Functional Characteristics of Four Novel Lone Atrial Fibrillation-Linked Connexin40 Mutants

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Abstract

Atrial fibrillation (AF) is the most common form of cardiac arrhythmia. Recently, four novel heterozygous Cx40 mutations, K107R, L223M, Q236H, and I257L were identified in 4 of 310 unrelated AF patients. To study possible alterations associated with these mutants, we studied their localization and function using gap junction (GJ)-deficient model cells. Cell pairs expressing Q236H alone or together with wildtype Cx43 showed a significantly lower coupling conductance. Impaired GJ function and dominant negative action on Cx43 of this mutant are consistent with previous findings on the majority of AF-linked Cx40 mutants. The remaining three novel AF-linked mutants did not show any apparent defects in our tested GJ or hemichannel assays, which may reflect the limitations of our experimental system.

Keywords

Atrial fibrillation, connexin40, gap junction, patch clamp, Vj-gating
Dedication

I would like to dedicate this to my family, Yara and my friends who have provided me with continuous support and motivation.
Co-Authorship Statement

Localization and electrophysiological data were collected by Mahmoud Noureldin. Honghong Chen generated vectors for all AF-linked Cx40 mutants cDNA and acquired AF-linked Cx40 mutant localization confocal images.
Acknowledgements

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>A</td>
<td>A Boltzmann fitting parameter to describe the slope of the fitted curve</td>
</tr>
<tr>
<td>AF</td>
<td>Atrial fibrillation</td>
</tr>
<tr>
<td>AP</td>
<td>Action potential</td>
</tr>
<tr>
<td>AV</td>
<td>Atrioventricular</td>
</tr>
<tr>
<td>CL</td>
<td>Cytoplasmic loop of a connexin</td>
</tr>
<tr>
<td>CT</td>
<td>Carboxyl terminal of a connexin</td>
</tr>
<tr>
<td>Cx</td>
<td>Connexin</td>
</tr>
<tr>
<td>DCC</td>
<td>Divalent cation containing</td>
</tr>
<tr>
<td>DCF</td>
<td>Divalent cation free</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle’s medium</td>
</tr>
<tr>
<td>E1</td>
<td>The first extracellular domain of a connexin</td>
</tr>
<tr>
<td>E2</td>
<td>The second extracellular domain of a connexin</td>
</tr>
<tr>
<td>ECS</td>
<td>Extracellular solution</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>Gj</td>
<td>Transjunctional conductance</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>$G_{J,ss}$</td>
<td>Normalized steady state junctional conductance</td>
</tr>
<tr>
<td>$G_{\text{min}}$</td>
<td>A Boltzmann fitting parameter to describe normalized voltage-insensitive residual conductance</td>
</tr>
<tr>
<td>GJ</td>
<td>Gap junction</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>$GJA1$</td>
<td>Gap junction alpha 1 gene</td>
</tr>
<tr>
<td>$GJA5$</td>
<td>Gap junction alpha 5 gene</td>
</tr>
<tr>
<td>HeLa</td>
<td>Human cervical cancer cells</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>$I_{j}$</td>
<td>Transjunctional current</td>
</tr>
<tr>
<td>$I_{ss}$</td>
<td>Steady state current</td>
</tr>
<tr>
<td>$I_{\text{peak}}$</td>
<td>Peak current</td>
</tr>
<tr>
<td>ICS</td>
<td>Intracellular solution</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>IRES</td>
<td>Internal ribosomal entry site</td>
</tr>
<tr>
<td>M1</td>
<td>The first transmembrane domain of a connexin</td>
</tr>
<tr>
<td>M4</td>
<td>The fourth transmembrane domain of a connexin</td>
</tr>
</tbody>
</table>
ms Millisecond
mV Millivolts
N2A Mouse neuroblastoma cells
nS Nanosiemens
NT Amino terminal of a connexin
PCR Polymerase chain reaction
PI Propidium Iodide
pIRES Plasmid containing internal ribosomal entry site
SA Sinoatrial
SEM Standard error of the mean
V_j Transjunctional voltage
V_m Transmembrane voltage
V_0 A Boltzmann fitting parameter to describe the voltage at which conductance is reduced by half
VCS The ventricular conducting system
YFP Yellow fluorescent protein
µm Micrometer
Chapter 1– Introduction

1.1 Connexins and gap junctions

Connexins are a family of transmembrane proteins that are expressed in every tissue of the human body. In the human genome, there are 21 identified connexin gene isoforms, each with a unique tissue expression pattern (Söhl & Willecke, 2004). Connexin nomenclature is based on their predicted molecular weight in kDa; for example, Cx40 has a calculated molecular weight of 40 kDa (Söhl & Willecke, 2003). Connexins can be grouped into 5 different subgroups based on their sequence identity: α, β, γ, δ, and ε (Söhl & Willecke, 2004). Generally, all connexins share a similar structural topology with four transmembrane domains (M1-M4), two extracellular domains (E1 and E2), one cytoplasmic loop (CL) and both amino terminal (NT) and carboxyl terminal (CT) located within the cytoplasm (Figure 1.1) (Zimmer et al., 1987). Six connexins oligomerize to form a hexamer structure called a connexon or a hemichannel (Falk et al., 1997). Connexin oligomerization is believed to occur somewhere between the endoplasmic reticulum and the trans Golgi network (Musil & Goodenough, 1993; Sarma et al., 2002). Hemichannels are then transported and inserted into the cell plasma membrane where they can function as channels (Laird, 2006). Two hemichannels on two adjacent cells can dock head-to-head at the E1 and E2 domains to form a gap junction (GJ) channel. All tissues in the human body express at least one type of connexin, allowing for a wide variation in the composition of both hemichannels and GJ channels. A hemichannel formed by identical connexin isoforms is called homomeric; otherwise it is referred to as
heteromeric. If the two docked hemichannels are identical, they form homotypic GJs, while non-identical hemichannels form heterotypic GJs (Figure 1.2) (Saez et al., 2003). GJ channels allow direct cell-to-cell communication with poor selectivity for ions and small molecules up to 1 kDa in size such as Na\(^+\), K\(^+\), Cl\(^-\), Ca\(^{2+}\), cAMP and inositol 1,4,5-triphosphate (IP\(_3\)) (Kumar & Gilula, 1996).

GJ channels are believed to have different functional roles in different tissues. The physiological importance of GJ channels are well reflected by genetic knockout studies in mouse models and numerous human diseases linked to connexin gene mutations. In an avascular organ such as the eye lens, GJs are believed to provide a pathway allowing lens fiber cells to transfer essential nutrients and metabolic wastes, maintaining homeostasis (Goodenough, 1979). Knockouts or mutations in either of the key lens connexin genes, Cx46 or Cx50, can lead to cataracts (Gong et al., 1997; White et al., 1998; Gao et al., 2010). Furthermore, more than a hundred genetic mutations within the coding region of Cx26, which form GJ channels in the inner ear cochlea, were found to be linked to deafness in humans (Lautermann et al., 1999; Thomas et al., 2003, 2004).
Figure 1.1 General structural topology of a connexin

All connexin isoforms generally share similar structural topology with Cx40 (shown here), with four transmembrane domains (M1-M4), two extracellular domains (E1 and E2), one cytoplasmic loop (CL), and both amino (NT) and carboxylic termini (CT) located within the cytoplasm. Adapted from (Bai, 2014).
Six connexins oligomerize to form a hexamer structure called a hemichannel. Two hemichannels on adjacent cells can dock together to form a GJ channel. A hemichannel formed from only one connexin isoform is called a homomeric hemichannel; otherwise it is called a heteromeric hemichannel. A GJ that is formed from two identical homomeric hemichannels is called a homotypic GJ channel; otherwise it is called a heterotypic GJ channel.

Figure 1.2 Structural models of hemichannels and gap junction channels
1.2 GJ regulation and modulation

1.2.1 Chemical regulation

Chemical factors such as an increase in intracellular calcium concentration were found to decrease GJ electrical currents by stabilizing a closed channel conformation (Wrigley et al., 1984; Noma & Tsuboi, 1987; Harris, 2001). Moreover, cell acidification due to an increase in CO₂ has been associated with a decrease in GJ coupling conductance (Wang et al. 1997; Turin & Warner, 1977; Spray et al., 1981). It is believed that the CT might play a role in chemical gating, as the introduction of positive charges in the CT reduced GJ channels’ sensitivity to CO₂ (Wang & Peracchia, 1997). The importance of connexins’ CT and CL domains in pH-gating have been highlighted through truncation studies involving Cx43 CT as well as chimeric experiments between Cx38 and Cx32 CL domains (EkVitorin et al., 1996; Morley et al., 1996; Wang et al., 1996). GJ blockers such as volatile anesthetics (halothane) and octanol were found to affect GJ opening without changing single channel conductance (Veenstra & DeHaan, 1988; Burt & Spray, 1989; He & Burt, 2000).

