Functional Properties of Mouse Connexin30.2 Expressed in the Conduction System of the Heart

Maria M. Kreuzberg, Goran Söhl, Jung-Sun Kim, Vytas K. Verselis, Klaus Willecke, Feliksas F. Bukauskas

Abstract—Gap junction channels composed of connexin (Cx) 40, Cx43, and Cx45 proteins are known to be necessary for impulse propagation through the heart. Here, we report mouse connexin30.2 (mCx30.2) to be a new cardiac connexin that is expressed mainly in the conduction system of the heart. Antibodies raised to the cytoplasmic loop or the C-terminal regions of mCx30.2 recognized this protein in mouse heart as well as in HeLa cells transfected with wild-type mCx30.2 or mCx30.2 fused with enhanced green fluorescent protein (mCx30.2-EGFP). Immunofluorescence analyses of adult hearts yielded positive signals within the sinoatrial node, atrioventricular node, and A-V bundle of the cardiac conduction system. Dye transfer studies demonstrated that mCx30.2 and mCx30.2-EGFP channels discriminate poorly on the basis of charge, but do not allow permeation of tracers >400 Da. Both mCx30.2 and mCx30.2-EGFP gap junctional channels exhibited weak sensitivity to transjunctional voltage (V_j) and a single channel conductance of ≈9 pS, which is the lowest among all members of the connexin family measured in HeLa cell transfectants. HeLa mCx30.2-EGFP transfectants when paired with cells expressing Cx40, Cx43, or Cx45 formed functional heterotypic gap junction channels that exhibited low unitary conductances (15 to 18 pS), rectifying open channel I-V relations and asymmetric V_j dependence. The electrical properties of homo- and hetero-typic junctions involving mCx30.2 may contribute to slow propagation velocity in nodal tissues and directional asymmetry of excitation spread in the AV nodal region. (Circ Res. 2005;96:1169-1177.)

Key Words: connexin ■ gap junctions ■ mCx30.2 ■ sinoatrial node ■ atrioventricular node

Gap junction (GJ) channels composed of connexin (Cx) molecules mediate direct intercellular diffusion of ions and small metabolites. Twenty connexin genes have been identified in the mouse genome.1 Depending on the expression pattern, connexins can form homotypic, heterotypic, or heteromeric channels with highly divergent conductance, permeability and gating properties.2 Cx40, Cx43, and Cx45 have been identified as the major connexins expressed in the heart.3,4 Cx43 is predominantly found in the atrial and ventricular working myocytes and, to a lesser extent, in peripheral Purkinje fibers, whereas Cx40 and Cx45 are expressed in the conduction system.5 Cx40 is preferentially found in the atrial myocardium, the A-V bundle and its branches, whereas Cx45 is mainly expressed in the sinoatrial (SA) and atrioventricular (AV) nodes, as well as the A-V bundle and its branches.6,7 Targeted deletion of Cx40 and Cx45 revealed the contribution of each of these connexins to heart development and function. Replacement of Cx43 by Cx40 or Cx40 by Cx45 showed that the function of certain cardiac connexins can be compensated by other connexins.12,13,14

Mouse connexin30.2 (mCx30.2) and its human orthologue, Cx31.9, have been reported to be expressed in vascular smooth muscle cells, brain, and testis.15,16 Furthermore, immunopositive signals for mCx30.2 were found in intercalated discs of the heart as well as in interstitial cells and cells of the seminiferous tubules of the testis.16 Functional analyses of Cx31.9 gap junction channels yielded a low unitary conductance (12 to 15 pS) and weak sensitivity to transjunctional voltage (V_j).15,17 However, functional properties of mCx30.2 GJ channels have not yet been described. Here we report that mCx30.2 is highly expressed in the cardiac conduction system and that mCx30.2 can form functional homotypic as well as heterotypic junctions with all the major cardiac connexins.

Materials and Methods
Expanded Materials and Methods are in the online data supplement at http://circres.ahajournals.org

Functional Cloning, Transfection, RT-PCR Analysis, and mCx30.2 Antibodies
The coding region of mCx30.2 was PCR-amplified. PCR-fragments were cloned into pRESpuro2 or pRESpuro2-EGFP vectors that...
were transfected into HeLa cells. Two peptides derived from the cytoplasmic loop and the C-terminal region of mCx30.2 were used to produce polyclonal mCx30.2 antibodies. Heart cryosections of adult mice and HeLa transfectants were exposed to mCx30.2, Cx40 or Cx43 antibodies.

Electrophysiology and Fluorescence Imaging

Xenopus oocytes were injected with mCx30.2 RNA to study junctional conductance (gj) by using a dual 2-electrode voltage clamp method. The dual whole-cell voltage clamp method combined with imaging, were used for electrophysiological and dye transfer studies.

