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References

1. Watanabe H, Nogami A, Ohkubo K, Kawata H, Hayashi Y, Ishikawa T, Makiyama T, Nagao S, Yagihara N, Takehara N, Kawamura Y, Sato A, Okamura K, Hosaka Y, Sato M, Fukae S, Chinushi M, Oda H, Okabe M, Kimura A, Maemura K, Watanabe I, Kamakura S, Horie M, Aizawa Y, Shimizu W, Makita N. Electrocardiographic characteristics and SCN5A mutations in idiopathic ventricular fibrillation associated with early repolarization. *Circ Arrhythm Electrophysiol.* 2011;4:874–881.
2. Richter S, Sarkozy A, Paparella G, Henkens S, Boussy T, Chierchia GB, Brugada R, Brugada J, Brugada P. Number of electrocardiogram leads displaying the diagnostic coved-type pattern in Brugada syndrome: a diagnostic consensus criterion to be revised. *Eur Heart J.* 2010;31:1357–1364.
3. Wilde AA, Antzelevitch C, Borggrefe M, Brugada J, Brugada R, Brugada P, Corrado D, Hauer RN, Kass RS, Nademanee K, Priori SG, Towbin JA. Proposed diagnostic criteria for the Brugada syndrome: consensus report. *Circulation.* 2002;106:2514–2519.
4. Hong K, Brugada J, Oliva A, Berruezo-Sanchez A, Potenza D, Pollevick GD, Guerchicoff A, Matsuo K, Burashnikov E, Dumaine R, Towbin JA, Nesterenko V, Brugada P, Antzelevitch C, Brugada R. Value of electrocardiographic parameters and ajmaline test in the diagnosis of Brugada syndrome caused by SCN5A mutations. *Circulation.* 2004;110:3023–3027.
5. Kawata H, Noda T, Yamada Y, Okamura H, Satomi K, Aiba T, Takaki H, Aihara N, Isobe M, Kamakura S, Shimizu W. Effect of sodium-channel blockade on early repolarization in inferior/lateral leads in patients with idiopathic ventricular fibrillation and Brugada syndrome. *Heart Rhythm.* 2012;9:77–83.
6. Hong K, Berruezo-Sanchez A, Poungvarin N, Oliva A, Vatta M, Brugada J, Brugada P, Towbin JA, Dumaine R, Pinero-Galvez C, Antzelevitch C, Brugada R. Phenotypic characterization of a large European family with Brugada syndrome displaying a sudden unexpected death syndrome mutation in SCN5A. *J Cardiovasc Electrophysiol.* 2004;15:64–69.
7. Poelzing S, Forleo C, Samodell M, Dudash L, Sorrentino S, Anaclerio M, Troccoli R, Iacoviello M, Romito R, Guida P, Chahine M, Pitzalis M, Deschenes I. SCN5A polymorphism restores trafficking of a Brugada syndrome mutation on a separate gene. *Circulation.* 2006;114:368–376.
8. Watanabe H, Yang T, Stroud DM, Lowe JS, Harris L, Atack TC, Wang DW, Hipkens SB, Leake B, Hall L, Kupershmidt S, Chopra N, Magnuson MA, Tanabe N, Knollmann BC, George AL Jr, Roden DM. Striking in vivo phenotype of a disease-associated human SCN5A mutation producing minimal changes in vitro. *Circulation.* 2011;124:1001–1011.
9. Medeiros-Domingo A, Tan BH, Crotti L, Tester DJ, Eckhardt L, Cuoretti A, Kroboth SL, Song C, Zhou Q, Kopp D, Schwartz PJ, Makielski JC, Ackerman MJ. Gain-of-function mutation, S422L, in the KCNJ8-encoded cardiac K ATP channel Kir6.1 as a pathogenic substrate for J wave syndromes. *Heart Rhythm.* 2010;7:1466–1471.
10. Antzelevitch C, Pollevick GD, Cordeiro JM, Casis O, Sanguinetti MC, Aizawa Y, Guerchicoff A, Pfeiffer R, Oliva A, Wollnik B, Gelber P, Bonaros EP Jr, Burashnikov E, Wu Y, Sargent JD, Schickel S, Oberheiden R, Bhatia A, Hsu LF, Haissaguerre M, Schimpf R, Borggrefe M, Wolpert C. Loss-of-function mutations in the cardiac calcium channel underlie a new clinical entity characterized by ST-segment elevation, short QT intervals, and sudden cardiac death. *Circulation.* 2007;15:442–449.
11. Burashnikov E, Pfeiffer R, Barajas-Martinez H, Delpon E, Hu D, Desai M, Borggrefe M, Haissaguerre M, Kanter R, Pollevick GD, Guerchicoff A, Laino R, Marieb M, Nademanee K, Nam GB, Robles R, Schimpf R, Stapleton DH, Viskin S, Winters S, Wolpert C, Zimmern S, Veltmann C, Antzelevitch C. Mutations in the cardiac L-type calcium channel associated with inherited J-wave syndromes and sudden cardiac death. *Heart Rhythm.* 2010;7:1872–1882.
12. Nam GB, Ko KH, Kim J, Park KM, Rhee KS, Choi KJ, Kim YH, Antzelevitch C. Mode of onset of ventricular fibrillation in patients with early repolarization pattern vs Brugada syndrome. *Eur Heart J.* 2009;31:330–339.
13. Kamakura S, Ohe T, Nakazawa K, Aizawa Y, Shimizu A, Horie M, Ogawa S, Okumura K, Tsuchihashi K, Sugi K, Makita N, Hagiwara N, Inoue H, Atarashi H, Aihara N, Shimizu W, Kurita T, Suyama K, Noda T, Satomi K, Okamura H, Tomoike H. Long-term prognosis of probands with Brugada-pattern ST elevation in V1–V3 leads. *Circ Arrhythmia Electrophysiol.* 2009;2:495–503.
14. Antzelevitch C, Yan GX. J wave syndromes. *Heart Rhythm.* 2010;7:549–558.

Cardiac connexins, mutations and arrhythmias

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Purpose of review
Connexins are the pore forming subunits of gap junction channels. They are essential for cardiac action potential propagation. Connexins are modified at the transcriptional or posttranslational levels under pathological states such as cardiac hypertrophy or ischemia, thus contributing to the arrhythmogenic substrate. However, the relation between nucleotide substitutions in the connexin gene and the occurrence of cardiac arrhythmias remains largely unexplored.

Recent findings
Recent studies have reported an association between nucleotide substitutions in the connexin40 (Cx40) and connexin43 (Cx43) genes (*GJA5* and *GJA1*, respectively) and cardiac arrhythmias. Of note, however, germline mutations in Cx43 are considered causative of oculodentodigital dysplasia, a pleiotropic syndrome wherein cardiac manifestations are notoriously absent.