1.2.2 Voltage regulation

All GJ channels studied so far are sensitive to transjunctional voltage ($V_j$), which is the voltage difference between the cytosol of two GJ-linked cells. $V_j$ can cause the GJ channel to transition from an open state to a much lower conducting or fully closed state, a property known as $V_j$-dependent gating or $V_j$-gating (Verselis et al., 1994). Generally,
most GJ channels are insensitive to low absolute $V_j$ (for example, 10 mV or lower), and more sensitive to higher $V_j$s. Different GJ channels have different $V_j$-gating properties depending on their connexin composition (Bukauskas & Verselis, 2004). Also, since GJ channels are located on the plasma membranes of two neighboring cells, they can also be influenced by the voltage difference between the inside and the outside of each coupled cell, known as the transmembrane voltage ($V_m$) (González et al., 2007). Only a small number of GJ channels, such as those formed by Cx26 or Cx43, are known to be sensitive to both $V_j$ and $V_m$ (Bukauskas & Weingart, 1994). Chimeric and mutational analyses were the first strategies used to determine molecular domains that form $V_j$-sensors and those responsible for the $V_j$-gating mechanism. The importance of NT and M1/E1 domains as $V_j$-sensors were based on studies involving Cx32 and Cx26. Gating polarity of both Cx32 and Cx26 was linked to the second amino acid residue of the NT, where a single mutation at this residue position reversed the gating polarity (Verselis et al. 1994). Also, in Cx26 and Cx32, a charge substitution of M1/E1 border amino-acids reversed gating polarity (Verselis et al. 1994). Thus, it was suggested that in Cx26 and Cx32 a charge complex between multiple domains (NT and M1/E1) could act as voltage sensor (Verselis et al. 1994). Moreover, the first GJ crystallization of Cx26 GJ at 3.5 Å resolution showed that Cx26 NT domain folds into the GJ pore and is involved in channel pore-lining, that could serve a role in $V_j$-gating (Maeda et al. 2009). This supports the model proposed by Maeda et al., where the NT plays a role in $V_j$-sensing (Maeda et al. 2009). Another theory, referred to as “the ball and chain model,” is based mainly on studies of Cx40 and Cx43 GJs, which suggests the importance of the CT for $V_j$-gating (Anumonwo et al., 2001; Moreno et al., 2002). This theory states that under specific
conditions, the CT acts like a ‘ball’ that blocks the channel by binding to specific residues lining the channel pore (Morley et al., 1996).

1.3 Cardiac gap junction channels

The human heart expresses three main connexin isoforms: Cx40, Cx43, and Cx45 (Figure 1.3). All three isoforms are expressed in the ventricular conducting system (VCS) (Davis et al., 1995; Van Kempen et al., 1995). Cx45 is expressed in low levels throughout the heart, but predominantly within the sinoatrial (SA) and atrioventricular (AV) nodes (Vozzi et al., 1999). Cx40 and Cx43 are co-expressed in the atria (Fishman et al., 1990; Kanagaratnam et al., 2002). Lastly, Cx43 is abundantly expressed within the ventricles (Kanter et al., 1994; Vozzi et al., 1999).

In a normal heart, action potential (AP) initiation is controlled by a group of pace-making cells located in the SA node (Gaztanaga et al., 2012). This initial AP rapidly travels throughout atrial cardiomyocytes leading to a synchronized atrial contraction. Then, the AP reaches the AV node, where a short conduction delay of the AP is observed (Severs et al., 2004). This delay is important to ensure a coordinated contraction between the atria and the ventricles. Following the AV node conduction delay, the AP travels through the ventricular conduction system to the ventricles, which causes ventricular contraction and pumps blood through the body’s circulatory system. Fast AP propagation between cardiomyocytes of the atria and ventricles is essential for proper rhythmic contractions in these chambers (Severs et al., 2004). Cardiac GJ channels play an important role in
synchronizing heart beats by providing a low resistance pathway for AP propagation between cardiomyocytes (Kanno & Saffitz, 2001; Gaztanaga et al., 2012).
There are three different connexin isoforms abundantly expressed within the human heart. Cx45 is mainly expressed within the sinoatrial (SA) and the atrioventricular (AV) nodes. Cx40 and Cx43 are co-expressed within the atria, and all three connexins are expressed in the ventricular conducting system (VCS). Also, Cx43 is abundantly expressed in the ventricles. Modified from (Severs et al., 2008).
1.3.1 Connexin40

Human Cx40 is encoded by the gap junction alpha 5 (GJA5) gene, which is located on chromosome number 1 (Willecke et al., 1990; Söhl & Willecke, 2004). Cx40 is widely expressed in human tissue cells such as the atrial cardiomyocyte, the heart’s conductive system, endothelial and smooth muscle cells of the vascular wall, as well as the kidneys (Little et al., 1995; van Kempen & Jongsma, 1999; Vozzi et al., 1999).

The importance of Cx40 in the heart has been investigated in both mice and humans. Past studies have shown that Cx40-deficient mice exhibited slower AP conduction velocity and were more vulnerable to inducible arrhythmias (Kirchhoff et al., 1998; Simon et al., 1998; Hagendorff et al., 1999). However, a more recent study of atrial synthetic culture strands obtained from Cx40 knockout mice showed opposite results; namely, an increase in conduction velocity (Beauchamp et al., 2006). Another study using human atria demonstrated an inverse correlation between Cx40 immunolabeled signal and AP conduction velocity (Kanagaratnam et al., 2002). Different GJA5 promoter polymorphisms were found to affect the levels of Cx40 mRNA and were linked to early onset of lone AF (Firouzi, 2004; Juang et al., 2007). A few human genetic studies have reported a link between somatic and/or germline mutations within the GJA5 gene and a common form of cardiac arrhythmias known as atrial fibrillation (AF) (Figure 1-4.) (Gollob et al., 2006; Yang et al., 2010; Sun et al., 2013). Functional characterization of most AF-linked Cx40 mutations showed impaired Cx40 GJ or hemichannel function (Gollob et al., 2006; Sun et al., 2013, 2014b).
1.3.2 Connexin43

Human Cx43 is encoded by gap junction alpha 1 (GJA1) gene, which is located on chromosome number 6 (Willecke et al., 1990; Söhl & Willecke, 2004). Cx43 is the most ubiquitously expressed connexin isoform in the human body. Cx43 protein is widely expressed in many human cell types such as astrocytes (Vis et al., 1998), skin cells (Guo et al., 1992), granulosa cells (Furger et al., 1996) and throughout the heart (Vozzi et al., 1999).

Mice lacking both copies of the Cx43 gene survived for only five hours after birth and died due to cardiac malformation (Reaume et al., 1995). More than 70 Cx43 mutations were found to be linked to oculodentodigital dysplasia (ODDD), a developmental human disease with a wide range of abnormalities in the teeth, fingers, eyes, and heart (Paznekas et al., 2003, 2009). In addition to ODDD, mutations in the Cx43 gene have been associated with other rare diseases. For example, Cx43R239Q is an autosomal recessive mutation was linked to craniometaphyseal dysplasia disease (Laird, 2014). Recent studies have shown a link between Cx43 mutants and cardiac arrhythmias. For instance, functional characterization of the Cx43 mutation (E42K) revealed GJ channel impairment, which was found to be associated with sudden infant death syndrome, presumably due to ventricular tachycardia (Van Norstrand et al., 2012). Non-familial lone atrial fibrillation was found to be associated with a somatic Cx43 mutation (c.932delC), which exhibited no electrical GJ coupling when expressed in mouse neuroblastoma cells (Thibodeau et al., 2010).
1.4 Atrial fibrillation

Atrial fibrillation (AF) is a heart disease characterized by a fast irregular atrial rhythm different from the normal cardiac rhythm controlled by the SA node (Severs et al., 2008; Tse, 2016). On an electrocardiogram, patients with AF display an absence of P waves and inconsistent R-R intervals. Clinically, AF is considered to be the most common sustained cardiac arrhythmia (Wakili et al., 2011b). Left untreated, the uncoordinated atrial contractions that occur in patients with AF can affect the ability of the heart to fully contract and pump blood to the ventricles. This can result in blood pooling and an increased chance of clot formation and embolic stroke (Rockson & Albers, 2004). In fact, AF accounts for 15% of all stroke cases, with a six-fold increase in stroke risk relative to people with normal sinus rhythm (Wolf et al., 1991).

AF increases in prevalence with age, ranging from 0.1% of the population younger than 55 years to 9% of individuals over 80 years (Go et al., 2001). Currently, an estimated 2.5 million people are affected by AF in the United States, with this number expected to double by 2050 (Go et al., 2001). Traditionally, AF has been considered as a complication to other diseases such as hypertension, valvular diseases, coronary artery disease, and diabetes (Xiao et al., 2011). However, in 30-45% of AF patients, AF is present with no identifiable underlying diseases, and is thus called idiopathic or lone AF (Lévy et al., 1999; Murgatroyd et al., 1999; Fuster et al., 2001; Nattel, 2002). Lone AF has been linked to multiple genetic mutations involving proteins that form ion channels, such as K+ channels, Na+ channels and GJs (Figure 1.4) (Xiao et al., 2011).
Summary of all reported lone atrial fibrillation linked connexin40 (Cx40) mutants. Germline mutations are presented as red circles, while somatic mutations are presented as greens circles. Previously studied mutants are identified in black font. The present study will focus on newly identified mutations depicted in red font. Modified from (Bai, 2014).
1.5 Rationale

Four novel AF-linked missense point mutations in Cx40 (K107R, L223M, Q236H and I257L) were recently identified in 4 of 310 unrelated lone AF patients and absent in 200 unrelated healthy individuals (Figure 1.4 and 1.5) (Shi et al., 2013). The studying of lone AF-linked Cx40 mutations could provide insight into the pathophysiological mechanisms behind AF without any confounding factors that may exist when other cardiovascular diseases are present. This provides us with a strong rationale to study these AF-linked Cx40 mutants.