Results

Expression of mCx30.2 Transcripts
After screening the HUSAR-derived mus musculus EST database (DDJB/GenBank/EMBL, Heidelberg), we identified a sequence tag (Acc. BF580638) with 91.2% similarity to the coding region of mCx30.2 (Acc. AL414561). This sequence comprised only \( \approx 160 \) bp of the complete coding sequence encompassing 837 bp, but contained an additional 34 bp upstream of the Kozak consensus motif. Most of this presumptive 5'-untranslated region (5'-UTR) could readily be localized \( \approx 700 \) bp further upstream of the mCx30.2 open reading frame within the genomic sequence (Acc. AL591067). Both the 3' part of this putatively spliced 5'-UTR as well as the 5' part, shortly upstream of the coding region, contained canonical splice donor and acceptor motifs, respectively.\(^\text{18}\) We analyzed expression of this splice isoform in heart and brain. Different primers spanning the intron were used to amplify mCx30.2 cDNA by RT-PCR (Figure 1A). Three out of 6 primers yielded amplicons of \( \approx 740 \) bp, 770 bp, and 825 bp in size (Figure 1B). Data obtained suggested that the 5’-untranslated sequence comprised at least 120 bp in both tissues. Furthermore, no putative splice acceptor site was identified within 240 bp further upstream. Thus, at least 1 spliced mRNA isoform is transcribed from the mCx30.2 gene containing one untranslated exon1 and the complete coding region on exon2 in tissues such as heart and brain.

To investigate the expression pattern of mCx30.2, we performed intron-spanning RT-PCR analyses in different mouse tissues. The expected fragment of 740 bp was found in heart, brain, kidney, lung, testis, and very faintly in liver (Figure 1C).

HeLa Cell Lines Stably Expressing mCx30.2 and mCx30.2-EGFP

To analyze functional properties of mCx30.2 GJs, HeLa cells were stably transfected with DNA coding for mCx30.2 or mCx30.2-EGFP. Expression of mCx30.2 and mCx30.2-EGFP mRNAs was verified after Northern blot hybridization (see Figure 2A). Immunoblots of different HeLaCx30.2 lysates revealed only one fragment of 28 kDa, which was absent in HeLa wild-type cells, whereas in HeLaCx30.2-EGFP cells the signal of the fusion protein was detected (Figure 2B).

Immunofluorescence of HeLaCx30.2 cells revealed a punctate staining pattern characteristic of gap junction plaques. Figure 2C (I-III) shows that in HeLaCx30.2-EGFP cells junctional plaques revealed by immunostaining (I) or by EGFP fluorescence (II) colocalize (III). HeLa cells expressing Cx40 (IV), Cx43 (V), and Cx45 (VI) when incubated with mCx30.2 antibodies did not show any punctate immunofluorescence and exclude cross-reactivity.

Expression of mCx30.2 Protein

Immunostaining of heart cryosections demonstrated that mCx30.2 is expressed in the cardiac conduction system, but not in working cardiomyocytes of atria or ventricles. Punctate mCx30.2 immunofluorescence was detected in areas of the SA node (Figure 3A and 3B), AV node (Figure 3A through 3F) and A-V bundle; squares indicate approximate regions from which immunofluorescence images were acquired. Immuno- fluorescence analysis in consecutive sections revealed that mCx30.2 and Cx40 were not coexpressed within the SA node and showed a rarely overlapping expression pattern within the AV node and A-V bundle. Cx43 (d,h,l) was not present in cardiac conduction system, but was predominant in the ventricular and atrial working myocardium. Because Cx45 antibodies show substantial cross-reactivity with other connexins, we used Cx45 \(^{-}\text{null}\) mice to analyze coexpression of mCx30.2 and Cx45. Figure 3B shows abundant immunostain-
Electrical Cell-Cell Coupling and Gating of Homotypic mCx30.2 Junctions

The ability of mCx30.2 to induce electrical coupling was examined in *Xenopus* oocytes and in HeLa cells expressing mCx30.2 or mCx30.2-EGFP. Pairs of *Xenopus* oocytes injected with mCx30.2 mRNA, displayed high levels of coupling ($g_j = 2.8 \pm 0.87 \mu S$). Normalized initial and steady state $G_j-V_j$ relations are plotted in Figure 4A. Initial $G_j$ (open circles) did not change significantly with $V_j$ of either polarity. Steady-state $G_j$ (filled circles) was insensitive to $V_j$ up to $\pm 60$ mV and declined only modestly with larger $V_j$. Representative record of transjunctional currents ($I_j$) measured at $V_j$ steps of both polarities illustrate the slow time course of the decline in $I_j$ (Figure 4B).

Similar to oocytes, coupling in HeLaCx30.2 and HeLa HeLaCx30.2-EGFP cell pairs showed weak voltage dependence (Figure 4C). Steady-state $G_j$ values obtained from HeLa cells expressing mCx30.2 (open circles) or mCx30.2-EGFP (solid circles) were indistinguishable. At modest $V_j$ of either polarity, $G_j$ showed a tendency to increase. $G_j$ decreased at $V_j > 80$ mV.

Heptanol (2 mmol/L), when applied to cell pairs expressing mCx30.2 or mCx30.2-EGFP, produced full uncoupling and relatively fast recovery on washout (Figure 4D). Application of 100% CO₂ also yielded rapid and full uncoupling between cells expressing mCx30.2 or mCx30.2-EGFP, but slow recovery (~5 minutes) during washout (data not shown).