Summary
Here, we review some of the current knowledge on the association between cardiac connexins and inherited arrhythmias.

Keywords
arrhythmogenic cardiomyopathy, arrhythmogenic right ventricular cardiomyopathy, connexin40 and connexin43, oculodentodigital dysplasia, progressive familial heart block, sudden infant death syndrome

INTRODUCTION

It is now 60 years since Silvio Weidman's classic study on 'the electrical constants of Purkinje fibers,' in which he beautifully demonstrated that electrotonic propagation in cardiac tissue extended well beyond the size of a single cell [1]. His observations provided the physiological evidence that cardiac cells are electrically coupled via low-resistive pathways. Electron microscopic observations followed, culminating with the elegant work of Revel and Karnovsky [2] showing that, at the site of close appositional membranes in the cardiac intercalated disc, the membranes were not fused but, instead, were separated by a gap with junctions that traversed it, thus leading Revel to later coin the term 'gap junctions.' Our understanding of the molecular nature of these structures, and their function in the normal heart, has expanded enormously since those (and many other) 'giants' of science first paved the way. Yet, it is fair to admit that the role of connexins and of gap junctions in cardiac arrhythmias can still be labeled, at least in some aspects, as 'controversial.' In fact, it is very likely that the role of connexins in arrhythmias is not only limited to their ability to form gap junctions (see, e.g. [3▪▪,4]). In the present review, we focus on the association between cardiac connexins (primarily Cx43 and Cx40) and inherited diseases. We begin by describing what may be the most noticeable paradox: that patients with the only inherited disease clearly ascribed to mutations in the gene coding for connexin43 (Cx43) actually do not present with cardiac arrhythmias. We will then summarize current knowledge on the association between variants/mutations in connexin genes and arrhythmias, and will conclude with the possible implication of connexins in the phenotype of an inherited arrhythmogenic disease (arrhythmogenic cardiomyopathy) wherein loss of gap junction plaques is secondary to disruption of a closely associated protein complex, namely the cardiac desmosome.

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KEY POINTS

- Atrium-specific genetic modifications in connexin40 (Cx40) or in connexin43 (Cx43) can be a substrate for atrial fibrillation, in part by a heterogeneous decrease in myocardial electrical coupling.
- A germline mutation in *GJA5*, the gene coding for Cx40, has been reported as a likely genetic cause of progressive familial heart block type I.
- Cx43 amino acid substitutions have been associated with two isolated cases of sudden infant death syndrome, yet
- Cx43 germline mutations are known as a cause of oculodentodigital dysplasia, a pleiotropic syndrome wherein cardiac manifestations are notoriously absent.
- Cx43 deficiencies secondary to desmosomal mutations are a likely component of the arrhythmia substrate in arrhythmogenic cardiomyopathy.
- Cx43 deficiencies affect not only electrical coupling but also the function of the sodium channel. The relation between Cx43 and the sodium channel is an exciting area that deserves further investigation.

Connexin43 germline mutations and oculodentodigital dysplasia

In 2003, Paznekas *et al.* [5] reported that Cx43 mutations cause the pleiotropic phenotype of oculodentodigital dysplasia (ODDD), an autosomal dominant syndrome that presents with craniofacial (ocular, nasal, dental) and limb dysmorphisms, as well as neurologic manifestations such as spastic paraplegia and neurodegeneration. In their study, the authors reported mutations in the *GJA1* gene, coding for the gap junction protein Cx43, in all 17 families studied. In a later review, the same group compiled a summary of all 62 known *GJA1* substitutions leading to mutations in Cx43 and the ODDD phenotype [6]. From the perspective of the present article, perhaps the most striking feature of Cx43-related ODDD is the absence of a cardiac arrhythmia phenotype in the patients. In fact, the only electrocardiological phenotype reported (a first-degree arrhythmogenic cardiomyopathy block) was later found in other family members not carrying the Cx43 mutation (see supplemental information in [6]). In contrast, in-vitro studies have reported important changes in gap junction channel function consequent to the mutations (see, e.g., [7–11]), and changes in electrical properties and arrhythmias have been reported in murine models of the disease [12,13]. The reason(s) for the lack of a cardiac electrical phenotype in ODDD patients remain unclear. A compensatory increase in other cardiac connexins (Cx40 and Cx45) cannot be discarded. Of note, these results contrast with a recent report ascribing disease causality to a Cx43 amino acid substitution observed in an isolated case of sudden infant death syndrome (see [14▪▪] and also section below).

Somatic mutations of connexin40 and connexin43 and atrial fibrillation

Atrial fibrillation is the most common sustained cardiac arrhythmia, may cause significant morbidity, and is a common cause of stroke [15]. Its prevalence increases with advanced age to about 6% in individuals older than 65 years. The pathogenesis of atrial fibrillation is complex, attributed to dynamic interactions among a wide range of structural, electrophysiological, inflammatory and genetic factors. Most patients with atrial fibrillation have associated cardiovascular diseases such as valve diseases or hypertension; atrial fibrillation can be concurrent with structural alterations of the atrium. However, some atrial fibrillation patients do not have structural alterations or identifiable underlying diseases, constituting a distinct atrial fibrillation subgroup, often referred to as idiopathic or lone atrial fibrillation. Nearly 30% of patients with atrial fibrillation (with or without structural heart disease) have a family history of the disease [16]. These findings suggest that genetic factors may determine whether atrial fibrillation develops or becomes sustained, even in patients with anatomical substrates. Recent genetic studies on familial atrial fibrillation have demonstrated mutations in the genes encoding cardiac ion channels [17–21]. Most of the mutations would be expected to shorten the duration of atrial action potentials and the effective refractory period, thereby predisposing affected individuals to reentry. In addition to cardiac ion channels, gap junctions are the principal determinant of action potential propagation and conduction velocity; atrial tissue-specific mutations in genes coding for connexins have been considered a potential substrate in some cases of idiopathic atrial fibrillation. Indeed, in an initial study, Gollob *et al.* [22] identified four missense mutations in *GJA5*, the gene encoding the gap junction protein Cx40, in the genomic DNA extracted from atrial specimens. Heterologous expression assays showed that mutation P88S in the gene coding for Cx40 prevented the formation of functional Cx40-mediated gap junction channels, and the expressed protein failed to reach the cell surface and assemble gap junction plaques. Interestingly, the atrial tissue of the P88S carrier showed a mosaic pattern comprising both areas of normal expression and others with

reduced expression and intracellular accumulation of the Cx40 protein. A separate patient exhibited two nonallelic mutations, G38D and M163V. These mutations, together with P88S, were found in DNA from atrial tissue but not from lymphocytes, suggesting a somatic source. When expressed in gap junction deficient neuroblastoma cell line N2A, the G38D mutant generated only sparse gap junction plaques, whereas most of the protein remained in the intracellular space. Junctional conductance in cells expressing G38D was significantly lower than wild type. Mutant M163V formed gap junctions with nearly normal distribution and electrical conductance, suggesting that this is most likely a benign polymorphism. In contrast, A96S was a germline mutation and resulted in apparently normal junctions, but their electrical coupling was markedly reduced as compared with wild type. Furthermore, when A96S-Cx40 was coexpressed with wild-type Cx40, junctional conductance was significantly less than that observed when only the wild-type protein was expressed. The results were not different if wild-type Cx43 was also expressed. This observation suggested that mutation A96S oligomerizes with the wild-type protein and acts as a dominant-negative unit. Overall, the data suggested that somatic Cx40 mutations can predispose patients to idiopathic atrial fibrillation, likely by impairing gap junction assembly and/or electrical coupling.

