Connexin40 Imparts Conduction Heterogeneity to Atrial Tissue


Abstract—Impulse propagation in cardiac tissue is a complex process in which intercellular coupling through gap junction channels is a critical component. Connexin40 (Cx40) is an abundant gap junction protein that is expressed in atrial myocytes. Alterations in the expression of Cx40 have been implicated in atrial arrhythmogenesis. The purpose of the current study was to assess the role of Cx40 in atrial impulse propagation. High-resolution optical mapping was used to study conduction in the right and left atrial appendages of isolated Langendorff-perfused murine hearts. Wild-type (Cx40+/+), heterozygous (Cx40+/−), and knockout (Cx40−/−) mice, both adult and embryonic, were studied to assess the effects of reduced Cx40 expression on sinus node function and conduction velocity at different pacing cycle lengths (100 and 60 ms). In both adult and late-stage embryonic Cx40+/− mice, heterogeneity in CV was found between the right and left atrial appendages. Either partial (Cx40+/−) or complete (Cx40−/−) deletion of Cx40 was associated with the loss of conduction heterogeneity in both adult and embryonic mice. Additionally, sinus node impulse initiation was found to be ectopic in Cx40+/− mice at 15.5 days postcoitus, whereas Cx40+/+ mice showed normal activation occurring near the crista terminalis. Our findings suggest that Cx40 plays an essential role in establishing interatrial conduction velocity heterogeneity in the murine model. Additionally, we describe for the first time a developmental requirement for Cx40 in normal sinus node impulse initiation at 15.5 days postcoitus. (Circ Res. 2008;103:1001-1008.)

Key Words: arrhythmia • conduction velocity • Cx40 • optical mapping • sinus node

Cardiac myocytes are connected electrically and metabolically through gap junction channels. A complete channel is composed of 2 opposing hexameric structures or connexons, each consisting of 6 protein subunits called connexins. At least 3 connexins are expressed in cardiac myocytes: connexin40 (Cx40), connexin43 (Cx43), and connexin45 (Cx45), each forming channels with unique electrophysiological and biophysical properties. Within the adult heart, connexin isoforms have distinct patterns of expression, which are generally well preserved among different species. Cx40 is expressed predominantly in atrial myocytes and in the ventricular conduction system; Cx43 is expressed in both atrial and ventricular myocytes; Cx45 is expressed mainly in the sinoatrial node, anteroventral node, and in the ventricular conduction system, with lower levels reported in the atrial myocardium.

Alterations in the expression of connexin proteins have been described in a variety of pathological conditions in both animal and human models. However, most studies that have investigated the importance of connexins have focused on ventricular conduction, whereas relatively few studies are available directly addressing their role in mediating atrial impulse initiation and propagation. Studies investigating the role of Cx40 in atrial arrhythmogenesis have found highly variable levels of expression. Conflicting results have also been reported with respect to the electrophysiological effects of Cx40 deletion on impulse conduction.

The lack of uniformity of these findings may be attributable, at least in part, to model-specific parameters. The purpose of the present study was to conduct a comprehensive investigation into the role of Cx40 in mediating impulse initiation and propagation in the intact mouse atria. To achieve this goal, we investigated atrial impulse initiation and chamber-specific conduction parameters in Cx40-deficient animals during embryonic development and over a wide range of ages. Our findings suggest an important role for Cx40 in establishing the site of impulse initiation during embryonic development and conduction heterogeneity within the atrial myocardium.

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Materials and Methods

Mice

All procedures were approved by the New York University School of Medicine Institutional Animal Care and Use Committee. Forty-nine adult (aged 2 to 11 weeks) and 80 embryonic mice (13.5 to 15.5 days postcoitus [dpc]) were included in this study. Interbreeding of the appropriate mice was performed to obtain heterozygous (Cx40+/−) and knockout (Cx40−/−) genotypes. Genotyping was performed using polymerase chain reaction (PCR) with genomic DNA extracted from tail tips as previously described.15 Wild-type (Cx40+/+) littermates were used as controls. The background strain of the mice was C57BL/6. Mice showing visible morphological abnormalities were excluded from the study (see supplemental material).