Conductance and Permeability of mCx30.2 Channels

We examined single channel conductance in HeLa cell pairs expressing mCx30.2 or mCx30.2-EGFP. Experiments were performed in weakly and well-coupled cell pairs with and without application of CO₂, respectively. Figure 4E shows $V_j$ and $I_j$ records during 2 $V_j$ steps and a ramp. A plot of $g_j$ over time indicates 4 equal conductance levels (horizontal lines) $\approx 9$ pS in magnitude, which we ascribe to single channels.

To assess permeability, we measured dye transfer and $g_j$ in HeLaCx30.2-EGFP cell pairs exhibiting at least 1 junctional plaque. Fluorescent dyes examined included Lucifer Yellow (443 Da, net charge, $z$, equals –2), Alexa Fluor-350 (326 Da, $z_1$), ethidium bromide (EtBr; 314 Da, $z_1$), 4',6-Diamidino-2-phenylindole (DAPI) (279 Da, $z_2$), and propidium iodide (PrI; 415 Da, $z_2$). Typically, a dye-filled patch pipette was used to load 1 cell of a pair with dye by establishing a whole-cell recording. After allowing ~10 minutes for dye transfer, a whole-cell recording was established in the second cell to measure $g_j$. In summary, mCx30.2-EGFP junctions demonstrated permeability to Alexa Fluor-350, EtBr, and DAPI (Figure 5A through 5C), but not to PrI and Lucifer yellow (not shown).

At least 5 cell pairs were examined for each dye.

Intercellular transfer of Neurobiotin (287 Da, $z_1$), was examined in monolayers of HeLa-WT, HeLaCx43, HeLaCx30.2, and HeLaCx30.2-EGFP cells. The cells were fixed 30 minutes after Neurobiotin microinjection into a single cell within each monolayer (asterisks in Figure 5D). Neurobiotin readily spread to first and second order neighbors in cells expressing Cx43, mCx30.2, and mCx30.2-EGFP, but not in HeLa-WT cells.
Properties of Heterotypic Junctions Formed of mCx30.2 and Other Cardiac Connexins

mCx30.2/Cx40 Heterotypic Junctions

In cocultures of HeLaCx30.2-EGFP and HeLaCx40 cells, we selected cell pairs in which only one cell exhibited EGFP fluorescence and at least 1 junctional plaque was visible in the junctional membrane. Fluorescence intensities of such plaques were approximately half of that of mCx30.2-EGFP homotypic junctions, consistent with fluorescence contributed from only one side of the junction. Formation of mCx30.2-EGFP/Cx40 junctional plaques in these cell pairs was abundant, suggesting that mCx30.2 and Cx40 hemichannels readily dock to form intercellular channels (see Figure II-S(A) in online data supplement).

We found that mCx30.2-EGFP/Cx40 junctions were functional and exhibited an asymmetric steady-state Gj-Vj relation.

Figure 3. Immunofluorescence analysis of mCx30.2 expression pattern and colocalization with other cardiac connexins. A, Shown are immunofluorescence images (green) of mCx30.2 (b,f,j), Cx40 (c,g,k) and Cx43 (d,h,l) in serial sections from adult mouse heart. Left column shows bright field images (a,e,i) stained for acetylcholine esterase (AChE); squares indicate approximate regions from which immunofluorescence images were acquired. Images in rows (a-d), (e-h) and (i-l) correspond to the regions of the SA node, AV node, and A-V bundle, respectively. Immunostaining of mCx30.2 is visible in the SA node (b), AV node (f), and A-V bundle (j). Cx40 immunostaining in the AV node and A-V bundle rarely overlaps with that of mCx30.2, but not within the SA node. Cx43 is not expressed in the SA node, AV node and A-V bundle, but in the atrial and ventricular working myocytes (d,h,l). Dashed lines separate AV-nodal region and A-V bundle from working myocardium. Used abbreviations: AVB, A-V bundle; AVN, AV node; CFB, central fibrous body; IVS, interventricular septum; RA, right atrium; SAN, SA node; SAJ, sinoatrial junction; TV-tricuspid valve. Nuclei of the cells were stained in red with propidium iodide. Scale bars, 20 μm.

B (Cx45) mCx30.2 LacZ (Cx45) mCx30.2

Coexpression of Cx45 and mCx30.2 in SA node (a,b) and AV node (c,d) of Cx45LacZ mouse. Immunofluorescence signals (b,d; in green) abundantly overlap with LacZ signals (a,c; in blue) in both nodal regions. Scale bars, 20 μm.

