>−/−</sup> and wild-type littermates and either whole or the top two-thirds of the ventricles were used. For primary culture, cells from genotyped neonatal mice were harvested and pooled after 48 h in culture. Tissues or cells were homogenized on ice in lysis buffer (50 mM Tris–Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 1% Triton X-100, 1 mM ethylene-diaminetetraacetic acid, 1 mM EGTA, 1 mM sodium fluoride, 1 mM sodium vanadate, and a cocktail of protease inhibitors (Mini-Complete protease inhibitors, Roche Applied Science, Mississauga, ON, Canada) and 10–30 μg of protein loaded on sodium dodecyl sulphate–PAGE gels. Western blots were performed using rabbit anti-Cx43 (dilution 1:5000, Sigma–Aldrich, St Louis, MO, USA). Membranes were exposed to fluororescent-tagged secondary antibodies (1:10 000, IR 800 anti-mouse or rabbit, Rockland Immunocchemicals, Gilbertsville, PA, USA or Alexa 680 anti-mouse or rabbit, Molecular Probes, Eugene, OR, USA). In other cases, Western blots were probed with rabbit anti-Cx40 (1:1000, Chemicon, Temecula, CA, USA): mouse anti-Cx45 (1:15, Fred Hutchinson Cancer Research Center Antibody Development Group, Seattle, WA, USA); rabbit anti-pCx43 (Ser 368) (1:1000, Cell Signaling Technology, Danvers, MA, USA), or mouse anti-GAPDH (1:5000, Chemicon) antibodies.

2.3 Reverse transcriptase-polymerase chain reaction

Total RNA was isolated from whole hearts of 3-week-old wild-type and Gja1<sup>−/−</sup> littermates using Trizol (Invitrogen, Burlington, ON, Canada) according to manufacturer’s instructions. Connexin cDNA was amplified using a two-step reverse transcription system (Promega, Madison, WI, USA). PCR conditions were as follows: 94°C for 2 min, then 94°C for 30 s, 55°C for 45 s, and 72°C for 45 s for either 25 or 26 cycles (for Cx43 and β-actin, respectively). Primer sequence was as follows: Cx43–5′-GGC TGC TCC TCA CCA ACG GCT-3′; 5′-AGG TCA TGC CCA CGA CGA CTG-3′; β-actin–5′–TAC ACA ACC ACC ACC ACC AAC AGA GGC ATC CTC ACC CT-3′; 5′-TAC ATG GCT GGG GTT TG TTG AA-3′. Samples were resolved on 1% agarose gel and analysed using Quantity One software (BioRad, Mississauga, ON, Canada).

2.4 Heart section preparation and immunohistochemistry

Whole hearts were excised from 3-week-old wild-type and Gja1<sup>−/−</sup> littermates and fixed in zinc fixative (0.1 M Tris-base, pH 7.4, 0.05% calcium acetate, 0.5% zinc acetate, and 0.5% zinc chloride) for 24 h at room temperature. Tissues were dehydrated and embedded in paraffin blocks. Sections of 5 μm were obtained from ventricles using a microtome and were mounted on slides. Slides were rehydrated, blocked in 2% bovine serum albumin (BSA) in phosphate-buffered saline for 1 h and probed using rabbit anti-Cx43 (1:500, Sigma–Aldrich) followed by anti-rabbit Texas red (1:500, Sigma–Aldrich) for 1 h each. Nuclei were labelled with Hoechst 33342 (10 μg/ml) in ddH<sub>2</sub>O.

Fluorescence was quantified in images using Image J program (National Institutes of Health, Bethesda, MD, USA, http://rsb.info.nih.gov/ij/). Equal background threshold was used in both wild-type and Gja1<sup>−/−</sup> heart image analysis.