In subsequent studies, Thibodeau *et al.* identified a novel single nucleotide deletion (c.932delC) of *GJA1*, the gene coding for Cx43, and studied it in the atrial tissue of a patient with idiopathic atrial fibrillation [23▪▪]. This mutation resulted in frameshift with 36 aberrant amino acids followed by a premature stop codon, leading to truncation of the C-terminal domain of Cx43. The mutation was absent from the lymphocyte DNA of the patient, indicating genetic mosaicism. Protein trafficking studies demonstrated intracellular retention of the mutant protein and a dominant-negative effect on gap junction formation of both wild-type Cx43 and Cx40. Electrophysiological studies revealed no electrical coupling of cells expressing the mutant protein alone and significant reductions in coupling when coexpressed with wild-type connexins. Atrial tissue of the affected patient showed a mosaic pattern of Cx43 immunostaining, with normal appearing intercalated disks and significant intracellular staining of Cx43 in adjacent cells. These data lead to the hypothesis that atrium-specific genetic modifications in Cx43 can be a substrate for atrial fibrillation, in part by a heterogeneous decrease in myocardial electrical coupling. Of interest, whether these Cx40 or Cx43 mutations can affect the function of other ion channels remains to be determined [3▪▪,24▪▪,25,26].

Connexin43 amino acid substitutions associated with two isolated cases of sudden infant death syndrome

Sudden infant death syndrome (SIDS) is defined as the sudden and otherwise unexplained loss of life of a child under the age of one. For the diagnosis of SIDS, the cause of death must remain unexplained after a thorough case investigation including medical autopsy, death scene investigation and detailed review of the clinical history. Although SIDS remains poorly understood and its cause is largely unknown, recent clinical and molecular evidence has implicated heritable arrhythmia syndromes due to mutations in cardiac ion channels as a cause of up to 10–15% of SIDS [27,28]. Studies in murine models have shown that complete loss of Cx43 expression leads to arrhythmias and sudden death. In a recent study, Van Norstrand *et al.* [14▪▪] reported two novel missense mutations in *GJA1* (amino acid substitutions E42K and S272P) in a group of 292 cases of SIDS. Analysis of the E42K victim's parental DNA demonstrated a de-novo mutation. Immunofluorescence demonstrated no trafficking abnormalities for either mutation and S272P demonstrated normal junctional conductance. However, junctional conductance measurements for the E42K mutation showed a loss of function that was not rescued by coexpression of the wild-type construct. Moreover, cardiac tissue obtained from the patient with the E42K mutation demonstrated a mosaic immunostaining pattern for Cx43 protein. These results open the possibility that mutations in *GJA1*, the gene coding for Cx43, may be associated with at least some cases of SIDS. It is interesting to note, however, that there are a number of reported cases of Cx43 mutations in patients afflicted with ODDD [6]. A number of those mutations are predicted to significantly affect the function of Cx43 channels [5]. Yet, cardiac arrhythmias and/or SIDS have not been reported to occur in the afflicted families. Whether the mutations reported by Van Norstrand *et al.* [14▪▪] were indeed causative of disease is an interesting question that deserves further investigation.

Connexin40 germline mutation and progressive familial heart block type-1

Although multiple mutations in genes coding for components of the voltage-gated channel complexes have been previously described in relation to arrhythmias and sudden death in the young [29], and connexin mutations have been implicated in atrial fibrillation [22,30] and in two isolated cases of SIDS [14▪▪], no study has identified an association between germline, cosegregated, inherited

mutations in gap junction proteins and inherited ventricular arrhythmias in humans.

Progressive familial heart block type I (PFHBI), also known as progressive cardiac conduction defect (PCCD) or Lenegre–Lev disease [31,32] is a dominant inherited disorder of the His–Purkinje system. Affected individuals show electrocardiographic evidence of bundle branch disease, that is, right bundle branch block, left anterior or posterior hemiblock, or complete heart block, with broad QRS complexes. The disease can progress from a normal electrocardiogram to right bundle branch block and from the latter to complete heart block. Affected individuals often present with family history of syncope, pacemaker implantation and/or sudden death [33]. Although structural abnormalities have been invoked as cause of the disease [31,32], a number of cases present with normal cardiac structure and contractile function. Mutations have been found in genes that influence cardiac excitability, such as cardiac Na channels (*SCN5A* [33] and *SCN1B* [34]) and transient receptor potential nonselective cation channel, subfamily M (*TRPM4* [35]). Recently, Makita *et al.* [36▪▪] reported that a germline mutation in *GJA5*, the gene coding for Cx40, also associated with PFHBI. Indeed, the authors screened 156 probands with diagnosis of PFHBI. In addition to 12 sodium channel mutations (*SCN5A* and *SCN1B*), they found a germline *GJA5* (Cx40) mutation (Q58L) in an afflicted family [36▪▪]. The disease had an early onset and was associated with otherwise unexplained cardiac sudden death in the proband and the proband's mother. The proband's sister is also affected. Heterologous expression of Cx40-Q58L in connexin-deficient N2A cells resulted in marked reduction of junctional conductance and diffuse localization of immunoreactive proteins in the vicinity of the plasma membrane without formation of gap junctions. Heteromeric co-transfection of Cx40-atrial fibrillation and Cx40-Q58L resulted in homogenous distribution of proteins in the plasma membrane rather than in membrane plaques in about 50% of cells; well-defined gap junctions were observed in other cells. Junctional conductance values correlated with the distribution of gap junction plaques. Mutation Cx40-Q58L impaired gap junction formation at cell–cell interfaces. Coexpression experiments indicated that Cx40-atrial fibrillation protein provided only partial rescue of the Cx40-Q58L cellular phenotype. This study represented the first demonstration of an association between germline, cosegregated (albeit in a small number of family members), inherited mutations in gap junction proteins and inherited ventricular arrhythmias in humans. The data also emphasize the importance of Cx40 in normal propagation in the specialized conduction system.