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We found that mCx30.2-EGFP/Cx40 junctions were functional and exhibited an asymmetric steady-state Gj-Vj relation.
with higher $V_j$-sensitivity at voltages relatively negative on the mCx30.2 side (Figure 6A). Open circles show steady-state (normalized) $G_j$ evaluated at the ends of the 20 s voltage steps. The solid line represents $G_j$ measured in response to the 2 slow $V_j$ ramps that changed from 0 to $-110$ mV over periods of 120 s. Representative recordings shown in Figure 6B demonstrate asymmetric $V_j$-gating in response to voltage steps of $-85$ mV and $-125$ mV as well as a ramp of $-110$ mV. Single channels were visualized during the recovery from full uncoupling with CO$_2$. Single channel conductance in these recordings was found to be $\approx 9$ pS (see horizontal lines).

Figure 6C shows a representative $I_j$ record at the single channel level in response to voltage steps ($\pm 85$ mV) and ramps ($\pm 105$ mV) applied to the HeLaCx40 cell of a Cx40/mCx30.2-EGFP cell pair. The open channel current, $I_j$, rectifies, decreasing significantly when the Cx40 side is made relatively more negative. On average, the conductance of the open state ($\gamma$) decreased from $\approx 27$ to $\approx 12$ pS when $V_j$ changed from $+100$ to $-100$ mV; $\gamma=\approx 18$ pS at $V_j=0$ mV. $V_j$-induced gating events occurred more often when the Cx40 side was relatively positive and gating transitions were mainly to a substate (see dashed arrows), with full closures occurring occasionally. Gating transitions for $V_j$s relatively negative on the Cx40 side were seldom observed, but mainly transited to the fully closed state and appeared to be slow (see asterisk). Therefore, the decrease in $G_i$ at positive $V_i$s in Figure 6A is mainly defined by rectification properties and the slow gating mechanism of mCx30.2 or Cx40, whereas at negative $V_i$s mainly by the fast $V_j$-sensitive gating mechanism of mCx30.2 and Cx40.

**mCx30.2/Cx43 Heterotypic Junctions**

In cocultures of HeLaCx30.2-EGFP and HeLaCx43-CFP cells, we selected cell pairs with junctional plaques exhibiting...
EGFP and CFP fluorescence (see Figure II-S(B) in online data supplement). The steady-state $G_j-V_j$ relation of this heterotypic junction is strongly asymmetric and exhibits an increase in $G_j$ when the cell expressing mCx30.2 is made more positive (Figure 7A). The $I_j$ record shown in Figure 7B demonstrates this asymmetry with long-duration $V_j$ steps of $-90$ and $-85$ mV applied to the HeLaCx40 cell. Repeated voltage ramps of $\pm 20$ mV were used to assess $g_j$ between the voltage steps. C, Representative $I_j$ and $V_j$ records measured during application of voltage steps and ramps to the HeLaCx40 cell. Gating transitions to the substate (dashed arrows) occurred more often when the mCx30.2 side was made relatively negative. The asterisk shows only one full closure of the channel that occurred at relative negativity of $V_j$ on the Cx40 side.

Figure 6. Electrophysiological characterization of heterotypic mCx30.2-EGFP/Cx40 channels examined in cocultured HeLaCx30.2-EGFP and HeLaCx40 cells. A, Normalized $G_j-V_j$ plot shows highly asymmetric $V_j$ gating. Data points (open circles) were measured at the ends of the voltage steps. Solid line was obtained applying $V_j$ ramps of $120$ s in duration. $V_j$ is defined relative to the mCx30.2-EGFP side. B, Example of $V_j-I_j$ and $g_j$ traces obtained to voltage steps of $85$ and $-85$ mV applied to the HeLaCx40 cell. Repeated voltage ramps of $\pm 20$ mV were used to assess $g_j$ between the voltage steps. C, Representative $I_j$ and $V_j$ records measured during application of voltage steps and ramps to the HeLaCx40 cell. Gating transitions to the substate occurred more often when the mCx30.2 side was made relatively negative. The asterisk shows only one full closure of the channel that occurred at relative negativity of $V_j$ on the Cx40 side.

Figure 7. Gating properties of heterotypic mCx30.2-EGFP/Cx43-CFP channels examined in cell pairs with junctional plaques formed between HeLaCx43-CFP and HeLaCx30.2-EGFP cells. A, Normalized $G_j-V_j$ plot; $V_j$ is defined relative to the mCx30.2-EGFP side. The solid line is a regression line of the fifth order fit to the data. B, Representative $V_j$ and $I_j$ records demonstrate asymmetric $V_j$ gating in response to consecutive steps of $-90, 90, -110,$ and $110$ mV and repeated ramps of $\pm 20$ mV applied to the HeLaCx30.2-EGFP cell. Negative $V_j$ steps induced a slow decrease in $I_j$ whereas positive ones initially increased and then slowly decreased $I_j$. C, Single channel record obtained by applying voltage ramps ($\pm 105$ mV) to the HeLaCx43-CFP cell shows a linear open channel $I-V$ relationship and a single channel conductance of $\approx 18$ pS.
mCx30.2/Cx45 Heterotypic Junctions

Junctional plaques were also observed between HeLa/Cx30.2-EGFP and N2A/Cx45-EGFP cells. A, Normalized G-J vs. Vj plot. The solid line is a fit of the data, shown in solid circles, to the Boltzmann equation. B-C, Examples of Vj and Ij records demonstrate asymmetric Vj gating in response to voltage steps applied to the Cx45-EGFP side. Repeated voltage ramps of ±100 mV (C) applied to the Cx45-expressing cell. For Vj steps positive on the Cx45 side, Ij decreased substantially and rapidly, whereas for positive Vj steps Ij increased. On termination of the positive Vj step, the increase in Ij slowly returned to the value preceding the steps (see insert). These data are consistent with Cx45 hemichannels being responsible for closure of the mCx30.2/Cx45 junctions observed at Vj relatively positive on the mCx30.2 side.