First, these recently identified mutants have to be biosynthesized, transported to the cell plasma membrane, and form GJ plaques between cells for GJ intercellular communication. If these mutants localize to the cell-to-cell interface to form GJ plaque-like structures, then we can study the functionality of these GJ channels. All of these Cx40 mutants are autosomal dominantly inherited (Shi et al., 2013), which means the mutant carriers have one copy of wildtype Cx40 and one copy of the mutant. Thus, the mutant could interact with wildtype Cx40 in different ways to assemble into GJ channels, including heteromeric and/or heterotypic interactions in the atria. Also, the co-expression of Cx40 and Cx43 within the atria raises the possibility of interaction between wildtype Cx43 and the mutants to form heteromeric mutant:Cx43 GJs or mutant/Cx43 heterotypic GJs. It would be interesting to investigate whether the mutants have any effects when co-expressed with the wildtype Cx43. Several connexin disease-linked mutations showed altered hemichannel function, which could contribute to the mechanism of the disease (Dobrowolski et al., 2007; Mese et al., 2011), including lone AF-linked Cx40 mutants.
(Sun et al., 2014a). Here, we study the hemichannel function of these novel AF-linked Cx40 mutants by investigating their ability to uptake a fluorescent dye, propidium iodide (PI).
Figure 1.5 Pedigree of families with lone atrial fibrillation

Squares represent males and circles represent females. Crossed out symbols represent dead family members. Probands are identified by arrows. Unaffected members are represented by an open symbol, while affected ones are represented by a closed symbol. Symbols with ‘+’ are carriers of the mutation while ‘-’ are non-carriers. Mutations K107R, L223M, Q236H and I257L were identified in families 1, 2, 3 and 4, respectively. Acquired from (Shi et al., 2013).
1.6 Hypothesis

We hypothesize that each one of the novel AF-linked Cx40 mutants will show impairment in their GJ and/or hemichannel function.

1.7 Objectives

1. Studying the AF-linked Cx40 mutants’ ability to form GJ plaques at the cell-to-cell interfaces in HeLa cells using fluorescent protein tagged mutants.

2. Studying the coupling conductance ($G_J$) and $V_J$-gating properties of AF-linked Cx40 mutants in homomeric homotypic GJs in N2A cell pairs using dual whole-cell patch clamp method.

3. Studying the $G_J$ and $V_J$-gating of homomeric heterotypic GJs formed between the AF-linked Cx40 mutants and the wildtype Cx40 in N2A cell pairs.

4. Exploring the effect of co-expressing the AF-linked Cx40 mutants with the wildtype Cx43 on the $G_J$ in N2A cell pairs.

5. Monitoring changes in the mutants’ $G_J$ in response to a simulated action potential protocol in N2A cells using dual whole-cell patch clamp method.

6. Assessing AF-linked Cx40 mutants’ hemichannel function using PI dye uptake assay in HeLa cells.
1.8 References


Laird DW (2014). Syndromic and non-syndromic disease-linked Cx43 mutations. In


Chapter 2 – Manuscript

2.1 Introduction

Atrial fibrillation (AF) is a heart disease that affects millions of people worldwide. It is clinically considered to be the most common sustained cardiac arrhythmia (Wakili et al., 2011b). With an overall prevalence of 0.95%, AF increases with age, starting from 0.1% in individuals younger than 55 years and reaching 9% in those over 80 years old (Go et al., 2001). AF prevalence is expected to triple by 2050, which in turn will increase the economic burden on the health system due to the high cost involved with patient care (Go et al., 2001). AF is characterized by a fast sporadic beating of the atria with the absence of P waves on the electrocardiogram (Wakili et al., 2011b). One of the serious consequences of living with untreated AF is that patients are at higher risk for stroke incidences (Wolf et al., 1991). Often, AF exists as a secondary disease to a wide range of other diseases such as hypertension, diabetes and coronary artery (Saffitz 2006). However, AF is found as the primary disease in 30–45% of AF patients, that are categorized as lone AF (Lévy et al., 1999; Murgatroyd et al., 1999; Fuster et al., 2001; Nattel, 2002). Lone AF has been linked to multiple genetic mutations within the proteins that form ion channels, such as potassium, sodium and gap junction (GJ) channels (Yang et al., 2004a, 2010b, 2010d; Hong et al., 2005; Gollob et al., 2006; Darbar et al., 2008; Lundby et al., 2008; Thibodeau et al., 2010; Sun et al., 2013, 2014b).

GJ channels are formed by a transmembrane protein called connexin (Cx). In humans, there are 21 different connexin isoforms that generally share similar structural topology
with four transmembrane domains (M1-M4), two extracellular domains (E1 and E2), one cytoplasmic loop (CL) and both the amino terminal (NT) and carboxylic terminal (CT) lying within the cytoplasm (Zimmer et al., 1987; Söhl & Willecke, 2004). Six connexins oligomerize to form a hexamer structure known as hemichannel or connexon that can function as a channel by itself (Evans et al., 2006). In addition, two adjacent cells each expressing one hemichannel can dock head to head using the E1 and E2 domains to form a GJ channel (Söhl & Willecke, 2004). The human heart expresses three different type of connexins, Cx40, Cx43, and Cx45, allowing for the formation of homomeric or heteromeric hemichannels and homotypic or heterotypic GJ channels. Cx45 is dominantly expressed in the sinoatrial (SA) and atrioventricular (AV) nodes, while Cx43 and Cx40 are both expressed and co-localized in the atrial myocardium and ventricular conduction system (Vozzi et al., 1999; Severs et al., 2004). In the atria, cardiac GJ channels provide a low resistance pathway for action potential (AP) propagation across cardiomyocytes (Kanno & Saffitz, 2001).

The importance of Cx40 and Cx43 in the heart has been highlighted in animal models and genetic mutation studies. It was found that Cx43 deficient mice died soon after birth due to cardiac malfunctions (Reaume et al., 1995). Another study reported that atrial synthetic strands from Cx43 deficient mice showed a decrease in conduction velocity (Beauchamp et al., 2006). Moreover, an early onset of lone AF was found to be associated with a somatic Cx43 mutant, which exhibited GJ impairment in mammalian cells (Thibodeau et al., 2010). Interestingly, viral expression of the exogenous wildtype Cx43 in the atria was found to prevent AF in pig models (Igarashi et al., 2012). For
Cx40, earlier studies reported that mice lacking the Cx40 gene exhibited a slower AP propagation and were more susceptible to inducible atrial arrhythmias (Kirchhoff et al., 1998; Simon et al., 1998; Hagendorff et al., 1999). On the other hand, recent studies reported different results. For example, Beauchamp et al., (2006) observed that atrial synthetic strands of Cx40 deficient mice showed an increase in the conduction velocity. Furthermore, Cx40 promoter polymorphisms resulted in different levels of Cx40 mRNA and were linked to an early onset of AF (Firouzi et al., 2006). Few somatic and germline mutations within human Cx40 gene were linked to lone AF (Gollo et al., 2006; Yang et al., 2010b, 2010c; Sun et al., 2013).

Here we investigated the functional characteristics of four novel lone AF linked Cx40 mutants, K107R, L223M, Q236H and I257L. These mutants were identified in 4 of 310 unrelated AF patients and absent in 200 unrelated healthy individuals (Shi et al., 2013). Studying lone AF-linked Cx40 mutants can help us understand and learn more about the etiology of AF without any confounding variables that could indirectly facilitate AF. In this study, we will be studying the GJ and hemichannel properties formed by the four AF linked Cx40 mutants. We hypothesize that these AF-linked Cx40 mutants will impair GJ and/or hemichannel function, which may predispose the mutant carriers to AF.
2.2 Methods

2.2.1 Plasmid construction

The C-terminal fusion fluorescent proteins tagged human Cx40-YFP as well as the untagged constructs, Cx40-IRES-GFP, Cx43-IRES-DsRed, Cx40-IRES-DsRed, were created as previously described (Sun et al., 2013; Jassim et al., 2016). Using QuikChange site-directed mutagenesis kit and using the corresponding tagged/untagged Cx40 as templates, the four AF-linked tagged/untagged Cx40 mutants were generated with the following primers:

K107R Forward: 5' CAGGAGAAGCGCAGGCTACGGGAGGCC 3'
Reverse: 5' GGCCTCCCGTAGCCTGCGCTTCTCCTG 3'

L223M Forward: 5' CTCCTCCTTAGCATGGCTGAACTCT 3'
Reverse: 5' AGAGTTCAGCCATGCTAAGGAGCAG 3'

I257L Forward: 5' CCCTCTGTGGCTACAGTXXAGAGCTGC3'
Reverse: 5' GCAGCTCTGGACTAGGCCCACAGAGGG3'

Q236H Forward: 5' GGAAGAAGATGATCAGACACCGATTTGTCAAACC3'
Reverse: 5' GGTGTGACAAATCGGTGTGATCTTCTTTCTCC3'
All of these Cx40 mutant constructs were sequenced to confirm the accuracy of the nucleotide sequence.