For both the relation between Cx43 and SIDS and that of Cx40 and PFHBI the challenge remains the limited (and admittedly weak) genetic evidence for the causal role of connexins in cardiac rhythm/conduction disorders. Functional studies in experimental models, though important, have a limited power to distinguish between functional polymorphisms and causal variants, even though the functional variants might contribute to the phenotype. Further research, exploring family-based associations between nucleotide substitutions in connexin genes and these or other disorders, will help clarify the role of connexin mutations as causative of arrhythmia disease.

Connexin43 deficiency secondary to mutations in desmosomal proteins: the case of arrhythmogenic cardiomyopathy

Arrhythmogenic cardiomyopathy (a term preferred to the more conventional one of 'arrhythmogenic right ventricular cardiomyopathy' or ARVC, given the common occurrence of left ventricular involvement; see [37▪▪,38]) is an inherited disease characterized by progressive myocardial loss, fibrosis, adiposis and severe ventricular arrhythmias. It is a frequent cause of sudden death in the young. Life-threatening arrhythmias often occur in the concealed phase of the disease, prior to overt structural damage [38]. Mutations in genes coding for desmosomal proteins are most commonly associated with the familial form of the disease. The question arises as to how mutations in proteins involved in mechanical coupling bring about electrical dysfunction and arrhythmias, particularly in cases wherein the lethal arrhythmias precede major disruption of the structural integrity of the heart. A first indication of involvement of ion channels as arrhythmia substrates came from the observations of Kaplan *et al.* [39,40], reporting loss of gap junction plaques in the hearts of patients affected with the disease. These results were confirmed and expanded in a follow-up study [41]. In-vitro studies have also shown that loss of expression of desmosomal proteins causes loss of gap junction plaques at sites of intercellular contact and Cx43 remodeling [42]. The intimate mechanisms leading to changes in Cx43 in arrhythmogenic cardiomyopathy remain unknown, though they may be related to the critical importance of mechanical attachment on Cx43 trafficking. Of note, however, several studies in murine models have demonstrated that a reduction in Cx43 abundance of over 50% does not lead to arrhythmias [43–46]. Additional studies have shown that loss of

desmosomal proteins also leads to a decrease in amplitude and a shift in gating kinetics of the sodium current [24▪▪,25]. The latter suggests that changes in both electrical coupling and cell excitability may provide a cellular substrate for the generation of arrhythmias in arrhythmogenic cardiomyopathy. Interestingly, recent studies have suggested a direct cross-talk between connexin and the sodium channel complex [3▪▪,24▪▪]. In fact, the loss of cell–cell coupling, as well as loss of Cx43 expression, causes a significant reduction in sodium current amplitude [3▪▪,26]. These results suggest that there is a gap junction-independent role for Cx43 in the heart, namely, the preservation of the functional integrity of the sodium channel complex, and that inherited or acquired diseases leading to Cx43 remodeling may, indirectly, disrupt the excitability of the cell (see also [47,48▪]). Whether this mechanism participates as a substrate for arrhythmias in patients with arrhythmogenic cardiomyopathy is a question for future investigation.

CONCLUSION

In summary, a series of studies have demonstrated an association between changes in the primary sequence of Cx43, or Cx40 and arrhythmias. The paradox remains, however, that patients with a variety of somatic mutations in Cx43 present a pleiotropic phenotype (ODDD) that does not include cardiac arrhythmias. Finally, Cx43 may be a critical component in the generation of arrhythmias observed in patients with desmosomal mutations, both by disrupting electrical coupling and by affecting electrical excitability. The role of desmosomes in electrical function, and the gap junction-independent functions of Cx43, are two very interesting topics that deserve future investigation.

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Conflicts of interest

There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- ▪ of special interest
- ▪▪ of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 321–322).

1. Weidmann S. The electrical constants of Purkinje fibres. J Physiol 1952; 118:348–360.
2. Revel JP, Karnovsky MJ. Hexagonal array of subunits in intercellular junctions of the mouse heart and liver. J Cell Biol 1967; 33:C7–C12.
3. ▪▪ Jansen JA, Noorman M, Musa H, *et al.* Reduced heterogeneous expression of Cx43 results in decreased Nav1.5 expression and reduced sodium current that accounts for arrhythmia vulnerability in conditional Cx43 knockout mice. Heart Rhythm (in press).

This study takes advantage of the murine model of conditional Cx43 knockdown to demonstrate a functional association between Cx43 and the sodium channel complex. It provides direct evidence that Cx43 affects the sodium current.

4. Danik SB, Rosner G, Lader J, *et al.* Electrical remodeling contributes to complex tachyarrhythmias in connexin43-deficient mouse hearts. FASEB J 2008; 22:1204–1212.
5. Paznekas WA, Boyadjiev SA, Shapiro RE, *et al.* Connexin 43 (*GJA1*) mutations cause the pleiotropic phenotype of oculodentodigital dysplasia. Am J Hum Genet 2003; 72:408–418.
6. Paznekas WA, Karczeski B, Vermeer S, *et al. GJA1* mutations, variants, and connexin 43 dysfunction as it relates to the oculodentodigital dysplasia phenotype. Hum Mutat 2009; 30:724–733.
7. Shibayama J, Paznekas W, Seki A, *et al.* Functional characterization of connexin43 mutations found in patients with oculodentodigital dysplasia. Circ Res 2005; 96:e83–e91.
8. Seki A, Coombs W, Taffet SM, *et al.* Loss of electrical communication, but not plaque formation, after mutations in the cytoplasmic loop of connexin43. Heart Rhythm 2004; 1:227–233.
9. Gong XQ, Shao Q, Langlois S, *et al.* Differential potency of dominant negative connexin43 mutants in oculodentodigital dysplasia. J Biol Chem 2007; 282:19190–19202.
10. Gong XQ, Shao Q, Lounsbury CS, *et al.* Functional characterization of a *GJA1* frameshift mutation causing oculodentodigital dysplasia and palmoplantar keratoderma. J Biol Chem 2006; 281:31801–31811.
11. Manias JL, Plante I, Gong XQ, *et al.* Fate of connexin43 in cardiac tissue harbouring a disease-linked connexin43 mutant. Cardiovasc Res 2008; 80:385–395.
12. Kalcheva N, Qu J, Sandeep N, *et al.* Gap junction remodeling and cardiac arrhythmogenesis in a murine model of oculodentodigital dysplasia. Proc Natl Acad Sci U S A 2007; 104:20512–20516.
13. Dobrowolski R, Sasse P, Schrickel JW, *et al.* The conditional connexin43-G138R mouse mutant represents a new model of hereditary oculodentodigital dysplasia in humans. Hum Mol Genet 2008; 17:539–554.
14. ▪▪ Van Norstrand DW, Asimaki A, Rubinos C, *et al.* Connexin43 mutation causes heterogeneous gap junction loss and sudden infant death. Circulation (in press).