Optical Mapping Studies

Details for the adult and embryonic heart isolation and perfusion are provided in the supplemental material. High-resolution optical mapping studies of adult atrial appendages were performed using an upright microscope (BX-51WI; Olympus Inc) equipped with a high-speed charge-coupled device camera (Dalsa Inc, Waterloo, Canada). Excitation light from a 100-W mercury arc lamp was passed through an interference filter (470±30 nm) together with a dichroic mirror (500 nm). The emitted fluorescent light was split with a second dichroic mirror (600 nm) and simultaneously collected and projected onto a CMOS video camera (Ultima-L; SciMedia, Inc). Recordings were obtained at 100 frames/s with a 100×100 pixel array at 14-bit resolution. Imaging studies were performed using 5×, 8×, and 10× objectives resulting in spatial resolution ranging from 20 to 40 μm/pixel.

Volume-Conducted Electrocardiograms

A bipolar electrode mounted on a micromanipulator was used to record biatrial electrograms from isolated hearts. Recordings were made using Ag-AgCl electrodes placed approximately 1 to 2 mm from the heart surface. Electrograms were amplified, low-pass-filtered at 300 Hz (CyberAmp; Axon Instruments), and digitally sampled at 5 kHz using an A/D digitizer (Digidata; Axon Instruments, Inc) and a commercially available software package (Axoscope; Axon Instruments, Inc). Continuous recordings were obtained through the entire experiment and stored for offline analysis. Recordings were electronically tagged to indicate when the hearts were paced and optically mapped.

Pacing of Murine Atrial Appendages

Average epicardial conduction velocity (CV) measurements were obtained by separately pacing the RAA and LAA (Figure 1A–C) with a platinum monopolar electrode (250 μm tip diameter; FHC Inc, Catalog #UE[GM1]). Details of the methods used to calculate CV are provided in the supplemental material. The appendages were paced at basic cycle lengths of 100 and 60 ms using pulses 2 ms in duration at 1.5 times diastolic threshold. This current amplitude was used to minimize virtual electrode effects. Activity during pacing was recorded for 2 seconds.

RNA Quantification

Total RNA was isolated using Trizol and quantified through spectrophotometry. Quantitative real-time PCR (qRT-PCR, ABI Prism 7700 Sequence Detection System; Applied Biosystems, Foster City, Calif) was used to determine the mRNA levels of Cx40, Cx43, and cyclophilin A. Detection of amplification is contingent on the specific hybridization of the fluorogenic probe and the flanking forward and reverse primers. The primer sequences for Cx40 and Cx43 were as follows: Cx40 forward primer: ATTCGATCCGCCACCACCT; Cx40 reverse primer: CATGCAAGGTATCACCAGGA AGA; Cx43 forward primer: TGCCCTTTCGTCAGCAGTACCT; Cx43 forward primer: TGAAAGGAAGAAGCAGTCCTT; Cx43 reverse primer: GAGAGATCCGAGTCTTTGGA; and Cx43 Taqman probe: ACGGCACCACCGCCACT.

The probe, labeled at the 5’ and 3’ ends with 6-carboxyfluorescein reporter and 6-carboxytetramethylrhodamine quencher, respectively, is hydrolyzed by the 5’ exonuclease activity of Taq DNA polymerase causing an increase in fluorescent signal that is collected and projected onto a CMOS video camera (Ultima-L; SciMedia, Inc). Recordings were obtained at 1000 frames/s with a 100×100 pixel array at 14-bit resolution. Imaging studies were performed using 5×, 8×, and 10× objectives resulting in spatial resolution ranging from 20 to 40 μm/pixel.
measured in real time after each cycle of PCR amplification. Fluorescence thresholds were set to 10 standard deviations above baseline fluorescence. Standard curves were constructed by plotting log_{10} RNA starting quantity versus cycle threshold. On the basis of appropriate serially diluted standard RNA, the amount of input standard RNA yielding the same amount of PCR product measured from an unknown sample was calculated. Measured RNA levels were normalized to cyclophilin A and expressed as a ratio of the gene of interest (ie, Cx40/cyclophilin A).18,19

**Immunohistochemistry**

Hearts were flash-frozen in liquid nitrogen and stored at −20°C. The tissue was later thawed and embedded in OCT before sectioning. Frozen sections were fixed in acetone followed by immunostaining.20 Sections were acquired to generate RAA and right atria, or LAA and left atria, or RAA and LAA of each heart in the same section for comparison. The primary antibodies used were the polyclonal anti-Cx40 available from Alpha Diagnostic and the monoclonal anti-Cx43 available from Zymed (Clone 3D8A5, Catalog #35–5000). Secondary antibodies Alexa594-conjugated antirabbit and Alexa488-conjugated antimouse IgG (Jackson ImmunoResearch Laboratories) were used, respectively. Sections were visualized with an Axioskop 2 Plus fluorescence microscope (Carl Zeiss, Munchen-Hallbergmoos, Germany). Samples processed without primary antibody served as negative controls. Colocalization methods were provided in the supplemental material.