2.2.2 Cell culture and transfection

Connexin deficient mouse neuroblastoma (N2A) and human cervical carcinoma (HeLa) cells (American Type Culture Collection, Manassas, VA) were cultured at 37°C with 5% CO₂. Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 1% penicillin, 1% streptomycin, 4.5 g/L D-(-)-glucose, 584 mg/L L-glutamine, and 110 mg/L sodium pyruvate. Twenty-four hours before cell transfection, N2A or HeLa cells were replated onto a 35-mm dish with 60% confluency. Transfection was performed the next day by adding 0.8–1 μg of DNA with 2 μL of the transfection reagent X-tremeGENE HP DNA (Roche Applied Sciences, Indianapolis, IN). To assess the effect of Cx40 mutants on wildtype Cx43, N2A cells were transfected in a 1:1 ratio of Cx40 mutants-IRES-GFP and Cx43-IRES-DsRed. Cell pairs co-expressing both GFP and DsRed were selected for dual whole cell patch clamp.

2.2.3 Localization experiments

HeLa cells were transiently transfected by either Cx40-YFP or one of the four Cx40 mutants-YFP. One day after transfection, cells were replated on 10 mm glass coverslips and incubated overnight. The number of successfully transfected cell pairs forming GJ plaque-like structures at the cell-cell interface were observed and counted. Confocal microscope (Zeiss LSM800 with Airyscan) was used to study localization of fluorescent protein tagged Cx40 mutants as described (Jassim et al., 2016).
2.2.4 Electrophysiology

On the experimental day, transfected N2A cells were replated on to glass coverslips to be incubated for 1.5 to 3 hours. Dual whole-cell patch clamp technique was performed on isolated cell pairs expressing Cx40 mutant with untagged GFP as a reporter. Junctional coupling conductance ($G_j$) and transjunctional voltage dependent gating ($V_j$-gating) were evaluated. Coverslips with cells were transferred into a recording chamber and bathed in extracellular solution (ECS) containing (in mM): 135 NaCl, 5 KCl, 10 Hpes, 1 MgCl$_2$, 2 CaCl$_2$, 1 BaCl$_2$, 2 CsCl$_2$, 2 Na Pyruvate and 5 D-glucose with pH and osmolarity of 7.2-7.4 and 310-320 mOsm, respectively. The recording chamber was placed on an upright fluorescent microscope (BX51WI, Olympus) to visualize GFP cell pairs. Two patch pipettes were filled with intracellular solution (ICS) containing (in mM): 130 CsCl, 10 EGTA, 0.5 CaCl$_2$, 4 Na$_2$ATP and 10 Hpes with pH and osmolarity of 7.2 and 290-300 mOsm, respectively. Initially cell pairs were both voltage clamped at 0 mV, then a 20 mV pulse was applied to one of the cell pairs (pulsing cell) while keeping the other clamped at 0 mV (the recording cell). If functional GJ channels exist between the cell pairs then a transjunctional current ($I_j$) can be measured at the recording cell. $G_j$ was calculated using this equation, $I_j / V_j$. $V_j$-gating properties were studied by applying a series of voltage pulses as described in previous studies (Bai et al., 2006; Sun et al., 2013). The $I_j$ was amplified using MultiClamp 700A (Molecular Devices, Sunnyvale, CA) and then digitized via a Digidata 1322A (Molecular Devices, Sunnyvale, CA).
2.2.5 Dye uptake assay

AF-linked Cx40 mutants hemichannel function were assessed using propidium iodide (PI) uptake assay. HeLa cells were transiently transfected with untagged Cx40 mutants, with GFP as the reporter protein. We used the previously characterized AF-linked Cx40 mutant L221I-IRES-GFP as the positive control and IRES-GFP vector as the negative control for these experiments. Divalent cation containing extracellular solution (DCC-ECS) is composed of (in mM): 142 NaCl, 5.4 KCl, 1.4 MgCl₂, 2 CaCl₂, 10 HEPES and 25 D-(-)-glucose adjusted with pH and osmolarity of 7.35 and ~298 mOsm, respectively. HeLa cells were incubated in a divalent cation free extracellular solution (DCF-ECS) containing PI (150 μM). Removal of both Ca²⁺ and Mg²⁺ ions including the addition of EGTA (2 mM) in the DCF-ECS facilitate the opening of GJ hemichannels. After incubation at 37°C for 15 minutes, cells were washed three times with DCC-ECS at room temperature prior to observation on a fluorescent microscope (DMIRE2, Leica). The number of transfected HeLa cells with or without PI uptake was counted and the percentage of cells with PI uptake was calculated. Only isolated individual HeLa cells were considered in the counting to prevent any errors, as paired cells could be connected intracellularly by GJ channels allowing the movement of PI from one cell to another.

2.2.6 Data analysis

Mann-Whitney U test was used for the following results, cell pair percentages with GJ plaques, coupling percentage, Gj, and PI-uptake percentages. Statistical significance is denoted with (*, P < 0.05 or **, P<0.01). The extent of Vj-gating was analyzed by
normalizing the steady state current to peak current for each current trace, which was used to obtain the normalized steady-state junctional to the peak conductance ($G_{j,ss}$). Then using a two-state Boltzmann equation, the $G_{j,ss}$–$V_j$ plots were fitted for both $V_j$ polarities. Student’s t-test was used to compare the wild type Cx40 and the AF-linked Cx40 mutants Boltzmann fitting parameters; the normalized voltage-insensitive residual conductance ($G_{\text{min}}$), voltage at which the conductance decreases by half ($V_0$) and the slope ($A$). Any other statistical tests used are indicated later.
2.3 Results

2.3.1 The four AF-linked Cx40 mutants formed GJ plaque-like structures at the cell-cell interface

The four AF-linked Cx40 mutants with YFP tagged at the carboxyl terminus were expressed in HeLa cells to study their localization and if morphological GJ plaques could be formed at the cell-cell interface. As shown in Figure 2.1A, the four AF-linked Cx40 mutants were localized in intracellular compartments and displayed GJ plaque-like structures at the cell-cell interfaces similar to that of Cx40-YFP. The percentages of observed GJ plaque-like structure in cell pairs expressing the four AF-linked Cx40 mutants and the wildtype Cx40 are summarized (Figure 2.1B). Our results indicate that cells expressing YFP-tagged K107R, L223M, Q236H and I257L were able to form GJ plaque-like structures at the cell-cell interface similar to that of wildtype Cx40.
Figure 2.1 AF-linked Cx40 mutants localization analysis in HeLa cells

(A) Fluorescent images of HeLa cell pairs expressing YFP-tagged Cx40, K107R, L223M, Q236H and I257L superimposed on their respective DIC images. Cells expressing the 4 AF-linked Cx40 mutants were able to form GJ plaque-like structures at the cell-cell interface similar to that of wildtype Cx40. Putative GJ plaques are highlighted using white arrows. Scale bar = 10 µm. (B) The bar graph summaries the percentage of cell pairs showing GJ plaque-like structures at the cell-cell interface for each of the respective mutants, with no significant difference between any of the mutants and the wildtype Cx40. The number of transfections (each with ~100 cell pairs positively expressing DNA constructs) is presented on each bar.
2.3.2 Electrophysiological studies

2.3.2.1 Homotypic Cx40 Q236H GJs showed a lower $G_j$ than that of Cx40 GJs

Dual whole-cell patch clamp was used to study the functionality of these untagged AF-linked Cx40 mutants. $I_j$ of cell pairs expressing one of the mutants were measured at a $V_j$ of 20 mV (Figure 2.2A). N2A cell pairs successfully expressing K107R, L223M, or I257L showed coupling percentages (68 %, 73 %, or 66 %, respectively) and $G_j$ (20.9 ± 5.2, 17.6 ± 5.2, or 26.9 ± 8.0 nS, respectively), which were not statistically different from those of wildtype Cx40 (Figure 2.2B and C). The coupling probability of Q236H (78%) was not statistically different from that of wildtype Cx40 (79%), however, Q236H $G_j$ (6.9 ± 2.1 nS, $P = 0.023$) was statistically lower than the wildtype Cx40 (12.7 ± 2.5 nS) (Figure 2.2B and C).
Figure 2.2 Coupling percentage and $G_j$ of AF-linked Cx40 mutants

(A) Dual whole-cell patch clamp technique was used to measure the $I_j$ from N2A cells expressing GFP untagged Cx40, K107R, L223M, Q236H or I257L at 20 mV $V_j$ pulse. 