Among 292 SIDS cases, two de-novo amino acid substitutions of Cx43 were identified. The challenge remains the absence of robust evidence for the causal role of Cx43 in sudden death and the lack of linkage of family-based association studies.

15. Wolf P, Abbott R, Kannel W. Atrial fibrillation as an independent risk factor for stroke: the Framingham Study. Stroke 1991; 22:983–988.
16. Fox CS, Parise H, D'Agostino RB Sr, *et al.* Parental atrial fibrillation as a risk factor for atrial fibrillation in offspring. JAMA 2004; 291:2851–2855.
17. Yang Y, Xia M, Jin Q, *et al.* Identification of a KCNE2 gain-of-function mutation in patients with familial atrial fibrillation. Am J Hum Genet 2004; 75:899–905.
18. Xia M, Jin Q, Bendahhou S, *et al.* A Kir2.1 gain-of-function mutation underlies familial atrial fibrillation. Biochem Biophys Res Commun 2005; 332:1012–1019.
19. Olson TM, Alekseev AE, Liu XK, *et al.* Kv1.5 channelopathy due to KCNA5 loss-of-function mutation causes human atrial fibrillation. Hum Mol Genet 2006; 15:2185–2191.
20. Darbar D, Kannankeril PJ, Donahue BS, *et al.* Cardiac sodium channel (SCN5A) variants associated with atrial fibrillation. Circulation 2008; 117:1927–1935.
21. Hodgson-Zingman DM, Karst ML, Zingman LV, *et al.* Atrial natriuretic peptide frameshift mutation in familial atrial fibrillation. N Engl J Med 2008; 359:158–165.
22. Gollob MH, Jones DL, Krahn AD, *et al.* Somatic mutations in the connexin 40 gene (*GJA5*) in atrial fibrillation. N Engl J Med 2006; 354:2677–2688.
23. ▪▪ Thibodeau IL, Xu J, Li Q, *et al.* Paradigm of genetic mosaicism and lone atrial fibrillation: physiological characterization of a connexin 43-deletion mutant identified from atrial tissue. Circulation 2010; 122:236–244.

This study reports a novel somatic mutation of Cx43 in an atrial specimen of a patient with idiopathic atrial fibrillation. When expressed heterologously, the mutant Cx43 channel showed a dominant negative effect on wild-type Cx43.

24. ▪▪ Sato PY, Coombs W, Lin X, *et al.* Interactions between ankyrin-G, Plakophilin-2, and Connexin43 at the cardiac intercalated disc. Circ Res 2011; 109:193–201.

Desmosomes, gap junctions and Na channels are structurally and functionally associated with each other at the intercalated disc. Ankyrin-G, a cytoskeletal adaptor protein, emerges as a possible common link. Sodium channel dysfunction may be a key arrhythmogenic substrate in arrhythmogenic cardiomyopathy.

25. Sato PY, Musa H, Coombs W, *et al.* Loss of plakophilin-2 expression leads to decreased sodium current and slower conduction velocity in cultured cardiac myocytes. Circ Res 2009; 105:523–526.
26. Lin X, Liu N, Lu J, *et al.* Subcellular heterogeneity of sodium current properties in adult cardiac ventricular myocytes. Heart Rhythm 2011; 8:1923–1930.

27. Tester DJ, Ackerman MJ. Sudden infant death syndrome: how significant are the cardiac channelopathies? Cardiovasc Res 2005; 67:388–396.

28. Schwartz PJ, Priori SG, Dumaine R, *et al.* A molecular link between the sudden infant death syndrome and the long-QT syndrome. N Engl J Med 2000; 343:262–267.

29. Ruan Y, Liu N, Priori SG. Sodium channel mutations and arrhythmias. Nat Rev Cardiol 2009; 6:337–348.

30. Firouzi M, Ramanna H, Kok B, *et al.* Association of human connexin40 gene polymorphisms with atrial vulnerability as a risk factor for idiopathic atrial fibrillation. Circ Res 2004; 95:e29–e33.

31. Lenegre J. Etiology and pathology of bilateral bundle branch block in relation to complete heart block. Prog Cardiovasc Dis 1964; 6:409–444.

32. Lev M, Kinare SG, Pick A. The pathogenesis of atrioventricular block in coronary disease. Circulation 1970; 42:409–425.

33. Probst V, Kyndt F, Potet F, *et al.* Haploinsufficiency in combination with aging causes SCN5A-linked hereditary Lenegre disease. J Am Coll Cardiol 2003; 41:643–652.

34. Watanabe H, Koopmann TT, Le Scouarnec S, *et al.* Sodium channel beta1 subunit mutations associated with Brugada syndrome and cardiac conduction disease in humans. J Clin Invest 2008; 118:2260–2268.

35. Kruse M, Schulze-Bahr E, Corfield V, *et al.* Impaired endocytosis of the ion channel TRPM4 is associated with human progressive familial heart block type I. J Clin Invest 2009; 119:2737–2744.

36. ▪▪ Makita N, Seki A, Sumitomo N, *et al.* A connexin 40 mutation associated with a malignant variant of progressive familial heart block type-1. Circ Arrhythm Electrophysiol (in press).

A novel germline Cx40 mutation was found as a likely cause of early-onset sudden death in a small number of family members with PFHB1.

37. ▪▪ Basso C, Pilichou K, Carturan E, *et al.* Pathobiology of Arrhythmogenic Cardiomyopathy. In: Corrado D, Basso C, Thiene G, guest editors. Arrhythmogenic Cardiomyopathy. Thakur RK, Natale A, editors. Cardiac Electrophysiology Clinics. Vol. 3. Philadelphia, PA: Elsevier; 2011. pp. 193–204.

In this and other publications, the authors make the case for replacing the term 'arrhythmogenic right ventricular cardiomyopathy' or 'ARVC' for the more general term 'arrhythmogenic cardiomyopathy' or 'arrhythmogenic cardiomyopathy,' thus emphasizing the common occurrence of left ventricular involvement.

38. Delmar M, McKenna WJ. The cardiac desmosome and arrhythmogenic cardiomyopathies: from gene to disease. Circ Res 2010; 107:700–714.

39. Kaplan SR, Gard JJ, Carvajal-Huerta L, *et al.* Structural and molecular pathology of the heart in Carvajal syndrome. Cardiovasc Pathol 2004; 13:26–32.

40. Kaplan SR, Gard JJ, Protonotarios N, *et al.* Remodeling of myocyte gap junctions in arrhythmogenic right ventricular cardiomyopathy due to a deletion in plakoglobin (Naxos disease). Heart Rhythm 2004; 1:3–11.

41. Asimaki A, Tandri H, Huang H, *et al.* A new diagnostic test for arrhythmogenic right ventricular cardiomyopathy. N Engl J Med 2009; 360:1075–1084.