Single channel recordings show higher open probability at Vj positive on Cx45-EGFP side (Figure 8D). As previously indicated, closure at Vj positive on mCx30.2 side represents closure of Cx45 hemichannels. The single open channel I-V curve is nearly linear; γ=≈17 pS.

Discussion

mCx30.2 Gene Structure and Protein Expression Pattern

Nielsen et al15 found that the human Cx31.9 gene is comprised of only 1 exon harboring the 5′-UTR, the coding region and the 3′-UTR, although a strong putative splice acceptor site was detected at position –5. We found that the mCx30.2 gene consists of at least 1 untranslated exon1 as well as exon2 containing the uninterrupted coding region of mCx30.2 and the 3′-UTR, as previously described for most other connexin genes.21 Because a splice donor site at the 3′ border of mCx30.2 corresponds to that found in Cx31.9 and exon1 is also located ≈730 bp upstream of the Cx31.9 coding region [Acc. AC080112], it is likely that the splicing pattern is conserved in both orthologous genes.

We detected mCx30.2 protein expression in the cardiac conduction system with particularly high levels in the SA and AV nodes (Figure 3). mCx30.2 signals were relatively intense within the SA node, which was immunonegative for Cx40 and Cx43. In the AV node, both mCx30.2 and Cx40 are expressed but Cx43 is not. Several reports indicate that Cx40 is preferentially expressed in the fast-conducting His bundle, the ensuing branches and Purkinje terminal fibers.8,6 Our immunostaining data show that mCx30.2 is predominantly expressed in slow-conducting regions, ie, the SA and AV nodes. We suggest that the low single channel conductance of mCx30.2 contributes to the slow propagation velocities within nodal regions.

In contrast to earlier reports,16 we did not detect mCx30.2 protein expression in vascular smooth muscle cells of several tissues that we analyzed by immunofluorescence (data not shown). It is possible that the low mCx30.2 expression levels in the cell types examined are below the detection limit of our mCx30.2 antibodies. Earlier reports of expression might have been because of cross reactivity of the mCx30.2 antibodies used.16 Nevertheless, our RT-PCR analyses revealed tran...
scripts of mCx30.2 in heart, brain, liver, lung, kidney, and testis. This expression pattern might be because of the expression of the mCx30.2 transcript in blood vessels of these tissues.

**Functional Properties of Homo- and Hetero-typic Junctions**

We demonstrated that mCx30.2 channels exhibit weak sensitivity to $V_j$ and a very low single channel conductance, $\approx 9$ pS. When expressed in *Xenopus* oocytes or HeLa cells, little if any reduction in $G_j$ was evident until $V_j$ exceeded $\pm 80$ mV. Attachment of EGFP to the carboxy terminus of mCx30.2 did not appear to have any measurable effects on single channel conductance or gating properties. Channels formed of human Cx31.9, the human orthologue of mCx30.2, were reported to exhibit similar properties, although the single channel conductance was somewhat larger, ie, 12 to 15 pS. Cx36, the major neuronal connexin, also forms channels with weak sensitivity to $V_j$ and a low single channel conductance (=$15$ pS) but exhibits only 30% to 40% sequence identity to mCx30.2 or Cx31.9. The low unitary conductance of mCx30.2 is reflected in the low unitary conductances of the heterotypic channels formed by pairing mCx30.2 with other cardiac connexins, ie, Cx40 (18 pS), Cx43 (18 pS), or Cx45 (17 pS). These conductances are close to the values of 17, 17, and 14 pS, respectively, predicted by the series arrangement of these heterotypic combinations according to $\gamma_A = 2 \cdot \gamma_A \cdot \gamma_B (\gamma_A + \gamma_B)$, where $\gamma_A$ and $\gamma_B$ represent the conductances of homotypic GJ channels of connexins A and B. Values of $\gamma$ for Cx40, Cx43, and Cx45 are $\approx 180$, 115, and 32 pS, respectively, as previously described.

Our dye transfer studies showed that mCx30.2 GJs are permeable to positively and negatively charged dyes such as Neurobiotin, EtBr, DAPI, Alexa Fluor-350. However, we did not detect transfer of Lucifer yellow or propidium iodide, suggesting that mCx30.2 channels, while not particularly selective on the basis of charge, are size-restrictive with a cut-off to tracers $>400$ Da molecular mass. Furthermore, this restricted permeability appears to be reflected in the efficacy of Neurobiotin transfer, which is low compared with that of Cx43-expressing cells (Figure 5D). Previous reports indicated that HEK cells expressing Cx31.9 lacked transfer of Lucifer yellow or ethidium bromide. Whether permeability of mCx30.2 channels to EtBr observed in this study represents a difference between Cx31.9 and mCx30.2 or is because of different detection sensitivity remains to be determined.