(B) Bar graph summarizes the coupling percentages of cell pairs expressing the AF-linked Cx40 mutants. No statistical difference was observed between each of the mutants and the wildtype Cx40. The number of transfections is indicated on each bar. (C) Bar graph illustrates the coupling conductance ($G_j$) of cell pairs expressing Cx40, Q236H, K107R, L223M, or I257L. Q236H showed a significantly lower $G_j$ than that of wildtype Cx40 (* $P < 0.05$). The number of cell pairs is indicated on each bar.
2.3.2.2 Homotypic Cx40 Q236H GJs showed a slightly altered Vj-gating property

To investigate the AF-linked Cx40 mutants Vj-gating properties, we measured Ij's in cell pairs at different Vj pulses. Cell pairs expressing untagged K107R, L233M, Q236H or I257L showed similar Vj-gating traces to those observed in wildtype Cx40 (Figure 2.3A). The normalized steady state conductance (Gj,ss), of each mutant (filled circles) at different Vjs was plotted and superimposed with that of wildtype Cx40 (open circles) (Figure 2.3B). The smooth black lines and grey dashed lines are the Boltzmann fitting curves for the mutants and wildtype Cx40, respectively (Figure 2.3B). Compared to the wildtype Cx40, GJ channels formed by Q236H showed a significant reduction in the Boltzmann fitting parameter V0 for both Vj polarities (Table 2.1).

To analyze Vj-gating kinetics, we fitted the Ij deactivation by a single exponential curve at high Vjs (60, 80 and 100 mV). As shown in Figure 2.4A, for each of the mutants a single exponential process well fitted the Ij deactivation at these Vjs. We found that as the absolute value of Vj increased the time constant, τ, decreased. At ±100 mV Vj, the τs of the mutant GJs reached 22.7 ± 3.3 ms (K107R), 26.7 ± 6.0 ms (L233M), 17.2 ± 3.3 ms (Q236H), and 31.4 ± 2.7 ms (I257L). None of the mutant τ values showed a statistical difference from that of wildtype Cx40 (32.0 ± 6.4 ms) at this or any of the other tested Vjs. The τ-Vj relationship for each mutant (black filled circles) was plotted and superimposed with that of wildtype Cx40 (grey open circles) (Figure 2.4B).
A

B

$G_{ij,ss}$

$V_i (mV)$

$V_j$

$+100 \text{ mV}$

$-100 \text{ mV}$

$V_i, V_j$

$V_i$

$n = 7$

$n = 5$

$n = 6$

$n = 5$
Figure 2.3 V$_j$-gating of the AF-linked Cx40 mutants

(A) Dual whole-cell patch clamp was used to measure I$_j$s in N2A cell pairs expressing Cx40, K107R, L223M, Q236H, or I257L in response to a series of V$_j$ pulses as indicated.

(B) The normalized steady state conductance, G$_{j,ss}$, of the Cx40 mutants (black filled circles) and the wildtype Cx40 (grey open circles) were plotted at different V$_j$s. Each of the mutant (smooth black lines) data were fitted using a two state Boltzmann equations at each V$_j$ polarity and then superimposed with that of wildtype Cx40 (grey dashed line). The n values indicate the number of cell pairs for each mutant and the wildtype Cx40.
Table 2.1 Boltzmann fitting parameters for the AF-linked Cx40 mutants

Student t-test was used to compare the Boltzmann fitting parameters of each mutant against those of wildtype Cx40 at the corresponding $V_j$ polarity. GJ channels formed by the mutant Q236H had a significantly lower $V_0$ values for both $V_j$ polarities than those of wildtype Cx40 (** $P < 0.01$).

<table>
<thead>
<tr>
<th>Cells expressing</th>
<th>$V_j$ polarity</th>
<th>$G_{\text{min}}$</th>
<th>$V_0$ (mV)</th>
<th>$A$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cx40</strong> (n = 7)</td>
<td>+</td>
<td>0.25 ± 0.02</td>
<td>40.2 ± 1.4</td>
<td>0.15 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.27 ± 0.02</td>
<td>42.92 ± 1.4</td>
<td>0.19 ± 0.07</td>
</tr>
<tr>
<td><strong>K107R</strong> (n = 5)</td>
<td>+</td>
<td>0.21 ± 0.03</td>
<td>38.60 ± 1.6</td>
<td>0.15 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.23 ± 0.03</td>
<td>41.07 ± 1.9</td>
<td>0.15 ± 0.05</td>
</tr>
<tr>
<td><strong>L223M</strong> (n = 5)</td>
<td>+</td>
<td>0.24 ± 0.02</td>
<td>40.2 ± 1.0</td>
<td>0.20 ± 0.10</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.31 ± 0.03</td>
<td>43.24 ± 1.8</td>
<td>0.17 ± 0.06</td>
</tr>
<tr>
<td><strong>Q236H</strong> (n = 6)</td>
<td>+</td>
<td>0.20 ± 0.02</td>
<td>33.33 ± 1.7**</td>
<td>0.19 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.24 ± 0.02</td>
<td>35.23 ± 2.0**</td>
<td>0.15 ± 0.04</td>
</tr>
<tr>
<td><strong>I257L</strong> (n = 5)</td>
<td>+</td>
<td>0.24 ± 0.03</td>
<td>40.94 ± 2.2</td>
<td>0.12 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>0.24 ± 0.03</td>
<td>43.43 ± 2.2</td>
<td>0.17 ± 0.08</td>
</tr>
</tbody>
</table>
A

Cx40

Normalized $I_j$ at 60 mV
Normalized $I_j$ at 80 mV
Normalized $I_j$ at 100 mV

K107R

L223M

Q236H

I257L

0.2 s

B

$\tau$ (ms)

$V_j$ (mV)

$n = 4$
Figure 2.4 AF-linked Cx40 mutant GJs V\textsubscript{j}-gating kinetics

(A) Dual whole-cell patch clamp was used to study V\textsubscript{j}-gating kinetics of GJs formed by Cx40, K107R, L223M, Q236H, or I257L. I\textsubscript{j} deactivates with time at V\textsubscript{j}s (60, 80 and 100 mV). I\textsubscript{j} deactivations were well fitted with a single exponential process (smooth black lines) on different grey data traces. (B) The time constant (τ) values were plotted on a semi logarithmic scale against different V\textsubscript{j}s. As the absolute V\textsubscript{j} values increased, the averaged τs of the mutants GJs (black filled circles) decreased similar to those observed for the wildtype Cx40 (grey open circles). At a specific V\textsubscript{j}, there was no statistical difference (two-way ANOVA test) between any of the mutants and the wildtype Cx40 τs. The n values indicate the number of cell pairs for each mutant and the wildtype Cx40.
2.3.2.3 Co-expression of Cx40 Q236H with Cx43 showed a lower \( G_j \) than the co-expression of wildtype Cx40 and Cx43

In order to investigate if AF-linked Cx40 mutants had a trans-dominant negative effect on wildtype Cx43, each of the mutants (or wildtype Cx40) was co-expressed with Cx43 (with an untagged reporter DsRed). Cell pairs successfully expressing both GFP and DsRed reporters were selected for dual whole-cell patch clamp (Figure 2.5A). Cell pairs successfully co-expressing K107R:Cx43, L223M:Cx43, or I257L:Cx43 showed coupling percentages (62 %, 51 % or 77 %, respectively) and \( G_j \) (33.4 ± 7.4 nS, 21.2 ± 9.5 nS or 27.3 ± 5.4 nS, respectively), which were not statistically different from those of wildtype Cx40:Cx43 (Figure 2.5B and C). The coupling percentage of Q236H:Cx43 (60 %) was not statistically different from that of wildtype Cx40:Cx43 (72 %). However, the averaged \( G_j \) (7.6 ± 2.4 nS) of cell pairs co-expressing Q236H:Cx43 was significantly lower than that of wildtype Cx40:Cx43 (23.0 ± 4.8 nS, \( P = 0.024 \)) (Figure 2.5B and C).
Figure 2.5 Coupling percentage and $G_j$ of cells co-expressing one of the AF-linked Cx40 mutants with the wildtype Cx43

(A) Dual whole-cell patch clamp technique was used to measure the $I_j$ from N2A cell pairs co-expressing Cx40, K107R, L223M, Q236H, or I257L (with an untagged reporter GFP) with wildtype Cx43 (with an untagged reporter DsRed). Representative $I_j$s from cell pairs co-expressing the mutant and Cx43 are illustrated. (B) Bar graph summarizes the coupling percentages of N2A cell pairs expressing each connexin combination. (C) Bar graph illustrates the $G_j$ of cell pairs co-expressing one of the Cx40 mutants (K107R, L223M, Q236H, or I257L) with Cx43. The $G_j$ of cell pairs co-expressing Q236H:Cx43 was significantly lower than that of wildtype Cx40:Cx43 ($P < 0.05$). The number of cell pairs is indicated on each bar.
2.3.2.4 L223M and Q236H formed heterotypic GJ channels with the wildtype Cx40

To evaluate if L223M or Q236H mutants (with untagged reporter GFP) are able to form functional heterotypic GJ channels with wildtype Cx40 (with untagged reporter DsRed), the Iᵢ in cell pairs expressing L223M/Cx40 or Q236H/Cx40 (Figure 2.6A) was measured. N2A cell pairs successfully expressing L223M/Cx40 or Q236H/Cx40 showed coupling percentages (54 % or 83 %, respectively) and Gj (18.1 ± 7.7 nS or 17.9 ± 4.4 nS, respectively), which were not statistically different from those of wildtype Cx40/Cx40 (79 % and 20.5 ± 6.1 nS, respectively) (Figure 2.6B and C).
Figure 2.6 Coupling percentage and \( G_j \) of heterotypic GJ channels L223M/Cx40 or Q236H/Cx40

(A) \( I_j \)s were measured from heterotypic N2A cell pairs expressing L223M (or Q236H with untagged reporter GFP) in one and Cx40 (with untagged reporter DsRed) in the other. (B) Bar graph summarizes the coupling percentages of heterotypic cell pairs with no statistical difference between the L223M/Cx40 (or Q236H/Cx40) and the wildtype Cx40/Cx40. The number of transfections is indicated on each bar. (C) \( G_j \)s of cell pairs expressing L223M/Cx40 or Q236H/Cx40 were not statistically different from that of Cx40/Cx40. The number of cell pairs is indicated on each bar.
2.3.2.5 Dynamic modulation of $G_j$ by simulated junctional potentials and $V_j$-gating recovery time course for homotypic Cx40 and Cx45 GJs.