**Western Blot Analysis**

A subset of optically imaged hearts (11 young and 11 adult) was examined by Western blot analysis. After the imaging studies, the hearts were flash-frozen in liquid nitrogen and stored at −20°C. To prepare samples, the hearts were later thawed, RAA and LAA isolated, and each manually Dounce-homogenized on ice in 50 mL of radioimmunoprecipitation buffer (20 mmol/L Tris, pH 7.4; 0.15 mol/L NaCl; 1% Triton X-100; 0.1% SDS) with 1 μL protease inhibitors, 1 μL 0.1 mol/L NaVO3, and 2.5 μL NaF. After tissue lysis, the samples were spun for 1 minute at 4°C and the supernatant was collected. Protein concentrations of each sample were measured using the Lowry method. Equal volumes of 2× SDS-PAGE loading buffer were added to the calculated volume of 5 μg of protein per sample and boiled at 95°C for 2 minutes. The final volumes of each atrial appendage sample were loaded on the premade linear 4% to 15% gradient gels (BioRad). Gels were run at a constant voltage of 100 V and transferred to nitrocellulose membrane using a constant current of 0.2 A (BioRad power supply). After blocking with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20 for 1 hour at room temperature, membranes were washed and incubated with 1:5000 rabbit polyclonal anti-Cx43 antibody overnight at 4°C. The rabbit polyclonal anti-Cx43 antibody (custom manufactured by Research Genetics, Huntsville, Ala) was directed against the same epitope used by Yamamoto and colleagues.21 Membranes were then treated with 1:1000 horseradish peroxidase-conjugated secondary goat antirabbit antibody (Jackson ImmunoResearch Laboratories). For loading controls and normalization, the same membranes were reblocked with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween-20, washed, and incubated with monoclonal anti-GAPDH antibodies (1:300) overnight at 4°C and subsequently blotted with antimouse secondary antibody. Immunoreactive proteins were visualized using chemiluminescent processing (ECL; Amersham Pharmacia Biotech) and autoradiography by exposure to Eastman Kodak X-AR films. Densitometry measurements of Cx43 protein levels were compared between RAA and LAA within each genotype, and between genotypes for RAA and LAA separately, for each age group using NIH ImageJ software.

**Statistical Analysis**

Statistical analysis was carried out with the Microsoft Excel software package. Values are reported as mean±SEM. Power analysis was used to determine the minimum detectable differences in CV measurements during the initial studies. When appropriate, statistical analysis was performed using analysis of variance or 2-tailed paired Student t test with P<0.05 considered significant. The Bonferroni correction was used to minimize Type I error when multiple statistical tests were performed using the same data set. Regression analyses were performed using a least-squares analysis in which the null hypothesis is confirmed when the slope is equal to zero.

**Results**

**Optical Mapping of Adult Hearts**

Representative atrial appendage activation maps are shown in Figure 2 for Cx40+/− (Figures 2A, D), Cx40−/− (Figures 2B, E), and Cx40−/− (Figures 2C, F) mice. Average CVs measured at basic cycle length 100 ms are shown in Figure 2G. Similar results were obtained at basic cycle length 60 ms (data not shown). In Cx40−/− mice, average CV in the LAA was significantly faster than in the RAA (0.63±0.004 and 0.53±0.004 m/s, respectively, at basic cycle length 100 ms, P=0.002; 0.61±0.004 and 0.51±0.004 m/s, respectively, at basic cycle length 60 ms, P=0.001). In both the heterozygous and homozygous Cx40-deficient mice, differences in CVs
between right and left atria were not found, indicating a loss of heterogeneity of CV in the Cx40-deficient atria. When CV was compared between mice of different genotypes, Cx40+/− mice were found to have slower LAA CV compared with Cx40+/+ mice (P<0.01). All other CVs were not significantly different between mice of different genotypes.