We have demonstrated expression of mCx30.2 in the SA node, AV node, and A-V bundle. It was previously reported that cells in the SA node express Cx45, cells in the AV node as well as in the A-V bundle express Cx40 and Cx45 and Purkinje cells express Cx40 and Cx43. Within the atrial and ventricular myocardium, atrial cardiomyocytes predominately express Cx40 and Cx43, whereas Cx43 is the major connexin in working ventricular myocytes. These patterns of expression suggest that mCx30.2 might interact with Cx40 or Cx45 within the SA and AV nodal regions and with Cx43 in adjacent regions that interconnect the atrial myocardium and the ventricular conduction system.

We have examined whether mCx30.2 was capable of interacting with the other cardiac connexins in heterotypic combinations after expression in HeLa cells. We found that all 3 possible heterotypic junctions with mCx30.2, mCx30.2/Cx40, mCx30.2/Cx45, and mCx30.2/Cx43, were functional under these conditions. In each case, the steady-state $G_j-V_j$ relations were asymmetric, but differed in the degree and direction of asymmetry. An important point in understanding the $G_j-V_j$ relations of these junctions is the small unitary conductance of the mCx30.2 hemichannel. When paired with a hemichannel that exhibits a larger unitary conductance, a larger fraction of $V_j$ will drop across the mCx30.2 hemichannel because of its low conductance (inferred to be $\approx 18$ pS, ie, 2-fold higher than that of the gap junction channel). This increases the fraction of $V_j$ that drops across the mCx30.2 hemichannel and thus decreases the probability that gating events will occur in Cx40, Cx43, or Cx45 hemichannels paired with mCx30.2. This effect would be smallest in Cx45/mCx30.2 heterotypic junctions because of the relatively low conductance of Cx45 hemichannels ($\approx 64$ pS). Similar effects of unitary conductance of component hemichannels on $V_j$-gating has been described for Cx43/Cx45 channels. Based on the $G_j-V_j$ dependent properties of the heterotypic junctions examined here and knowing that both Cx43 and Cx45 have a negative gating polarity, $25$, whereas Cx40 exhibits a positive one, we ascribe a negative gating polarity to mCx30.2.

Our data show that mCx30.2/Cx40 junctions in HeLa transfectants exhibit significant rectification (Figure 6B and 6C). This property would facilitate the spread of excitation from the cells expressing Cx40 to neighboring cells expressing mCx30.2 and impede spread in the opposite direction. In the heart, normal signal propagation is oriented downstream along atrium, AV node, A-V bundle and branches, Purkinje terminals, and working myocytes of ventricles. Along this pathway, action potentials might encounter several types of heterotypic and heteromeric junctions containing mCx30.2. Transjunctional voltage at the edge of an excitation front could be relatively high in regions with slow velocities of propagation, such as the AV node. Such $V_j$s across junctions with strong $G_j-V_j$ dependence, eg, mCx30.2/Cx45 could modulate $g_j$, thereby impeding signal transfer in one direction and facilitating it in the opposite direction. Formation of heterotypic junctions containing mCx30.2 could contribute, at least in part, to electrical signal transfer from the AV node to ventricular working myocytes. However, to what extent heterotypic junctions form within the cardiac conduction system is not known. Furthermore, it is possible that mCx30.2 can form heteromeric channels with coexpressed Cx45. Future studies will have to show whether such heteromeric channels will also exhibit a low unitary conductance.

In summary, we have analyzed expression of the mCx30.2 gene and found that mCx30.2 is abundantly expressed in the nodal regions of the mouse heart. mCx30.2 can form heterotypic channels with all major cardiac connexins in transfected HeLa cells and is likely to influence and regulate the propagation of electrical signals in the heart.
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**Expanded Materials and Methods**

*Functional cloning of mCx30.2 and transfection.* The coding region of mCx30.2 was PCR-amplified from mouse genomic DNA (C57BL/6) using the upstream primer, mCx302KXho, and one of two downstream primers, mCx302EcoSTOP or mCx302EcoGO. The upstream primer included a 5'-Xhol restriction site followed by the optimized translation initiation motif. Both downstream primers contained a 5'-EcoRI restriction site and permitted to arrange the presence (mCx302EcoSTOP) or the deletion (mCx302EcoGO) of the native stop codon of mCx30.2. A PCR was performed using 1 unit of *Taq* DNA Polymerase (Promega, Mannheim, Germany) in reaction buffer containing 1.5 mmol/L MgCl₂, 0.4 mmol/L dNTPs, 0.4 µmol/L of each primer and 4% dimethylsulfoxide. PCR-fragments were gel purified, digested with *Xhol* and *EcoRI* and cloned into the *EcoRV/EcoRI* digested vectors pIRESpuro2 or pIRESpuro2-EGFP (Clontech, Palo Alto, CA, USA). The resulting plasmids, pIRESpuro2-mCx30.2-Stop and pIRESpuro2-mCx30.2-Go-EGFP, were transfected into coupling-deficient HeLa cells (ATCC CCL1; ATCC, Rockville, USA) as described earlier.