The kinetics of $I_j$ deactivation ($V_j$-gating) at large $V_j$s could have a time constant of ~32 ms, which could dynamically deactivate some GJ channels. Assuming cardiac AP possesses an instantaneous onset membrane depolarization of ~100 mV, a plateau, a much slower repolarization back to resting membrane potential, and a short junctional delay ($\Delta t$) at the cell-cell junctions (Figure 2.7A). Accordingly, we designed a $V_j$ protocol to simulate voltage differences across the GJs between two cells and then we monitored changes in $G_j$ (Figure 2.7B). When such $V_j$ protocol was applied to homotypic Cx45 or Cx40 GJs at $\Delta t = 10$ ms and 3 Hz, we observed substantial $G_j$ reduction or no reduction, respectively (Figure 2.7C). This protocol was repetitively applied to GJ-coupled cell pairs at 1 Hz (equivalent to a normal heart rate of 60 beats / minute) or 3 Hz (equivalent to tachycardia conditions 180 beats / minute) and $G_j$s were monitored at different junctional delays ($\Delta t = 1, 3, \text{or } 10$ ms) (Figure 2.8). Cell pairs expressing Cx45 GJ showed consistent significant reduction in $G_j$ at $\Delta t = 3$ ms for both 1 and 3 Hz; as well as $\Delta t = 10$ ms for both 1 and 3 Hz in a 50 pulse-protocol. With the exception of $V_j$-protocol $\Delta t = 10$ ms and 1 Hz, no significant $G_j$ reduction was observed for cell pairs expressing Cx40 (Figure 2.8). Similar to the wildtype Cx40, cell pairs expressing the AF-linked Cx40 mutants did not show reduction in $G_j$ when tested with $V_j$-protocol of $\Delta t = 10$ ms and 3 Hz repeating frequency (Figure 2.9).

To investigate the underlying mechanisms responsible for the differences between Cx40 and Cx45 GJs, we studied the recovery time course of Cx40 or Cx45 GJs after $V_j$-gating
(Figure 2.10A). We used a large $V_j$ (100 mV) to deactivate GJ channels, and then following different time interval of no $V_j$ (0 mV), a test $V_j$-pulse (100 mV) was then given to test the $I_j$ peak amplitude (reflecting the functionally recovered GJ channels after deactivation) (Figure 2.10A). We were able to observe a slow recovery time course for Cx45 GJs, however, the recovery time of Cx40 GJs was much shorter and we could not fit the data with a single exponential time course (Figure 2.10). Majority of GJ channels in cell pairs expressing Cx40 recovered (88%) when a very short (0.1 second) interval was provided (Figure 2.10B). A much faster recovery from the deactivated Cx40 GJs may be responsible for the observed non-consistent reduction in the $G_j$ during the repetitive $V_j$-pulses at different $\Delta ts$ and repeating frequencies.
Figure 2.7 Cx45 and Cx40 G_j dynamic modulation by simulated junctional potentials

(A) A large transient V_j, followed by a smaller longer V_j in opposite polarity, could be imposed at the GJ between two cells when a cardiac action potential (AP) propagates from cell 1 to cell 2 with a junctional delay (Δt). (B) V_j changes were simplified as a brief -100 mV V_j pulse, followed by a prolonged V_j of Δt*100/70 mV for 70 ms (which is caused by AP repolarization phase between two cells). We used a test pulse of 10 mV V_j to monitor change in G_j. When a V_j-protocol (Δt = 10 ms at 3 Hz for 50 times) was applied to a cell pair expressing Cx45, we observed a progressive reduction of I_j, (traces of 1, 10, 20, 30, 40, and 50). However, no obvious reduction of I_j was observed when the same V_j protocol was applied to the cell pairs expressing Cx40 (inset). (C) Normalized conductance (G_j/G_j ini) was plotted with time during the 50 V_j-pulses. No change in the normalized conductance was observed in cell pair expressing Cx40 (open circles). However, cell pair expressing Cx45 (filled circles) showed a progressive G_j reduction during 50 V_j-pulses.
Figure 2.8 Cx45 and Cx40 $G_j$s dynamic modulation at different junctional delays and repeating frequencies

$V_j$ protocol as described in Figure 2.7B with different junctional delay ($\Delta t = 1, 3$ or 10 ms) and repeating frequency (1 or 3 Hz) was applied to cell pairs expressing untagged Cx45 (black circles) or Cx40 (open circles) for 50 cycles. Cell pairs expressing Cx45 showed consistent and significant $G_j$ reduction (last 10 points as indicated by gray background color) at $\Delta t = 3$ ms 1 and 3 Hz; 10 ms 1 and 3 Hz. However, no consistent $G_j$ reductions were observed in cell pairs expressing untagged Cx40 in these series of experiments. The number of cell pairs is indicated for each experiment. One sample t-test was used to compare statistical differences between the average of the last 10 points and theoretical value of one.
Figure 2.9 AF-linked Cx40 mutants $G_j$ dynamic modulation at different junctional delays and frequencies

V$_j$ protocol as described in Figure 2.7B was applied to cell pairs expressing AF-linked Cx40. Similar to the wildtype Cx40 (open circles), no consistent reduction in the normalized $G_j$ was observed in cell pairs expressing any of the AF-linked Cx40 mutants (filled circles) The number of cell pairs is indicated for each experiment. Statistical difference of the last 10 points average (grey column) to one was calculated using a one sample t-test.
Figure 2.10 Cx40 and Cx45 $V_J$-gating recovery time course

(A) A large $V_J$-pulse (100 mV) was applied to deactivate GJ channels in cell pairs expressing Cx45 or Cx40. After $V_J$-dependent deactivation the $V_J$ was eliminated for different durations, then a test $V_J$-pulse was applied to measure $I_J$ amplitude (functionally recovered GJs). (B) Average recovery percentage of cell pairs expressing Cx40 (open circles) or Cx45 (filled circles) plotted against the time intervals. The recovery of Cx45 GJ $V_J$-gating could be nicely fitted by a single exponential process; however, Cx40 GJ data could not be fitted using a single exponential process.
2.3.3 Hemichannel function

Propidium iodide (PI) uptake assay was used to investigate the AF-linked Cx40 mutants hemichannel function. Figure 2.1A, shows the fluorescent images of individual Hela cells expressing L221I, Cx40, K107R, L223M, Q236H or I257L under DCF-ECS conditions. The percentage of individual cells showing PI uptake in cells successfully expressing K107R, L223M, Q236H or I257L (17 %, 10 %, 12 % or 21 %, respectively) was not significantly different from either the wildtype Cx40 (15 %) or the negative control (expressing GFP, 9 %). However, the PI uptake percentage of wildtype Cx40, negative control or each Cx40 mutants was statistically lower than the positive control L221I (69 %, P < 0.001) (Figure 2.11B). These results suggest that the Cx40 mutants and the wildtype Cx40 failed to show PI uptake in DCF-ECS conditions.
Figure 2.11 AF-linked Cx40 mutants PI uptake under DCF conditions

(A) Green cells shown in the second column indicate successfully transfected HeLa cells with empty vector (GFP), L221I, Cx40, L223M, Q236H, K107R or I257L. In the third column, PI uptake (red fluorescence) can be seen in cells expressing L221I only. The scale bar = 50 μm. (B) Bar graph summarizes PI uptake percentage of isolated individual cells expressing Cx40 mutants, Cx40, or GFP. PI uptake percentage for each of the AF-linked mutants was not statistically different from that of wildtype Cx40 or the empty vector. The number transfection is indicated on each bar (with observations of 60-180 individual cells per transfection).
2.4 Discussion

In this study, we examined morphological and functional characteristics of four recently identified lone AF-linked Cx40 mutations (K107R, L223M, Q236H and I257L). Our localization experiments showed that YFP-tagged K107R, L223M, Q236H and I257L were able to form GJ plaque-like structures at the cell-cell interface in HeLa cells. Electrophysiological experiments revealed that only homotypic Q236H GJs exhibited a significantly lower G_j when compared to that of wildtype Cx40. Moreover, mutant Q236H exhibited a trans-dominant negative effect when co-expressed with Cx43. All GJs of recently identified AF-linked Cx40 mutants showed similar V_j-dependent gating in tested V_jS as those of Cx40 GJs with an exception of Q236H, which showed a reduced V_0. The recovery time course of V_j-gating of Cx40 GJs appeared to be faster than that of Cx45 GJs. In response to the simulated cardiac AP V_j-protocol of 10 ms and 3 Hz, neither the wildtype Cx40 nor mutants showed any consistent reduction in G_j. Under our experimental conditions, cells expressing any of the four AF-linked Cx40 mutants displayed little PI uptake, which was similar to that of wildtype Cx40.