2.5 Primary cardiomyocyte cultures

Whole hearts were excised from neonatal Gja1<sup>+/+</sup> and Gja1<sup>−/−</sup> littermates. Hearts were first minced, then serially digested with 4 × 15 min rounds of a collagenase enzyme mixture (22.5 μg/ml Liberase Blendzyme 4, Roche, QC) diluted in Hank’s balanced salt solution (Sigma–Aldrich). Cells from the third and fourth digest were centrifuged and resuspended in M199 medium (Invitrogen), supplemented with 1% penicillin/streptomycin (Invitrogen) and 10% foetal BSA and plated for 1 h. Unattached cells were re-plated on 12 mm glass coverslips previously coated with human fibronectin (BD Biosciences, Mississauga, ON, Canada).

2.6 Immunofluorescence of cultured cells

Primary cultures of cardiomyocytes were fixed in ice-cold 80% methanol/20% acetone for 15 min at 4°C prior to immunostaining.
Localization of Cx43 was analysed using rabbit anti-Cx43 (1:500, Sigma-Aldrich) or rabbit anti-pCx43 (serine 368) (1:100, Cell Signalling Technology) antibodies. Mouse anti-GM130 was used as a marker for a resident protein of the Golgi apparatus (1:100, BD Bioscience). Coverslips were exposed to primary antibodies and fluorescent-tagged secondary antibodies (anti-mouse or rabbit FITC or Texas Red, Jackson ImmunoResearch, West Grove, PA, USA) for 1 h at room temperature. Cell nuclei were stained for 10 min with Hoechst 33342 (10 μg/ml) in ddH2O prior to mounting in Airvol (Air Products and Chemicals, Inc., Allentown, PA).

2.7 Analysis of cardiomyocyte beat frequency
Cardiomyocytes cultured for 48 h were observed using a Leica DM IRE2 inverted epifluorescence microscope equipped with a heated stage. Spontaneous synchronous beating of groups of at least 20 cells was observed and the number of beats per 60 s was counted. Littermates from four distinct litters were counted using three measurements per well and three wells per animal.

2.8 Dye transfer
Cultured cardiomyocytes were randomly pressure-injected using an Eppendorf Femtojet automated pressure microinjector with 1% Lucifer yellow CH lithium salt dye dissolved in ddH2O (Molecular Probes) until the cell brightly fluoresced. Images were collected 1 min after injection using OpenLab software (Quorum Technologies Inc., Guelph, ON, Canada). The instances of dye transfer and the order of dye transfer (cells directly touching the microinjected cell being of first order) were both quantified.

2.9 Dual whole cell patch-clamp recording
The dual whole cell patch-clamp technique was used to measure the electrical coupling level between pairs of neonatal cardiomyocytes isolated from wild-type and mutant hearts. Cultures from individual mice were used at 48 h after plating, and only the cardiomyocyte pairs that contracted were selected for recordings. After genotyping, the junctional conductance results obtained from Gja1+/+ and Gja1 Jrt/+ mice were pooled. Off-line series resistance compensation was applied as previously described.17

2.10 Statistics
Unless stated otherwise, results were analysed using a Student’s two-tailed independent sample t-test for significance using a value of P < 0.05 [asterisks (*) in figures]. Graphs represent mean ± standard error.

3. Results
3.1 The Cx43G60S mutant dominantly inhibits the ability of Cx43 from reaching the most phosphorylated state in the heart with no effect on the levels of Cx40 or Cx45
The original characterization of Gja1 Jrt/+ mice indicated that Cx43 levels were severely reduced in at least some tissues.22 Immunoblot analysis of Cx43 revealed that brain, heart, and skin tissues from Gja1 Jrt/+ mice had reduced levels of Cx43 when compared with wild-type littermates (Figure 1). Given the documented importance of Cx43 in the heart, we focused our attention on examining the consequence of a Cx43 mutant being expressed in cardiac tissue. Western blots revealed that the level of Cx43 protein was reduced in 3-weeks old Gja1 Jrt/+ mice by almost 80% and 60% in ventricle and atria lysates, respectively (Figure 2A and B). Densitometry revealed that the slower migrating P (phosphorylated) species of Cx43 was reduced by more than 80% in Gja1 Jrt/+ ventricles and atria (Figure 2C). The amount of the faster migrating species of Cx43 (designated as P0) did not differ between Gja1 Jrt/+ and Gja1 Jrt/+ littermates in both ventricular and atria lysates (Figure 2D). It had previously been shown that at least one phosphorylated species of Cx43 migrates together with this apparently non-phosphorylated Cx43 in