*Northern Blot and RT-PCR analysis.* Total RNA was purified from HeLa cells or adult mouse tissues using the TRIzol®-reagent following the manufacturer’s instructions (Invitrogen Life Technologies, Karlsruhe, Germany). Northern blot analysis was carried out using the ULTRAHybTM hybridization solution (Ambion, Austin, Texas) as recommended. The hybridization probe of 532 bp consisted of the 5'-terminal fragment of the mCx30.2 coding region. Reverse transcription of RNA was performed according to Söhl et al. One twentieth of the transcribed cDNA was amplified by PCR as described above. The following mCx30.2 specific primers were designed for the PCR reaction: P1 (5'-CTC AG C TCT AAG GCC CAG GTC CCG), P2 (5'-CAG GTC CCA AGC TGT CTC TCA AGG TC), P3 (5'-CTA TCA TCC ATC CCG GCC AAA GCC), P4 (5'-GAC CAC AGG TCA CCC TGT CCC), P5 (5'-GGG TAA GGG ATG GAA AGA GAG), P6 (5'-CCA TGA TTG ATC TCT CTG GCT CCC) and C-TER-DSP (5'-CCG CGC TGC GAT GGC AAA GAG).
**mCx30.2 antibodies, immunoblotting and immunocytochemistry.** Two peptides derived from the cytoplasmic loop (APPCARGRAEAPCSPC) or the C-terminus (GDSEGEGGSGHSKAS) of mCx30.2 were commercially synthesized, coupled to keyhole limpet hemocyanin, and injected into rabbits following the “double X procedure” (Eurogentec, Seraing, Belgium). Sera were affinity purified and characterized by using immunoblot as well as immunofluorescence analysis in HeLa cells stably expressing mCx30.2 and mCx30.2-EGFP. As both antibodies when applied alone showed similar quality in immunoblotting and immunocytochemistry, a mixture of them (1:1) was used in all experiments. HeLa cells were harvested and lysed in Laemmli sample buffer. Protein lysates were electrophorezed, transferred onto a nitrocellulose membrane (Amersham Biosciences), blocked with 5% skim milk and incubated with polyclonal mCx30.2 antibodies (1:200) overnight at 4°C. After washing, membranes were incubated for 1 h at room temperature with secondary antibodies (1:15.000; horseradish peroxidase-conjugated goat anti-rabbit; Dianova, Hamburg, Germany). Antibodies were detected using the SuperSignal® West Pico Chemiluminescent detection kit (Pierce, Rockford, IL, USA). Immunoblots were standardized using the mouse monoclonal anti-β-actin (1:500; Sigma) and the horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (1:15.000; Dianova). Heart cryosections (12-20 µm) of adult mice were fixed in 96% ethanol, washed in PBS® and preincubated for 1 h in blocking reagent (PBS® containing 5% NGS). Slides were incubated for 2 hours with polyclonal anti-mCx30.2 (1:100), anti-Cx40 (1:150; Alpha Diagnostics, USA) or anti-Cx43 (1:300; 3) antibodies. Connexins were visualized using the secondary antibodies, Alexa (488)-conjugated goat anti-rabbit (1:800). Slides were washed in PBS® and mounted in Permafluor aqueous mounting medium (Beckman Coulter, Marseille, France). Nuclei were stained with propidium iodide. Fixation and immunostaining of HeLa cells grown on glass coverslips (~70% confluence) were performed as described for cryosections using the secondary antibodies, Alexa (594)-goat anti-rabbit (MoBiTec). Fluorescent signals were recorded using Zeiss confocal (LSM 510) and Zeiss Axiophot microscopes.
Acetylcholine esterase histochemistry and LacZ staining. Heart cryosections (12-20 µm) were fixated in 0.1 mol/L HNaPO₄/Na₂PO₄ buffer (pH 7.3) containing 4% PFA and 0.22 mol/L sucrose for 10 min at room temperature. After two washing steps in PBS slices were incubated in staining solution (65 mmol/L sodium acetate (pH 5.0), 1.73 mmol/L acetylthiocholine iodide, 15 mmol/L sodium citrate, 3 mmol/L CuSO₄, 0.5 mmol/L isoOMPA, 5 mmol/L K₃[Fe(CN)₆], 1% Triton-X 100) for 2h at 37°C. Slices were washed twice with PBS and stained for 5 min in 0.1% Eosin. LacZ staining of heart cryosections was carried out as described previously.