2.4.1 Defects associated with AF-linked Cx40 mutants

The AF-linked Cx40 mutants investigated in this study were able to form GJ plaque-like structures. However, other AF-linked Cx40 mutants, such as Q49X and P88S, displayed localization impairments which appear to be responsible for the failure of GJ channels formation (Gollob et al., 2006; Sun et al., 2014b). Our results indicated that Cx40 Q236H homomeric homotypic GJ channels showed a significantly reduced G_j, which is
consistent with most of previously characterized lone AF-linked Cx40 mutations with impairments in GJ function. Similar to Q236H, AF-linked Cx40 mutations, such as I75F and A96S, showed no apparent defects in their ability to form GJ plaque-like structures. However, electrophysiological studies showed either Gj elimination (I75F) or significantly reduced Gj (A96S) in cell pairs expressing these mutants (Gollob et al., 2006; Sun et al., 2013). In addition, similar to Q236H, AF-linked Cx40 mutants G38D and M163V have shown an altered V0 parameter with a ~10 mV decrease and ~5 mV increase, respectively (Patel et al., 2014). Interestingly, the AF-linked Cx40 mutant G38D displayed a reduced ability to form GJ plaque-like structures and an overall reduction in the macroscopic conductance (Gollob et al., 2006). However, two studies reported an increase in the single channel conductance (Patel et al., 2014; Cruz et al., 2015).

In the present study, the co-expression of Cx40 Q236H with Cx43 significantly reduced coupling conductance, indicating a trans-dominant negative action, which is similar to what was observed in other lone AF-linked Cx40 mutants such as I75F, P88S, Q49X, and L229M (Gollob et al., 2006; Sun et al., 2013, 2014b). We investigated L223M/Cx40 and Q236H/Cx40 heterotypic GJ channels. Currently, only one other study focused on heterotypic interactions between lone AF-linked Cx40 mutants (I75L and L229M) and the wildtype Cx40 (Sun et al., 2013). Our current results are similar to L229M previous findings, as L229M mutant was able to form functional heterotypic with Cx40. However, I75L/Cx40 GJs displayed asymmetrical Vj-gating properties at 80 mV (Sun et al., 2013).
GJ hemichannel assessment of some disease-linked connexin mutations revealed hemichannel impairments when compared to their wildtype. For instance, Cx43 mutations, linked to oculodentodigital dysplasia (ODDD), showed a gain of hemichannel function (Dobrowolski et al., 2007, 2008). A decrease of hemichannel function was also observed for a few other ODDD-linked Cx43 mutants (Lai et al., 2006). Moreover, a gain of hemichannel function was observed in several Cx26 mutations that were associated with hearing loss as well as skin diseases (Mese et al., 2011; Mhaske et al., 2013). In regards to AF-linked Cx40 mutants, despite exhibiting an apparently normal GJ function, L221I and V85I displayed an increase in hemichannel function via an elevated PI-uptake (Sun et al., 2014a). However, in this study the AF-linked Cx40 mutants did not show any obvious changes in PI-uptake.

2.4.2 AF-linked Cx40 mutants and their possible role in AF pathogenesis

The exact molecular mechanisms underlying how Cx40 mutant GJ channel impairments may cause AF are not fully understood. One study highlighted that human atrial AP conduction velocity is dependent on the relative abundance of Cx40 and Cx43. It was found that AP conduction velocity decreased as the relative quantities of immunolabeled Cx40 increased \([\text{Cx40}/(\text{Cx40}+\text{Cx43})]\) or Cx43 decreased \([\text{Cx43}/(\text{Cx40}+\text{Cx43})]\) (Kanagaratnam et al., 2002). Based on our results, we speculate that a decrease in Q236H \(G_j\) and/or its trans-dominant negative effect on Cx43 \(G_j\) can lead to a decrease in the coupling conductance, which could decrease AP conduction velocity in cells expressing this mutant. This in turn could increase the heterogeneity of AP conduction velocity, promoting AF (Kléber & Rudy, 2004). In addition, a decrease in AP propagation velocity
can act as a functional substrate promoting a re-entry circuit, which could facilitate AF (Wakili et al., 2011a).

The relationship between a gain in Cx40 hemichannel function and lone AF is also not quite clear. However, several possibilities associated with gain of hemichannel function can contribute to AF. Firstly, ATP is known to pass through GJ hemichannel pores, thus a gain of hemichannel function can allow an increase of ATP movement to the extracellular space (Cotrina et al., 1998). In the extracellular space ATP can bind to purinergic receptors, and through a series of signaling pathways the release of Ca$^{2+}$ from the sarcoplasm reticulum may occur (Piazza et al., 2007; Song et al., 2007). Prolonged periods of intracellular Ca$^{2+}$ elevation could lead to cell apoptosis (Kass & Orrenius, 1999), which may act as a structural substrate to promote re-entry leading to AF (Tse, 2016). Secondly, the opening of hemichannels enables the leakage of both Ca$^{2+}$ and Na$^{+}$ ions down their concentration gradients. This in turn could depolarize the cell membrane and trigger premature focal ectopic AP, which can act as the trigger and the driver for circus re-entry and AF (Wakili et al., 2011b; Gaztanaga et al., 2012).

2.4.3 Other AF-linked genetic factors

Genetic factors linking to AF are not just limited to mutations within connexin genes. The first genetic mutation linked to AF was identified in the KCNQ1 gene, which encodes a potassium channel subunit (Chen, 2003). Since then, nine more AF-linked mutations in different potassium channel subunits have been identified (Brugada, 2003; Yang et al., 2004b, 2009, 2010a; Xia et al., 2005). In addition, seven mutations within
three genes that encode different sodium channel subunits were linked to AF (Olson et al., 2005; Ellinor et al., 2008; Makiyama et al., 2008; Watanabe et al., 2009). Furthermore, mutations in a transcription factor (Naiche et al., 2005), Ca²⁺ handling protein (Bhuiyan et al., 2007), nucleoporins (Zhang et al., 2008), and atrial natriuretic peptide (Ren et al., 2010) have been linked to lone AF. In this study, we add the mutant Q236H to the list of AF-linked Cx40 mutations that exhibit functional GJ channel impairment showing an important link between Cx40 mutations and lone AF.

2.4.4 Conclusion

In summary, our results indicate that the AF-linked Cx40 Q236H mutation exhibited GJ function impairment by reducing the overall G_j when expressed alone or with the wildtype Cx43, which might play a role in AF pathogenesis. The three other mutants, K107R, L223M and I257L, did not exhibit any apparent GJ or hemichannel functional impairments in our expression system. One explanation could be that these mutants exhibit impairments in a parameter that we did not test in this study. Although GJ-deficient model cells are an excellent model system for the characterization of connexin mutations, these cells could have some characteristics and signaling pathways that might be different from the native cardiomyocytes, where these mutants would be expressed and show impairments. Another possibility could be that these three mutants are not directly causing AF and other unidentified genetic factors in the patients could be responsible for AF onset.
2.5 References


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Chapter 3–Discussions

3.1 Summary of AF-linked Cx40 mutant

AF-linked Cx40 mutant impairments can be divided into two main categories; GJ impairments and hemichannel impairments. GJ impairments can be further divided into two subcategories: localization impairments and functionality impairments (Figure 3.1). Unlike the AF-linked Cx40 mutants investigated in this study, each of the following Cx40 mutants, G38D, Q49X and P88S, exhibited localization impairments, which appear to be responsible for the failure of GJ formation (Gollob et al., 2006; Sun et al., 2014b) (Figure 3.1). Other AF-linked Cx40 mutants were able to successfully form GJ plaque-like structures (in GJ-deficient model cells), however, these GJs were found to be non-functional. For example, homotypic GJs formed by the mutants I75L and A96S displayed G_j impairments, which is similar to what was observed with Q236H homotypic results (Gollob et al., 2006; Sun et al., 2013) (Figure 3.1). Furthermore, some AF-linked Cx40 mutants showed dominant (I75F and Q49X) and trans-dominant negative effects (I75F, Q49X, L229M) on Cx40 and Cx43, respectively (Sun et al., 2013, 2014b). Q236H falls into the latter group (Figure 3.1). Interestingly, the mutant L229M did not exhibit any GJ impairment when expressed alone or with Cx40, being the first AF-linked Cx40 mutation to have a potent effect on Cx43, but not on Cx40. In addition, AF-linked Cx40 mutants V85I and L221I appeared to have normal GJ channel function, but displayed a gain of hemichannel function under DCF conditions (Figure 3.1) (Sun et al., 2014a).
AF-linked Cx40 mutants can impair GJ or hemichannel function. GJ impairment can be classified as localization impairment or channel function impairment. Some AF-linked Cx40 mutants exhibit dominant or trans-dominant negative effects on Cx40 or Cx43, respectively. Modified from (Sun et al., 2014a).