**Cx40 and Cx43 Expression**

Analysis of Cx40 and Cx43 mRNA levels by qRT-PCR was performed to determine whether connexin mRNA levels correlate with chamber-specific CV differences (Figure 3A–B). In the Cx40+/+ mice, significant right–left heterogeneity in Cx40 mRNA expression was detected with higher levels of mRNA being expressed in the LAA compared with RAA (P=0.03). This right–left heterogeneity in mRNA expression was lost in the Cx40−/− mice. Cx40 mRNA expression was significantly reduced in the Cx40−/− mice compared with the Cx40+/+ mice in both the RAA (P<0.001) and LAA (P=0.001). Cx43 mRNA was not found to be heterogeneously expressed in the RAA and LAA of Cx40+/+, Cx40−/−, or Cx40+/− mice, nor were differences in Cx43 mRNA expression detected in mice of different genotypes. These data suggest that the CV heterogeneity detected in Cx40+/+ mice and the loss of CV heterogeneity detected in Cx40 mutants can be explained by heterogeneity in Cx40 mRNA expression.

Analysis of Cx40 protein levels was performed to determine whether gradients in Cx40 expression were present in the mouse atria (Figure 3C). Visual inspection comparing samples of Cx40+/+ mice revealed no overt right–left differences. Immunostaining of Cx43 was also performed in both Cx40+/+ and Cx40−/− mice to exclude the possibility of compensatory changes in Cx43 (Figure 3D). Cx43 expression did not appear altered in the Cx40−/− compared with the Cx40+/+ mice nor were right–left differences detected in mice of either genotype. Finally, colocalization of Cx40 and Cx43 immunosignal by immunofluorescence microscopy was evaluated and no statistical differences between the right and left atria of Cx40+/+ mice were identified (data not shown).

Next, Cx40 protein expression was quantified by Western blotting (Figure 3E–F). Although there was a trend toward greater Cx40 protein expression in the LAA compared with the RAA in both Cx40+/+ and Cx40−/− mice, these values were not significantly different. Significantly reduced expression of Cx40 was found in Cx40−/− compared with Cx40+/+ mice in both the RAA and LAA (P>0.05).
At 13.5 dpc, both Cx40^+/− and Cx40^−/− mice. Bar=0.5 mm. G, Representative pseudoelectrocardiogram showing normal sinus rhythm. A=atrial depolarization; V=ventricular depolarization. Bar=200 ms. H, Average CVs in the RA and LA of Cx40^+/− (n=11), Cx40^+/- (n=5), and Cx40^−/− (n=5). Significantly faster CVs were recorded in the RA compared with LA of Cx40^+/− hearts only (P=0.01).

**Age Dependence**
Regression analysis was performed comparing age (in days) versus right–left CV differences to determine if CV heterogeneity was age-dependent. The heterogeneity of CVs in Cx40^+/− mice was not found to be associated with age. Similarly, the loss of heterogeneity in Cx40^−/− mice did not show age-dependent changes.

**Optical Mapping of Embryonic Hearts**
Next, we investigated whether the heterogeneity observed in Cx40^+/− adult mice was also present in embryonic mice. As shown in Figure 4, mice at 15.5 dpc demonstrated a similar pattern to the adults with CV heterogeneity found in Cx40^+/− mice and lost in Cx40^+/- and Cx40^−/− mice. However, unlike their adult counterparts, CV heterogeneity in embryonic Cx40^+/− mice was established by faster RAA CV relative to the LA. Although there appears to be some heterogeneity in CV in the embryonic Cx40^−/− mice, this difference was not significant and is likely attributable to difficulty in measuring CV in embryonic hearts due to their small size, greater curvature of tissue, and variations in heart rate. Interestingly, no right–left heterogeneity was seen in Cx40^+/− mice at 13.5 dpc (data not shown), suggesting that this narrow period of time between 13.5 and 15.5 dpc may represent an important window during which Cx40 contributes to the development of interatrial CV heterogeneity.