42. Oxford EM, Musa H, Maass K, *et al.* Connexin43 remodeling caused by inhibition of plakophilin-2 expression in cardiac cells. Circ Res 2007; 101:703–711.

43. Morley GE, Vaidya D, Jalife J. Characterization of conduction in the ventricles of normal and heterozygous Cx43 knockout mice using optical mapping. J Cardiovasc Electrophysiol 2000; 11:375–377.

44. Thomas SP, Kucera JP, Bircher-Lehmann L, *et al.* Impulse propagation in synthetic strands of neonatal cardiac myocytes with genetically reduced levels of connexin43. Circ Res 2003; 92:1209–1216.

45. Danik SB, Liu F, Zhang J, *et al.* Modulation of cardiac gap junction expression and arrhythmic susceptibility. Circ Res 2004; 95:1035–1041.

46. Eckardt D, Theis M, Degen J, *et al.* Functional role of connexin43 gap junction channels in adult mouse heart assessed by inducible gene deletion. J Mol Cell Cardiol 2004; 36:101–110.

47. Delmar M. Connexin43 regulates sodium current; ankyrin-G modulates gap junctions: the intercalated disc exchanger. Cardiovasc Res 2012; 93:220–222.

48. ▪ Delmar M, Liang FX. Connexin43, and the regulation of intercalated disc function. Heart Rhythm (in press).

A review on the molecular associations of Cx43 with desmosomes and sodium channels, forming a 'protein interacting network' at the cardiac intercalated disc.

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A Novel Disease Gene for Brugada Syndrome: Sarcolemmal Membrane-Associated Protein Gene Mutations Impair Intracellular Trafficking of hNav1.5

Running title: *Ishikawa & Sato et al.; SLMAP mutations in Brugada syndrome*

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Abstract:

Background - Mutations in genes including *SCN5A* encoding the α subunit of the cardiac sodium channel (hNav1.5) cause Brugada syndrome (BrS) via altered function of cardiac ion channels, but over two-thirds of BrS remains pathogenetically elusive. T-tubules and sarcoplasmic reticulum (SR) are essential in excitation of cardiomyocytes and sarcolemmal membrane-associated protein (SLMAP) is a protein of unknown function localizing at T-tubules and SR.

Methods and Results - We analyzed 190 unrelated BrS patients for mutations in *SLMAP*. Two missense mutations, Val269Ile and Glu710Ala, were found in heterozygous state in two patients, which were not found in healthy individuals. Membrane surface expression of hNav1.5 in the transfected cells was affected by the mutations, and silencing of mutant *SLMAP* by small interfering RNA rescued the surface expression of hNav1.5. Whole-cell patch clamp recordings of hNav1.5 expressing cells transfected with mutant *SLMAP* confirmed the reduced hNav1.5 current.

Conclusions - The mutations in *SLMAP* may cause BrS via modulating the intracellular trafficking of hNav1.5 channel.

Key words: arrhythmia (mechanisms); genes; ion channels; sarcoplasmic reticulum

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Introduction

Brugada syndrome (BrS) is a cardiac channelopathy characterized by specific findings in the ECG such as accentuated J wave and ST-segment elevation in the right precordial leads, which is often accompanied by syncope and sudden cardiac death (SCD) due to ventricular arrhythmias.[1,2] Worldwide prevalence of BrS is approximately 1 in 10,000, but it is much higher in Asian countries, reaching 5 to 10 in 10,000.[3-5] About one-third of BrS patients have a family history of BrS and/or SCD, which is consistent with the autosomal dominant inheritance, suggesting that genetic abnormalities cause BrS.[6]

Mutations in 12 different genes have been reported in BrS.[7-13] The major disease gene for BrS is *SCN5A* that encodes the pore-forming α subunit of the cardiac sodium channel hNav1.5, and the *SCN5A* mutations reduce the availability of sodium channels leading to the diminished peak of inward sodium current (I_{Na}) and/or the voltage-dependent shift in activation or inactivation profile, due to the structural changes in the channel molecule or from trafficking abnormalities.[14,15] Mutations in genes encoding auxiliary proteins regulating sodium channel function, such as glycerol-3-phosphate dehydrogenase 1-like enzyme (GPD1-L) and small subunits of sodium channel (hNavβ1 and hNavβ3) are also associated with BrS and the loss of hNav1.5 function.[16-18]

Excitation-contraction coupling is indispensable for the excitation of cardiomyocytes and regulated by the functional association of T-tubules and sarcoplasmic reticulum (SR).[19] It has

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been reported that abnormalities of T-tubules or SR can cause ventricular arrhythmias.[20-22] One of the components of T-tubules and SR is sarcolemmal membrane-associated protein (SLMAP), of which the gene *SLMAP* maps to chromosome 3p14.3-21.2 and encodes several isoforms of SLMAP via alternative splicing.[23] SLMAP is composed of several functional domains including a forkhead-associated domain, a RecN domain, two leucine zipper domains and a tail anchor domain that is expressed as a mutually exclusive TM1 or TM2 domain. The tail anchor domains play a pivotal role in subcellular targeting of SLMAP.[24] It is known that a ubiquitously expressed isoform, SLMAP3, is encoded by an open reading frame from the start codon in exon 1, whereas the other isoforms, SLMAP1 and SLMAP2, expressed abundantly in striated muscles including heart, are encoded by the other overlapping reading frames from different start codons.[25] Although the functional involvement of SLMAP in cardiac pathophysiology is largely unknown, SLMAP is a candidate gene to search for mutations in arrhythmias including BrS of unknown etiology.

In this study, we analyzed BrS patients for *SLMAP* mutations and investigated the functional significance of the identified mutations. The disease-associated *SLMAP* mutations decreased the cell surface expression of hNav1.5 and reduced the I_{Na} in transfected cells. This is the first report demonstrating the functional association of SLMAP with hNav1.5 and a novel pathogenic substrate for BrS.

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Materials and Methods

Subjects

We studied 190 genetically unrelated patients with BrS. All patients manifested with a BrS diagnostic ECG pattern and were all free from mutations in *SCN5A* (BrS1).[26] Control subjects were 94-380 ethnic-matched healthy individuals. Blood sample was obtained from each subject after an informed consent for gene analysis was given. Data from public available databases as the 1000 genome project (http://www.1000genomes.org/) were also analyzed as controls. The research protocol was approved by the Ethics Review Committee of Medical Research Institute, Tokyo Medical and Dental University, the Mayo Foundation Institutional Review Board, and the Medical Ethical Committee of Fondazione IRCCS Policlinico San Matteo.