Figure 3.1 Summary of AF-linked Cx40 mutants

AF-linked Cx40 mutants can impair GJ or hemichannel function. GJ impairment can be classified as localization impairment or channel function impairment. Some AF-linked Cx40 mutants exhibit dominant or trans-dominant negative effects on Cx40 or Cx43, respectively. Modified from (Sun et al., 2014a).
3.2 AF-linked Cx40 mutant interaction with Cx43

The nature of interaction between Cx40 and Cx43 in atrial cardiomyocytes remains unclear, as there are conflicting results regarding the ability of Cx40 and Cx43 to form functional heterotypic GJ channels (Bruzzone et al., 1993; Valiunas et al., 2000; Lin et al., 2014). In rodent aortic smooth muscle cells, Cx43 antibodies immunoprecipitated both Cx40 and Cx43. Single channel analysis in A7r5 cells co-expressing Cx40 and Cx43 revealed different levels of unitary channel conductance that were not seen in either Cx40 or Cx43 homotypic GJs. Both of these functional and co-immunoprecipitation studies suggest the possible formation of heteromeric GJ channels between both connexins (He et al., 1999). Each one of the four recently identified AF-linked Cx40 mutants are inherited in an autosomal heterozygous manner, meaning that the mutant carriers have one copy of the mutant gene and one copy of the wildtype gene (Shi et al., 2013). Furthermore, Cx40 and Cx43 are co-expressed together in the atria. Therefore, there is a possibility that the mutant form heteromeric GJ channels with wildtype Cx43. In this study, only cell pairs co-expressing Q236H:Cx43 had a significantly lower Gj compared to cell pairs co-expressing the wildtype Cx40:Cx43. As mentioned before, our Q236H results are consistent with the majority of lone AF-linked Cx40 mutants, such as I75F, P88S, Q49X, or L229M (Gollob et al., 2006; Sun et al., 2013, 2014b). The effect of Q49X on the wildtype Cx43 reveals one possible mechanism through which AF-linked Cx40 mutations can have a dominant or trans-dominant negative effect on wildtype Cx40 or Cx43, respectively. Q49X localization studies showed that unlike wildtype Cx40, the mutant Q49X was retained within the endoplasmic reticulum. One of the proposed
mechanisms responsible for the dominant and trans-dominant negative properties of this mutant might be its ability to bind and sequester Cx40 and Cx43 within the endoplasmic reticulum; thus preventing them from being transported to the plasma cell membrane and forming GJs (Sun et al., 2014b).

3.3 AF-linked Cx40 mutant simulated cardiac AP $V_j$-protocol and the role of cardiac connexins in AP propagation

To test these AF-linked Cx40 mutants under more physiologically relevant conditions, we designed a simplified $V_j$-protocol (repeated 50 times) simulating the voltage difference across GJs as APs propagate from one cardiomyocyte to another. We were interested in observing whether these Cx40 mutants displayed different GJ properties (specifically $G_j$) compared to wildtype Cx40. No $G_j$ reduction was observed in cell pairs expressing either the wildtype Cx40 or any of the four AF-linked Cx40 mutants in response to the simulated cardiac AP $V_j$-protocol. However, as previously observed (Ye, Yue, Bai unpublished observations), cell pairs expressing Cx45 exhibited a junctional delay ($\Delta t$) and frequency-dependent $G_j$ reduction starting from $\Delta t = 3$ ms and 1 Hz. Interestingly, our recovery time course results revealed a difference in Cx45 and Cx40 properties, as Cx40 GJs recovered faster than Cx45 GJs. If the recovery time course of Cx40 is shorter than the difference between any two subsequent pulses of the 50 pulse $V_j$-protocol (1 s or 0.33 s in 1 Hz or 3 Hz, respectively), then we do not expect to see any $G_j$ reduction, since our results revealed that it takes only 0.1 s for 88% of Cx40 GJs to recover after deactivation.
3.4 K⁺ and Na⁺ channels link to AF

Other ion channels, such as K⁺ and Na⁺ channels, are necessary for the generation of cardiac APs and have also been associated with lone AF. K⁺ channels are responsible for outward currents and the regulation of the AP cardiac repolarization phase. KCNH2 gene encodes the α-subunit (Kv1.1) of the K⁺ channel I_Kr, which is responsible for the rapid component of the delayed rectifier outward current. KCNH2 missense mutation N588K was identified in patients with hereditary short-QT syndrome and AF (Brugada et al., 2004). Functional characteristics of N588K revealed a gain of function, with an increase in the repolarization currents during the early phase of the AP, which resulted in AP abbreviation. Another gene linked to lone AF is KCNA5, which encodes the α-subunit (Kv1.5) of the ultra-rapid delayed rectifier potassium channel (I_Kur). Three KCNA5 mutations, T527M, A567V, and E610K, were identified in four unrelated AF patients (Yang et al., 2009). Functional analysis of these mutants revealed a loss of function by decreasing the ultra-rapid activating delayed rectifier currents (Yang et al., 2009). This is believed to prolong AP duration, therefore increasing the tendency for early after-depolarization and AF (Olson et al., 2006).

I_{Na} is responsible for inward currents and the regulation of the AP depolarization phase. The missense mutation M1875T in the SCNA5 gene that encodes the α-subunit of I_{Na} channel was associated with familial AF (Makiyama et al., 2008). Functional analysis of M1875T revealed a gain of function by increasing the inward Na⁺ current, which may lead to early after-depolarization and AF (Makiyama et al., 2008). Another SCNA5 mutation, N1986K, has been identified in a family with lone AF (Ellinor et al., 2008).
Electrophysiological studies of N1986K revealed a hyperpolarization shift in the channel steady-state inactivation, which was expected to result in AF through the loss of \( I_{Na} \) function (Ellinor et al., 2008).

### 3.5 Limitations and future studies

To investigate the AF-linked Cx40 mutant effects on Cx43 we co-transfected the mutants with the wildtype Cx43 in a 1:1 ratio. Although cell pairs with apparent similar expression of the reporter genes were chosen for patch clamping, this did not guarantee that transfected cells received an equal ratio of both DNA vectors, or if the received ratio was consistent between the tested cell pairs. For future studies, a bi-directional vector containing both connexin sequences sub-cloned within it would theoretically ensure that each of the transfected cells receive a 1:1 ratio of the mutant and wildtype Cx43 DNA.

When AF-linked Cx40 mutants, such as Q49X, I75F, P88S, and A96S, were co-expressed with wildtype Cx40, they showed a dominant negative effect and decreased the overall GJ \( G_j \) (Gollob et al., 2006; Sun et al., 2013, 2014b). The three AF-linked Cx40 mutants that did not exhibit any apparent impairments may have a dominant negative effect when co-expressed with the wildtype Cx40. Due to the low expression efficiency of our DsRed untagged Cx40, we were unable to test the effect of co-expressing the newly identified AF-linked Cx40 mutants with the wildtype Cx40. Our RFP-tagged Cx40 construct displays higher expression efficiency compared to the DsRed untagged Cx40 construct. In a future study, RFP-tagged Cx40 could be co-expressed with YFP-tagged
AF-linked Cx40 mutants to investigate if any of these four mutants exhibit a dominant negative effect on the wildtype Cx40.

Similar to the mutants K107R, L223M, and I257L, the AF-linked Cx40 mutant M163V was able to form GJ plaque-like structures and displayed comparable G_j to that of wildtype Cx40, suggesting that it might be a benign variant (Gollo et al., 2006). Cruz et al. (2015), reported similar electrophysiology results, however, their dye flux studies showed an increase in permeability for both anionic (lucifer yellow) and cationic (ethidium bromide) probes (Cruz et al., 2015). They suggested this enhanced cationic permeability could increase Ca^{2+} transfer between cells resulting in an increase in Ca^{2+} intracellular concentration through calcium induced calcium release, which could promote arrhythmias. It would be interesting to investigate if any of the four AF-linked Cx40 mutants exhibit different ionic or metabolic permeability (Cruz et al., 2015).

Three of the four AF-linked Cx40 mutants did not exhibit any apparent defects in our GJ-deficient cell model. One possible future study could investigate the localization and GJ properties of Cx40 mutants K107R, L223M, and I257L in a more physiologically relevant cell line such as mouse neonatal cardiomyocyte cells. Furthermore, the characteristics of these AF-linked Cx40 mutants in animals such as mice could be tested. Knockout and knock-in genetic engineering techniques could be used to replace the wildtype Cx40 gene with one of these AF-linked Cx40 mutants. Mice carrying the AF-linked Cx40 mutants could be characterized by immunochemical and electrophysiological analysis.
3.6 References


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