**Impulse Initiation in the Embryonic Heart**
Given our findings suggesting that the development of CV heterogeneity in Cx40^+/− mice is a Cx40-dependent process occurring as early as 15.5 dpc, we next investigated whether Cx40 also influenced the site of sinus node impulse initiation in embryonic mice. Figure 5A–D shows representative color maps of dV/dt activity during normal sinus rhythm, whereas Figure 5E–H is a schematic indicating the site of impulse initiation in Cx40^+/− and Cx40^−/− mice at 13.5 and 15.5 dpc. At 13.5 dpc, both Cx40^+/− and Cx40^−/− mice showed normal sinus node activation originating near the crista terminalis. At 15.5 dpc, normal sinus node activation was maintained in Cx40^+/− mice but was lost in Cx40^−/− mice with atrial impulse initiation occurring at multiple ectopic sites, including both appendages and the left atrial myocardium. Additionally, the site of impulse initiation often varied from beat to beat. These data suggest that there is a developmental requirement of Cx40 between 13.5 and 15.5 dpc for normal impulse initiation in the sinus node region.

**Discussion**
Although Cx40 has been implicated in mediating cardiac arrhythmogenesis, its exact role in this respect is not fully understood. The principal findings of this study are: 1) both adult and embryonic Cx40^+/− mice exhibit interatrial CV heterogeneity with the right and left atria conducting at significantly different velocities; 2) this heterogeneity in CV between the atrial chambers is lost with complete or partial (heterozygous) deletion of Cx40 in both adult and embryonic mice; 3) Cx40 mRNA levels showed significant interatrial heterogeneity in Cx40^+/− mice with higher levels in the adult LAA compared with RAA; 4) this heterogeneity in Cx40 mRNA expression was lost with partial or complete deletion of Cx40; 5) the interatrial CV heterogeneity seen in embryonic (and then later in adult) Cx40^+/- mice is absent at 13.5 dpc and develops by 15.5 dpc; and 6) development of normal sinus node impulse initiation is independent of Cx40 expression at 13.5 dpc and becomes dependent by 15.5 dpc. These findings demonstrate for the first time that Cx40 imparts interatrial CV heterogeneity in Cx40^+/− adult and embryonic mice. Furthermore, there is a developmental requirement of Cx40 for normal impulse initiation to occur within the sinus node region.

Several important aspects of this study contribute to our understanding of the role Cx40 plays in atrial conduction heterogeneity. This is the first study to include mice from a wide range of ages, allowing for comparisons between embryonic and adult mice. Additionally, CV was quantified...
Figure 5. Impulse initiation in Cx40-deficient embryonic mice. A–D, Representative dF/dt activity maps for Cx40+/+ mice at 13.5 dpc (A), Cx40+/- mice at 13.5 dpc (B), Cx40-/- mice at 15.5 dpc (C), and Cx40-/- mice at 15.5 dpc. Bar=0.5 mm. E–H, Schematic showing normal sinus node activation originating near the crista terminalis in (E) Cx40+/+ mice at 13.5 dpc (n=8), (F) Cx40+/- mice at 13.5 dpc (n=14), and (G) Cx40-/- mice at 15.5 dpc (n=6) and originating ectopically in (H) Cx40-/- mice at 15.5 dpc (n=10). SVC=superior vena cava; IVC=inferior vena cava; CT=crista terminalis; SAN=sinoatrial node.
at each stage in a chamber-specific manner allowing for the detection of regionally specific effects of Cx40 deletion. Finally, morphologically abnormal hearts were excluded from this study. Studies have found the incidence of major cardiac malformations in Cx40-deficient mice to be as high as 33% with such malformations including tetralogy of Fallot, double-outlet right ventricle, endocardial cushion defects,22 cardiac hypertrophy, and ventricular septal defects.23 In addition, unique defects in mice doubly deficient in 3 different cardiac connexins have been reported.24,25 The high rate and variability of morphological anomalies seen in connexin-deficient mice raises the possibility that inclusion of such animals could influence analysis of impulse initiation and conduction parameters. Although additional work is needed to determine whether anatomic changes can lead to functional changes in CV, preliminary experiments in our laboratory using morphologically abnormal Cx40-deficient hearts support this idea. These hearts showed decreased atrial CV compared with morphologically normal age-matched controls (0.48±0.16 versus 0.57±0.09 m/s, respectively; n=3).