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Figure 1  Connexin43 (Cx43) is differentially expressed in organs of wild-type and Gja1 Jrt/+ mice. Various organs were excised from 3-week-old wild-type and Gja1 Jrt/+ mouse littermates and assessed by western blots for Cx43 as normalized to a β-actin loading control. Pooled results are shown as a percentage of wild-type expression. Brain, heart, and skin from mutant mice have significantly reduced Cx43 levels (intestines, n = 4; brain, heart, n = 6; thymus, lungs n = 7; skin n = 8; where n = number of animals). *P < 0.05 by the Student’s t-test.
the P₀ band, which includes Cx43 being phosphorylated at serine 368. By including an antibody specific for Cx43 when phosphorylated at serine 368, we determined that the level of Cx43 phosphorylated at serine 368 was not changed in mutant mouse ventricles or atria (Figure 3A). To test if the reduction in Cx43 abundance in Gja1/Jrt/+ hearts was at the transcription level, we used semi-quantitative RT-PCR to investigate Cx43 mRNA levels. As indicated in Figure 3B, there was no change in the level of Cx43 mRNA in Gja1/Jrt/+ mouse hearts compared with wild-type littermates.

Cardiac tissues express multiple connexins, including Cx43, Cx40, and Cx45, and we postulated that Cx40 and/or Cx45 might be up-regulated to compensate for the reduction in Cx43 levels. However, western blotting (Figure 4A) and corresponding densitometric measurements (Figure 4B and C) revealed no significant change in the level of Cx40 or Cx45 in Gja1/Jrt/+ mouse ventricles or atria compared with Gja1/Jrt/+ littermates. Collectively, these data suggest that the presence of the Cx43G60S mutant causes a post-transcriptional, preferential down-regulation of the highly phosphorylated species of Cx43 in both major chambers of the heart with no compensatory up-regulation of Cx40 or Cx45 protein levels.

3.2 Cx43 gap junctions are less evident in ventricles from mutant mice

In order to assess if the reduction in total Cx43 protein can be translated into a change in the number of gap junctions found in cardiac tissue, we immunolabelled ventricles from wild-type and mutant mice at the age of 3 weeks. Immunolocalization studies revealed abundant Cx43 at locations of intercalated discs in ventricle sections of wild-type mice (Figure 5A, arrows). In contrast, far fewer Cx43 gap junctions were seen at the intercalated discs of Gja1/Jrt/+ mice while intracellular Cx43 staining was observed in paranuclear regions (Figure 5B, arrowheads). In some regions of the Gja1/Jrt/+ heart, plaque-like structures could be readily detected (Figure 5B, insert) although the intensity of the Cx43 immunostaining in these regions was reduced and there was some evidence for Cx43 being found in lateral regions of the cell. This result extends the original characterization of the Gja1/Jrt/+ mouse where few gap junctions were seen in the hearts of 11-week-old mice. Quantification studies revealed a severe reduction in the total amount of Cx43-specific fluorescent signal and in the number of Cx43 plaque-like structures in Gja1/Jrt/+ hearts (Figure 5C and D).

3.3 Intracellular Cx43 is localized to the Golgi apparatus in cardiomyocytes

In order to determine the localization of intracellular Cx43 in mutant mice, cardiomyocytes were cultured from neonatal Gja1/Jrt/+ and wild-type littermates. Surprisingly, there was no significant difference in total Cx43 levels between Gja1/Jrt/+ and wild-type littermates in tissue lysates or primary cultures from neonatal hearts (Figure 6A) but there was a loss of the most highly phosphorylated species of Cx43...
in cardiomyocytes from mutant mice heart tissues (Figure 6A). In addition, we observed a three- to four-fold elevation of total Cx43 in cardiomyocytes cultured for 2 days in comparison with Cx43 levels within neonatal heart tissue (Figure 6A). In comparison with wild-type cells (Figure 6B), cultured cardiomyocytes from Gja1/Jrt/+ littermates revealed fewer Cx43 plaques between cells (Figure 6D, arrow) with prevalent intracellular Cx43 staining (Figure 6D, arrowheads). However, immunolabelling with an antibody specific to Cx43 when phosphorylated at serine 368, revealed similar Cx43 localization patterns in cardiomyocytes from both wild-type and mutant mice (Figure 6C and E). This result suggests that the phosphorylation of Cx43 by protein kinase C at serine 368 is not affected by any trafficking defects caused by the Cx43G60S mutant.