Electrophysiological measurements. mCx30.2 RNA was prepared with the mMessage mMach ine kit from Ambion (Austin, TX, USA). Each oocyte was injected with 50 nL of mCx30.2 RNA (~1 µg/µL) together with an antisense oligonucleotide (~0.5 pmol/L/nL) complementary to XenCx38. Approximately 24 hours after injection, oocytes were devittelinated and paired. Recordings of mCx30.2 junctional currents between Xenopus oocyte cell pairs were obtained with a dual two-electrode voltage clamp as previously described. Transjunctional voltage (Vₖ) steps preceded by a small, brief pre-pulse of constant amplitude (10 mV), were used to measure junctional conductance (gₖ) so that a family of transjunctional currents (Iₖ) could be normalized. Only cell pairs with gₖ values not exceeding 5 µS were used to avoid effects of series access resistance on voltage dependence. For simultaneous electrophysiological and fluorescence recording of HeLa transfectants, cells were grown on coverslips and transferred to an experimental chamber mounted on the stage of a Olympus IX70 inverted microscope. Bath and recording electrode solutions are as previously described. The gₖ was measured using the dual whole-cell voltage clamp. A Boltzmann relation was used to fit experimental data describing gₖ - Vₖ dependence for mCx30.2/Cx45 heterotypic junctions, 

\[ G_j = \frac{(G_{\text{max}}-G_{\text{min}})}{1+\exp[-A(V_j-V_0)]]}+G_{\text{min}}. \]

The G_max is the maximal conductance, G_min is the minimal or residual conductance, A is a measure of voltage sensitivity in mV⁻¹ and V_0 is the V_j at which G_j is halfway between the maximum and minimum values.

Fluorescence imaging and dye transfer studies. Fluorescence signals were recorded and analyzed using a Hamamatsu cooled digital camera mounted on an Olympus IX70 microscope.
and UltraVIEW software (Perkin-Elmer Life Sciences, Boston, MA). Intercellular dye transfer was measured after loading one cell with dye through the patch pipette and monitoring the dynamics of fluorescence intensity in both cells of the cell pair. Typically, one cell of a pair was loaded with dye by establishing a whole-cell recording with a dye-filled pipette and at least 5 cell pairs were examined for each dye. After allowing ~10 min for dye transfer, a whole-cell recording was established in the second cell to measure $g_j$. This delay avoided dye diffusion into the pipette of cell 2. Fluorescent images were acquired throughout at 2 sec intervals with 200 ms exposure times. All examined cell pairs demonstrated full uncoupling during application of heptanol that excluded presence of cytoplasmic bridges. All data are plotted as background-subtracted intensities. To measure the extent of dye transfer from one cell to its neighbors, cells were grown in 35 mm dishes up to 90% of confluence. Dyes were injected into one cell iontophoretically through a microelectrode. Intercellular transfer of Neurobiotin was evaluated 30 min after microinjection.

**Expanded Figures.**

Fig. 1-S. Colocalization of mCx30.2 and Cx45 in the conduction system of the heart in a Cx45<sup>+/LacZ</sup> mouse. (I) Left column (A,D,G) shows bright field images stained for β-galactoside (blue) to reveal regions of Cx45 expression. Middle and right columns show fluorescence images of consecutive sections immunostained for mCx30.2 (green), enlarged from the regions indicated by squares in the left column. (A-C) mCx30.2 and Cx45 are abundantly coexpressed in regions ascribed to the SA node (SAN) and the junction between the superior vena cava and the right atrium (SAJ). (D-F) and (G-I) illustrate colocalization of mCx30.2 and Cx45 in AV node (AVN) with posterior nodal extension (PNE) and the A-V bundle (AVB) with left bundle branch (LBB), respectively. The distance between the parallel sections D (posterior) and G (anterior) is ~1.2
mm. (II) β-galactoside staining (blue; J,L,N,P) and mCx30.2 immunostaining (green; K,M,O,Q) of consecutive sections located in between the sectional planes shown in D and G. Cx45 and mCx30.2 are coexpressed throughout the AV node (J-M) and in the A-V bundle (N-Q). The distances between D and J and J and L are ~150 µM. The distances between L and N, N and P and P and G are ~300 µm. Other abbreviations: IVS, interventricular septum; MV, mitral valve; RA, right atrium; SVC, superior vena cava; TV, tricuspid valve. Scale bars, 20 µm.

Fig. 2-S. Selected examples of images demonstrating cell pairs examined for functional properties of heterotypic junctions. Images were acquired by using monochromatic CCD camera. Pseudocolours (a feature of UltraView imaging software) were used to demonstrate expression of different fusion proteins. (A) Phase-contrast (top) and fluorescence (bottom) images of a HeLaCx30.2-EGFP/HeLaCx40 cell pair from coculture of HeLaCx30.2-EGFP and HeLaCx40 cells. (B) Fluorescence image of HeLaCx43-CFP (green) and HeLaCx30.2-EGFP (red) cell pair forming junctional plaque in apposition between cells. (C) Heterotypic mCx30.2-EGFP/Cx45-EGFP junctions were examined in cocultured HeLaCx30.2-EGFP and N2ACx45-EGFP cells. The absorption/emission maxima for EGFP and CFP are 490/510 and 430/475 nm, respectively. Arrows point to junctional plaques.

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