Animal and human studies that have investigated the relation between atrial fibrillation and Cx40 expression have found conflicting results.8–14 Additionally, evidence of an association between pacing-induced atrial fibrillation in goats and the development of multiple small areas of atrial tissue deficient in Cx40 suggests that Cx40 expression itself may be influenced by chronic exposure to arrhythmias.26 From these studies it seems likely that remodeling of Cx40 is highly dependent on model-specific parameters and more work is needed to determine the underlying factors that influence connexin expression. Prior studies have also found conflicting results related to the effects of Cx40 deletion on atrial CV. A recent study reported faster CVs are associated with reduced expression of Cx40 in neonatal atrial myocyte monolayers.15 Conversely, Cx40 deletion has been shown to slow CV in the right atrial free wall during epicardial pacing in intact adult murine atria.16 Together, these studies suggest Cx40’s role in mediating normal and abnormal atrial impulse propagation may vary depending on age and/or structural parameters. The difference in the direction of interatrial CV heterogeneity dependence on Cx40 could be explained by heterogeneity in Cx40 expression. Although Western blot and immunohistological analysis did not demonstrate significant right–left differences in Cx40 protein expression in the Cx40−/− mice, quantification of Cx40 mRNA expression found chamber-specific differences that followed a similar pattern to the CV findings. It is recognized that qRT-PCR is an assay that is less variable than Western blot.28 Heterogeneity in Cx40 mRNA expression (LAA>RAA) was detected in Cx40+/+ mice and lost with partial deletion of Cx40, mirroring CV findings.

Variations in connexin expression likely play an important role in the development of atrial arrhythmias associated with both structural heart disease and normal aging.29,30 Our findings could explain an increased incidence of arrhythmias through at least 3 mechanisms. First, because cardiac disease and human polymorphisms of Cx40 would be unlikely to result in the complete absence of Cx40 expression, the heterozygous (Cx40+−) mouse is likely to be a more realistic model. The current study found that heterozygous deletion of Cx40 resulted in significant slowing of LAA CV in adult mice. Such slowing of CV within the left atrium could act as a substrate for increased arrhythmic propensity humans. Given the arrhythmogenic potential of the pulmonary vein region, changes in Cx40 expression in this area may have important clinical implications. The second possibility relates to the interatrial heterogeneity found in the Cx40−/− mice, which was lost in Cx40−/− and Cx40−/− mice. It is possible that overexpression of Cx40 could contribute to greater heterogeneity than that observed in the Cx40−/− mice. Although this interpretation is an extrapolation of our findings, the importance of electrophysiological heterogeneities in mediating cardiac arrhythmogenesis is well established.31 Increased CV heterogeneity with overexpression of Cx40 could increase the likelihood of significant arrhythmogenic events, consistent with human studies demonstrating a link between Cx40 upregulation and atrial fibrillation.8,10 Finally, it is possible that the interatrial CV heterogeneities found in this study may be amplified in larger animals. Because myocyte size is relatively stable across different species, greater numbers of gap junction channels are recruited during electric propagation as heart size is increased. Consequently, changes in Cx40 expression may have a greater impact on CV in larger hearts. Future studies are needed to better understand the complex interplay among cardiac connexin isoforms, CV, and arrhythmogenesis.

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Disclosures
None.

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Methods

Adult Heart Isolation and Perfusion

Heparin sodium (1000 U/kg) was administered intraperitoneally followed by exposure to 100% CO₂ and cervical dislocation. The hearts were quickly removed via thoracotomy and placed in Tyrode’s solution containing (in mM): NaCl, 130; NaHCO₃, 24; KCl, 4.7; CaCl₂, 1.8; KH₂PO₄, 1.2; NaH₂PO₄, 1.2; MgCl₂, 1.0; glucose, 11.1; Albumin, 0.052 g/l equilibrated with a 95/5% O₂/CO₂ gas mixture to achieve a pH of 7.4. The aorta was cannulated and the heart was Langendorff perfused at a constant pressure of 68-74 mm Hg (1-2 ml/minute) at 37°C. Hearts were stained with a bolus injection of 2 ml of 21 µM di-4-ANEPPS (Molecular Probes) and allowed to equilibrate for 10 minutes.