To further determine the subcellular localization of Cx43, cardiomyocytes from wild-type and mutant mice were double-labelled for Cx43 and GM130, a resident protein of the Golgi apparatus. While the majority of Cx43 in wild-type cardiomyocytes was found in punctate gap junction structures (Figure 7, arrows), Cx43 was found co-localized with GM130 in cardiomyocytes from both wild-type and mutant mice (Figure 7, arrowheads). Minimal Cx43 was detected in the endoplasmic reticulum, lysosomes or mitochondria of cardiomyocytes from both wild-type and mutant mice (see Supplementary material online, Figure S1).
3.4 Cultured cardiomyocytes from Gja1Jrt/+ mice beat and are dye-coupled but exhibit decreased electrical coupling

Cardiomyocytes have the unique property of beating spontaneously in culture and those isolated from wild-type mice displayed a beating frequency of approximately 84 b.p.m. on average. Despite the changes in phosphorylation species and localization of Cx43, cultured cardiomyocytes from Gja1Jrt/+ mice still beat with a significantly faster rate with an average of 126 b.p.m. (Figure 8A) which is $1.5 \times$ faster than wild-type cardiomyocytes under the same conditions.
To determine the approximate level of GJIC between cardiomyocytes from both wild-type and Gja1<sup>Jrt/+</sup> mutant mice, dye-transfer studies were performed (Figure 8B). Cardiomyocytes from Gja1<sup>Jrt/+</sup> mice were able to pass Lucifer yellow dye with similar incidence (Figure 8C) and to the same extent (Figure 8D) as that of wild-type cardiomyocytes. Since dye-coupling experiments provide an estimate of the ability of gap junctions to transfer only small molecules, cultured pairs of ventricular myocytes from wild-type and mutant mice littermates were also subjected to double whole cell patch-clamp recording to assess electrical conductance. Cardiomyocytes from wild-type mice exhibited coupling conductances of approximately 13.2 nS while cardiomyocytes from mutant mice exhibited significantly reduced coupling of approximately 6.3 nS (Figure 8E). Collectively, these studies suggest that gap junction coupling
in mutant cardiomyocytes is substantial but significantly less than that observed in normal cardiomyocytes.

4. Discussion

In this study we set out to determine the consequences of mice harbouring a disease-causing Cx43 mutant on Cx43 levels, localization and function. The Gja1Jrt/þ mouse, which expresses a dominant-negative Cx43 G60S mutant, was created through an ENU mutagenesis screen.22 These mice suffer from symptoms which phenotypically resemble ODDD, a human disease linked to autosomal dominant Cx43 mutations.12 Initial characterization of Gja1Jrt/þ reported a severe down-regulation of total Cx43 levels in the heart and ovaries of ageing mice and some cardiac defects.22 In our current studies, mouse organs like thymus and lungs, which are rich in the P0 species of Cx43 typically associated with an intracellular pool,25 revealed no mutant-induced overall loss of Cx43. In other cases, tissues that expressed the P (most phosphorylated) species of Cx43 more commonly associated with gap junction plaques25 (e.g. skin and heart), were more dramatically affected by the presence of the Cx43G60S mutant. These observations would suggest that the Cx43G60S mutant preferentially impairs the endogenous wild-type Cx43 from reaching a more highly phosphorylated state.

There are different possibilities as to why the Cx43G60S mutant is not found in a highly phosphorylated state.