Mutational Analysis of SLMAP in BrS

Genomic DNA extracted from peripheral blood leukocytes of each individual was subjected to polymerase chain reaction (PCR) using primer pairs for *SLMAP* (Table S1). PCR products from Japanese patients were analyzed by direct sequencing method, while those from Caucasian patients underwent denaturing high-performance liquid chromatography (DHPLC) and direct sequencing.[27] The sequencing of PCR products were done using Big Dye Terminator version 3.1 (Applied Biosystems, CA, USA) and ABI3100 DNA analyzer (Applied Biosystems). The patients carrying rare non-synonymous variations were also analyzed for mutations in the all known BrS susceptibility gene (Table S2).

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Constructs for SLMAP, hNavβ1 and hNav1.5

We obtained cDNA fragments for human SLMAP by reverse transcription (RT)-PCR from human heart cDNA. Wild type (WT) cDNA fragment for SLMAP with TM1 or TM2 domain were amplified, and equivalent cDNA fragments containing a G to A substitution in codon 269 (for V269I), a C to A substitution in codon 288 (for H288Y) or an A to C substitution at codon 710 (for E710A) were created by the primer-mediated mutagenesis method (Supplemental Table S3). The cDNA fragments of SLMAP were cloned into pEGFP-C1 for EGFP-SLMAP, pcDNA3.1 for pcDNA3.1-SLMAP, and pIRES-CD8 for pIRES-CD8-SLMAP. A cDNA fragment of hNavβ1 was cloned into pcDNA3.1-myc, His-B to obtain myc, His-hNavβ1. The cDNA fragment of human *SCN5A* was a gift from Dr. AL George (Vanderbilt University). A Flag-tagged hNav1.5 was constructed by inserting a Flag epitope (DYKDDDDK) into the extracellular linker 1 between S1 and S2 in D1 domain after the position of aa154 in the hNav1.5 construct (L1-Flag-hNav1.5).[28] All constructs were sequenced to ensure that no errors were introduced.

Immunofluorescence Microscopy

HEK293 or H9c2 cells were seeded onto culture slides (BD Biosciences, CA, USA), and 24 hours later, L1-Flag-hNav1.5 plus each EGFP-SLMAP with or without pcDNA3.1-SLMAP were transfected. After 48 h of the transfection, the cells were permeabilized and incubated with the primary rabbit anti-Flag polyclonal Ab (Sigma, CA, USA) and secondary Alexa fluor 568 goat

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anti-rabbit IgG (Molecular Probes, OR, USA). Images of cells were collected and analyzed with LSM510 laser-scanning microscope. To quantify membrane expression of hNav1.5, fluorescence intensity at the entire cell area and the plasma membrane region (2μm) in the middle *xy* images of *z* series stack were measured, and the ratios of peripheral to total cell area fluorescence intensity (PTAFI) were calculated as described previously.[16] Analyses of labeled cells were performed using ImageJ software (NIH, MD, USA).[29]

Silencing of Transfected SLMAP by Small Interfering RNA (siRNA)

Pre-designed siRNA for human SLMAP (siRNA ID: s15435) and non-silencing siRNA as a negative control were purchased from Ambion (TX, USA). HEK293 cells were seeded onto poly-D-Lysine coated dishes or slides. After 24 h, the cells were co-transfected with the combination of each EGFP-SLMAP and L1-Flag-hNav1.5 with the pre-designed siRNA or non-silencing siRNA. After 48 h of the transfection, the cells were lysed and subjected to Western blot (WB) analyses.

Electrophysiological Studies

We used the tsA-201 cell line, a derivative of HEK293 cell line, in our electrophysiological study, as described previously.[28] In brief, the cells were transfected transiently with either WT or mutant EGFP-SLMAP or pIRES-CD8-SLMAP in combination with pcDNA3.1-Nav1.5. Sodium currents were recorded from the cells that were positive for EGFP or labeled with CD8-Dynabeads using the whole-cell patch clamp techniques.

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Statistical Analysis

Numerical data were expressed as means ± SEM. The normal distributions and equal variances of the data in this study were confirmed by using Shapiro-Wilk test or F-test, respectively. Statistical differences were analyzed using one-way analysis of variance followed by Dunnett's test and Student's *t* test. A *p*- value less than 0.05 was considered to be statistically significant.

Results

Mutational Analysis of SLMAP

We analyzed 190 BrS patients for mutations in *SLMAP*, and eight synonymous and five non-synonymous genetic variants were detected (Table 1, Figure 1A). Among them, five variants had been registered in a public database of polymorphism (dbSNP database, Table 1). A non-synonymous variant p.Tyr68Phe (c.203A>T) was a polymorphism found in both patients and controls at similar frequencies in the Japanese population. The other four synonymous variants were rare, but may not be disease-causing mutations, since no functional impact was deduced.

Three other variants were identified in the heterozygous state in each one patient, p.Val269Ile (c.805G>A), p.His288Tyr (c.862C>T) and p.Glu710Ala (c.2129A>C) (Figure S1A and Table 1). The p.Val269Ile and p.Glu710Ala missense mutations (V269I and E710A, respectively) were found in a 46-year-old and a 57-year-old male patients, respectively, who both experienced syncope and showed spontaneous saddle-back (V269I) or coved (E710A) type ST

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elevation on ECG, while the p.His288Tyr variant (H288Y) was found in a 51-year-old male patient who developed a diagnostic BrS pattern only after the infusion of class Ic drugs. ECG records of the patients with V269I or E710A showed no apparent conduction delay (Figures 1B and 1C, Table S4). In addition, both of them did not show obvious cardiac structural and functional abnormalities. All these substitutions were predicted to affect evolutionary conserved residues of SLMAP (Figure S1B). Both V269I and H288Y should be expressed only in SLMAP3, whereas E710A would be expressed in all SLMAP isoforms (Figure 1A). Because these variants were found in Japanese patients, we analyzed 380 Japanese individuals selected at random. V269I and E710A were not detected in the controls, while H288Y was observed in one control (Table 1). In addition, V269I and E710A were absent among the 1094 individuals (381 Europeans, 246 Africans, 181 Admixed Americans, and 286 East Asians), while H288Y was reported in the 1000 genome project. The patients carrying these variants had no mutation in all the known BrS susceptibility genes and no family history of arrhythmia or SCD, and family studies were not done.