Embryonic Heart Isolation

Pregnant mice were euthanized with 100% CO₂ followed by cervical dislocation and embryos were collected. The embryos were stored in HBSS (Invitrogen) at 4°C. Hearts were removed, cleaned, and superfused with warm (37°C) HBSS. The isolated hearts were stained with 2 ml of 21 µM di-4-ANEPPS and allowed to equilibrate for 5 minutes. Hearts were maintained at 37°C using a heated stage system (Bioscience Tools).
Colocalization Methods

Colocalization of Cx40 and Cx43 was studied by co-staining tissue sections with primary antibodies, followed by appropriate staining with secondary antibodies conjugated to green or red fluorophores. The sections were imaged and digitally stored. Thresholding was performed for green and red images from each section using a standardized threshold algorithm (Adobe Photoshop). Digital image processing for qualitative colocalization analysis was then performed in a blinded fashion with WCIF ImageJ 1.37c (Tony Collins, Wright Cell Imaging Facility, Toronto, Canada) using previously described techniques. The degree of colocalization of red and green signals were determined using Manders' Calculator, which generates a coefficient (Manders' coefficient, $R$) that varies from $R = 0$ (no colocalization) to $R = 1$ (full colocalization).

Calculation of CV

The background fluorescence was subtracted from each frame to obtain the voltage dependent signal. Ten to fifteen activation sequences were averaged to improve the signal-to-noise ratio of the fluorescent signal and to establish a more representative activation pattern minimizing beat-to-beat variability. On average, the signal-to-noise ratio of the unprocessed movies was $5.59 \pm 0.55$. The improvement in signal-to-noise ratio after averaging was proportional to the square root of the number of averaged beats. A digital low pass filter ($6^{th}$ order Butterworth; 200 Hz cutoff frequency) was applied to each pixel. Regions of interest were identified and a mask was used in order to exclude all other
regions. Activation times were assigned to each pixel, with pixels considered to be activated when action potential amplitudes reached 50% of the maximum value (Figure 1D-E). This time point was obtained from a linear interpolation of the two sample points closest to the 50% intensity value. The interpolation was done using floating point precision and resulted in a unique activation time for each pixel. For display purposes, activation times were binarized into 256 colors. Conduction vectors were determined for each pixel by analyzing the activation times of an array of the neighboring 5x5 pixels. The details of this method have been published elsewhere. The average magnitude of all conduction vectors was calculated and recorded as the average CV.

Histological Analysis
Alterations in Cx40 expression have been associated with atrial fibrosis and increased atrial dimension size in both animal and human models. The current study excluded mice with congenital or morphologic abnormalities. Here we show representative histological sections demonstrating similar morphology and fibrosis in Cx40+/+, Cx40 +/- and Cx40 -/- hearts. Spontaneously contracting tissue was arrested in diastole with a 1 mL perfusate of 50 mM KCl and then perfused with 1 mL 4% paraformaldehyde (in PBS) at 20ºC. Tissue was allowed to stand in 4% paraformaldehyde at 4 ºC for 24 hours. Tissue was then serially dehydrated in progressively increasing concentrations of ethanol and then allowed to stand in 100% ethanol overnight. Following fixation in xylene, tissue was embedded in paraffin and sectioned at 10 µm. Sections were stained with
hematoxylin and eosin to assess morphology and Masson’s trichrome to assess for collagenous components, as previously described. As shown in the figure (A-F), similar levels of fibrosis were detected in Cx40<sup>+/+</sup>, Cx40<sup>+-</sup> and Cx40<sup>-/-</sup> hearts. Additionally, the figure (G-L) demonstrates similar morphology among mice of all three genotypes.

**Figure Legend**

**Online Figure 1.** Masson’s Trichrome and hematoxylin and eosin stained sections. A-F. Representative sections stained with Masson’s Trichrome, showing similar collagen deposition in Cx40<sup>+/+</sup> (A and D), Cx40<sup>+-</sup> (B and E), and Cx40<sup>-/-</sup> (C and F) hearts (n=2). G-L. Representative sections stained with hematoxylin and eosin, showing similar morphology in Cx40<sup>+/+</sup> (G and J), Cx40<sup>+-</sup> (H and K), and Cx40<sup>-/-</sup> (I and L) hearts. Two hearts were evaluated for each genotype. Bar = 2mm. RA, right atria; LA, left atria; RV, right ventricle; LV, left ventricle.

**References**