Figure 7 Connexin43 (Cx43) in cardiomyocytes localizes to the Golgi apparatus. Cultured cardiomyocytes isolated from neonatal wild-type and Gja1Jrt/þ littermates were double-labelled for Cx43 (red) and GM130 (green), a resident protein of the Golgi apparatus. Nuclei were labelled with Hoechst stain (blue). In wild-type littermates (+/+) Cx43 was found assembled as punctate gap junctions (arrows) and co-localized with GM130. In the case of mutant (Jrt/+) cardiomyocytes, a substantial amount of Cx43 co-localized with GM130 (yellow; arrowheads). Bar = 20 μm (n = 5).
Figure 8 Cultured cardiomyocytes from Gja1<sup>+/+</sup> mice are dye-coupled but have a faster colony beating rate and reduced intercellular electrical coupling. (A) Cultured cardiomyocytes isolated from neonatal wild-type and Gja1<sup>+/+</sup> mice (each dot) were found to spontaneously beat and mutant mice exhibited an increased average beat rate (*P < 0.05, B). Cardiomyocytes were microinjected with Lucifer yellow dye as seen in representative post-injection and phase-contrast images (B). Injected cells are marked with asterisks; Bar = 20 μm. Cardiomyocytes from Gja1<sup>+/+</sup> mice were able to pass Lucifer yellow dye with similar incidence (C) and to the same order of contacting cells (D) as wild-type cardiomyocytes. (E) Double patch-clamp recording traces obtained from neonatal wild-type and Gja1<sup>+/+</sup> cardiomyocyte pairs responding to −20 mV impulses. The Gja1<sup>-/-</sup> cell pair exhibited markedly reduced electrical coupling than the Gja1<sup>+/+</sup> cell pair. Pooled peak junctional conductance results indicated that the electrical coupling level between Gja1<sup>-/-</sup> cardiomyocytes was reduced by more than 50% in comparison with Gja1<sup>+/+</sup> cardiomyocytes. *P < 0.05.
One is that the Cx43 mutant may be a poor substrate for phosphorylation. This is unlikely considering that the G60S amino acid substitution is located in the first extracellular loop while all the known phosphorylation sites are on the C-terminal tail.\(^{28}\) In addition, the Cx43\(^{G60S}\) mutant was found to be a suitable phosphorylation substrate for PKC. Another plausible explanation is that the Cx43\(^{G60S}\) mutant acts to impair the delivery of Cx43 to the cardiomyocyte cell surface. In the event that the Cx43 mutant and its co-expressed wild-type Cx43 counterpart co-oligomerize in the Golgi apparatus,\(^{17}\) this may reduce their collective ability to reach the cell surface, and thus fail to enter the compartment where they would become a substrate for extensive phosphorylation. This is supported by Cx43 being localized in the Golgi and by similar apparent intracellular accumulation of Cx43 in wild-type and mutant-cultured cardiomyocytes when exposed to either lysosomal or proteasomal inhibitors (see Supplementary material online, Figure S2). Previously, we demonstrated that the Cx43\(^{G21R}\) and Cx43\(^{G138R}\) mutants can co-immunoprecipitate with wild-type Cx43,\(^{17}\) thus we speculate that the Cx43\(^{G60S}\) mutant and wild-type Cx43 interact and co-oligomerize. This mechanism of Cx43\(^{G60S}\) mutant acting on wild-type Cx43 while a resident of the Golgi apparatus is likely distinct from cardiomyocytes obtained from other ODDD mouse models. For example, the poorly phosphorylated Cx43\(^{G138R}\) mutant was found abundantly within cell surface gap junctions, but were non-functional.\(^{24}\) In the case of the Cx43\(^{I130T}\) mutant mouse, where the phosphorylation of Cx43 at serines 325 and 365 was absent, there was a reduction in the cell surface population of Cx43 in cardiomyocytes.\(^{23}\) Thus, the Cx43\(^{I130T}\) mutant, similar to the Cx43\(^{G60S}\) mutant, exhibits a possible trafficking defect. Collectively, these studies also lead to the conclusion that extensive Cx43 phosphorylation is not a prerequisite for gap junction plaque formation. Finally, since similar steady-state Cx43 mRNA levels were found in the heart and skin (data not shown) of the Gja1\(^{I130T}\) mice and their wild-type littermates, we conclude that the dominant-negative effect of the Cx43\(^{G60S}\) mutant on the overall level of Cx43 occurs at the post-transcriptional level.