Decreased Cell Surface Expression of hNav1.5 in the Presence of Mutant SLMAPs

Because the majority of BrS-associated mutations are known to affect the hNav1.5 properties including loss of cell surface expression, we tested whether the SLMAP mutations would affect the subcellular localization of hNav1.5. We examined expression of SLMAPs in cell lines available for transfection experiments and found the endogenous expression in HEK293, tsA-201,

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and H9c2 cells (Supplemental Figure S2). We then analyzed the cell surface expression of hNav1.5 in HEK293 cells co-transfected with L1-Flag-hNav1.5 and EGFP-SLMAP3 or EGFP-SLMAP1 with TM1 or TM2 domain (Figure 2). The ratio of peripheral to total cell area fluorescence intensity (PTAFI ratio) of hNav1.5 in the co-transfected cells of L1-Flag-hNav1.5 and EGFP-SLMAP3-TM1-WT was similar to the PTAFI ratio in the transfectants of L1-Flag-hNav1.5 and EGFP-SLMAP3-TM2-WT. On the other hand, the PTAFI ratios in the transfected cells of L1-Flag-hNav1.5 with EGFP-SLMAP3-TM1-V269I, -TM2-V269I, -TM1-E710A, or -TM2-E710A were significantly decreased, while the PTAFI ratios in the transfectants of L1-Flag-hNav1.5 with EGFP-SLMAP3-TM1-H288Y or -TM2-H288Y were not significantly altered (Figure 2, Table S5). E710A in SLMAP3 should also be expressed as E261A in SLMAP1, and we found that the PTAFI ratios in the L1-Flag-hNav1.5 transfected cells of either EGFP-SLMAP1-TM1-E261A or -TM2-E261A were significantly decreased.

To mimic a heterozygous state of *SLMAP* mutations, HEK293 cells were co-transfected with L1-Flag-hNav1.5, SLMAP3-TM1-WT, and each mutant SLMAP construct (Figure 3, Table S5). The PTAFI ratios in the L1-Flag-hNav1.5-transfected cells with both WT and mutant SLMAP were similar to those in the L1-Flag-hNav1.5-transfected cells with each mutant SLMAP, suggesting that V269I and E710A reduced the surface expression of hNav1.5 by a dominant-negative mechanism.

We also investigated the reduction of hNav1.5 expression by the *SLMAP* mutations in a

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rat cardiomyocyte-derived cell line, H9c2. H9c2 cells were transiently transfected with L1-Flag-hNav1.5 and either WT or mutant EGFP-SLMAP3-TM1. It was found that V269I and E710A mutations, but not H288Y, diminished the surface expression of hNav1.5 (Figure S3).

Silencing of Mutant SLMAPs Rescued the Cell Surface Expression of hNav1.5

To demonstrate the effect of *SLMAP* mutations on the surface expression of hNav1.5 by another method, we investigated whether silencing of the SLMAP mutants could rescue the decreased surface expression of hNav1.5. Silencing efficacy of pre-designed siRNA for human *SLMAP* were evaluated, and it was found that administration of siRNA at a final concentration of 30nM completely inhibited the SLMAP expression (Figure 4). HEK293 cells were transfected with the combinations of L1-Flag-hNav1.5, each EGFP-SLMAP construct, and pre-designed siRNA, to analyze the localization of hNav1.5.

The PTAFI ratios in the transfected cells of L1-Flag-hNav1.5 with each EGFP-SLMAP3 or -SLMAP1 were not changed in the presence of non-silencing siRNA (Figure 4A). The PTAFI ratios in the cells expressing L1-Flag-hNav1.5 with EGFP-SLMAP3 of either WT or H288Y with TM1 or TM2 domain were not significantly different between the presence of pre-designed siRNA and non-silencing siRNA (Figure 4B, Table S6). However, the PTAFI ratios in the cells expressing L1-Flag-hNav1.5 with EGFP-SLMAP3-TM1-V269I or EGFP-SLMAP3-TM2-V269I was significantly higher in the presence of pre-designed siRNA than in the presence of non-silencing siRNA. Similarly, the ratios in the transfectants or

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L1-Flag-hNav1.5 with EGFP-SLMAP3-TM1-E710A or EGFP-SLMAP3-TM2-E710A were significantly higher in the presence of pre-designed siRNA than in the presence of non-silencing siRNA. The pre-designed siRNA could also suppress the impaired surface expression of hNav1.5 caused by the E261A mutation in SLMAP1 (Figure S5 and Table S5). The rescued expression levels, however, were similar to those in the L1-Flag-hNav1.5 transfected cells of EGFP-SLMAP1-WT with either pre-designed siRNA or non-silencing siRNA. These data indicated that the decreased surface expression of hNav1.5 was caused by the *SLMAP* mutations.

Altered Electrophysiological Characters Caused by the SLMAP Mutations

Because the impaired intracellular trafficking of hNav1.5 should result in the reduced hNav1.5 function, we investigated potential effects of the SLMAP mutants on the hNav1.5 kinetics. Whole-cell patch clamp recordings were obtained from tsA-201 cells transiently transfected with pcDNA3.1-hNav1.5 in combination with EGFP-SLMAP3-WT or -SLMAP1-WT carrying either TM1 or TM2 domain. Peak current density of I_{Na} (pA/pF) recorded from the cells co-transfected with pcDNA3.1-hNav1.5 and EGFP-C1 was used as a control (Figure 5 and Figures S5 and S6). It was found that the peak current densities recorded from the transfected cells of pcDNA3.1-hNav1.5 with EGFP-SLMAP3 or EGFP-SLMAP1 in TM1 or TM2 domain were not significantly different from the control. In addition, they did not show any significant changes in the activation and inactivation kinetics of I_{Na}, and the time course of recovery from inactivation, as compared to EGFP only (Table 2).

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When we analyzed the effect of mutant SLMAP3 carrying V269I, H288Y or E710A on the kinetics of hNav1.5 in the transfected cells (Figure 5, Figure S5), the peak current densities recorded from the cells co-transfected with pcDNA3.1-hNav1.5 and EGFP-SLMAP3-H288Y were similar to those recorded from the cells co-transfected with pcDNA3.1-hNav1.5 and EGFP-SLMAP3-WT. In clear contrast, the peak current densities of I_{Na} recorded from the cells co-transfected with pcDNA3.1-hNav1.5 and EGFP-SLMAP3-V269I with TM1 or TM2 domain were significantly smaller than those recorded from the cells co-transfected with pcDNA3.1-hNav1.5 and EGFP-SLMAP3-WT with TM1 or TM2 domain by 56.5 % and 51.9 %, respectively (Table 2). In addition, the peak current densities recorded from the cells co-transfected with pcDNA3.1-hNav1.5 and EGFP-SLMAP3-E710A with TM1 or TM2 domain were significantly smaller than those recorded from the cells co-transfected with pcDNA3.1-hNav1.5 and EGFP-SLMAP3-WT with TM1 or TM2 domain by 49.7 % and 40.7 %, respectively. On the other hand, none of the EGFP-SLMAP3-V269I, -H288Y and -E710A caused significant changes in the activation and inactivation kinetics of I_{Na}, and the time constants for recovery from inactivation.

We also investigated whether E261A mutation in SLMAP1 would show an effect on hNav1.5 kinetics as E710A mutation in SLMAP3 did. It was demonstrated that the peak current densities recorded from the cells co-transfected with pcDNA3.1-hNav1.5 and EGFP-SLMAP1-E261A were significantly lower than those recorded from the cells