The critical importance of Cx43 in heart development and normal conduction has been demonstrated using both germ line\(^{8,10}\) and cardiac-specific Cx43 knockout mice.\(^{27,31}\) Yet, 3-week-old Gja1\(^{I130T}\) mice, expressing only 20–40% of normal Cx43 levels in ventricles and atria are viable, exhibit near normal Mendelian genetics at birth (data not shown) and many live more than 1 year. Intriguingly, cultured cardiomyocytes from mutant mice retain their dye-coupling ability owing, in part, to the fact that the mutant phenotype, as assessed by the levels of Cx43, was less severe under these cultured conditions. Nevertheless, electrical conductance measurements revealed that there was a significant reduction in coupling in neonatal cardiomyocytes from Gja1\(^{I130T}\) mice similar to what has been reported for the Cx43\(^{I130T}\) mice.\(^{23}\) It is also notable that there was no compensatory expression change in the levels of either Cx40 or Cx45, which are the two other most prominent connexins of the mouse heart. One interesting difference in mutant-expressing cardiomyocytes is that, despite the vast reduction in Cx43 in Gja1\(^{I130T}\) mice, cultured cardiomyocytes have a higher beat frequency than cardiomyocytes from wild-type littermates. Dobrowolski et al.\(^{24}\) observed a similar increase in cultured cardiomyocyte beating rates from the Cx43\(^{G138R}\) mice and suggested that this finding may be because of an increase in ATP release from hemichannels generated from the Cx43\(^{G138R}\) mutant. This mechanism may be less likely for the Cx43\(^{G60S}\) mutant as its steady-state distribution favours its localization within the Golgi apparatus. Moreover, the Cx43\(^{G60S}\) mutant mouse appears to be more susceptible to arrhythmias than the Gja1\(^{I130T}\) mice.

Our study is in accordance with the concept suggesting that the level of Cx43 that is synthesized in the heart is in excess of what is required for heart development and synchronized myocardium beating.\(^{26}\) Evidence for this position was presented in Cx43\(^{+/−}\) mice that exhibited essentially normal heart function with a 50% complement of Cx43.\(^{10}\) While Cx43 may be expressed in excess of what is required under normal conditions, this may not be the case when additional pathologies are present. In several cardiac diseases, especially those leading to arrhythmias, decreased levels of Cx43 or change in its distribution have been observed.\(^{32,33}\) One interesting finding from our localization studies of Cx43 within cardiac tissue was that there was considerable variability within the heart sections. In some areas of the ventricles, small but clearly stained gap junction plaques could be found with variable amounts of lateral staining while in other areas of the ventricle only intracellular Cx43 was detected. To some degree, this heterogeneity is reminiscent of Cx43 remodelling and down-regulation in the heart after ischaemia or in hypertrophic cardiomyopathy.\(^{29,34}\) In other cases, Cx43 may re-localize to lateral connections between cardiomyocytes in diseased cardiac tissue.\(^{29,35}\) Thus during disease, further reduction of Cx43 in cardiac tissue of Gja1\(^{I130T}\) mice may result in dangerously low gap-junction coupling, which may in turn lead to lethal arrhythmias.

In summary, our findings from the Gja1\(^{I130T}\) mice suggest that Cx43 is normally biosynthesized in excess of what is necessary for basic cardiac tissue function and mouse survival. Even though Cx43 favours a Golgi apparatus distribution profile in cardiomyocytes from mutant mice and has an impaired ability to form gap junctions or reach a normal phosphorylation status, there is sufficient residual Cx43 function or increased channel activity provided by other cardiac connexins to maintain synchronized cardiomyocyte function. Finally, we propose that ODDD patients may have greatly reduced Cx43 levels and be less-tolerant to cardiac pathologies.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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