Closing the Gap on Autosomal Dominant Connexin-26 and Connexin-43 Mutants Linked to Human Disease*

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Cells within the vast majority of human tissues communicate directly through clustered arrays of intercellular channels called gap junctions. Gene ablation studies in mouse models have revealed that these intercellular channels are necessary for a variety of organ functions and that some of these genes are essential for survival. Molecular genetics has uncovered that germ line mutations in nearly half of the genes that encode the 21-member connexin family of gap junction proteins are linked to one or more human diseases. Frequently, these mutations are autosomal recessive, whereas in other cases, autosomal dominant mutations manifest as disease. Given the broad and overlapping distribution of connexins in a wide arrangement of tissues, it is hard to predict where connexin-linked diseases will clinically manifest. For instance, the most prevalent connexin in the human body is connexin-43 (Cx43), yet autosomal dominant mutations in the GJA1 gene, which encodes Cx43, exhibit modest developmental disorders resulting in a disease termed oculodentodigital dysplasia. Autosomal recessive mutations in the gene encoding Cx26 result in moderate to severe sensorineural hearing loss, whereas autosomal dominant mutations produce hearing loss and a wide range of skin diseases, including palmoplantar keratoderma. Here, we will focus on autosomal dominant mutations of the genes encoding Cx26 and Cx43 in relation to models that link genotypes to phenotypic outcomes with particular reference to how these approaches provide insight into human disease.

Overview of Connexin-linked Diseases

A little over a decade ago, the first mutations in the gene encoding Cx32 were found to be linked to the X-linked form of Charcot-Marie-Tooth disease (1–3). Now, numerous mutations in the gene encoding Cx32 have been linked to this neuropathy associated with the demyelination of peripheral axons (4). Once it was established that connexin gene mutations could lead to disease, this brought in a new era of connexin studies that included the structure and function analysis of disease-linked mutants. Mutations in the gene encoding Cx47 have been attributed to Pelizaeus-Merzbacher-like disease, which is another disease characterized by defective myelination (5, 6). Sensory organ diseases, including congenital cataracts, have been attributed to mutations in the genes encoding Cx46 and Cx50 (7), whereas moderate to severe sensorineural hearing loss has been assigned to mutations in upwards of five different connexin family members, including Cx26, Cx30, Cx30.3, Cx31, and Cx43 (8–10). Interestingly, mutations in Cx26 alone are thought to account for 35–45% of all congenital sensorineural hearing loss in some populations (8). The same subset of connexin family members linked to sensorineural hearing loss is, in some cases, responsible for a wide array of skin diseases (11–13). Until ODDD was linked to mutations in the GJA1 gene encoding Cx43 (14), it was expected that germ line mutations in this connexin would likely be lethal given the critical role Cx43 plays in cardiac function and its widespread expression in >35 distinct tissue environments (15, 16). In addition to reports linking connexin gene germ line mutations to disease, other studies have suggested that somatic mutations in the gene encoding Cx40 may be linked to atrial fibrillation (17), and recently, polymorphisms in Cx50 have been proposed to be linked to schizophrenia (18). These latter associations need to be interpreted cautiously and will likely require the study of large cohorts of patients before they can unequivocally be considered causal of human disease.

Autosomal Dominant Mutations

Once molecular geneticists had identified mutations in the genes encoding connexins and linked these results to a cohort of patients, these findings inevitability led to the need for a rigorous set of studies to establish how these genotypic changes lead to disease outcomes. This process is particularly intriguing when considering autosomal dominant mutants, in which both wild-type and mutant proteins are predicted to be produced in equal quantities in all cells where the promoter is temporally and spatially activated. Notably, not only do these molecular studies provide reference points to understanding the manifestations of the disease but offer tremendous potential to assign functional importance to specific domains and motifs that house the mutation. In fact, the use of disease-causing Cx43 mutants provides an experimental setting that cannot be recapitulated by gene ablation or replacement approaches. Although deletion of a connexin gene (knock-out) or substitution of one connexin gene for another (knock-in) in the mouse is an excellent means to define the essential properties of a specific connexin, it does not provide a realistic representation of connexins in human health and disease, where patients rarely suffer from a condition in which a connexin is ablated. The closest comparison with connexin ablation is found in a few cases in which the connexin gene harbors a nucleotide duplication or deletion resulting in a severe truncation or deletion of the protein (19, 20).

There are at least 14 autosomal dominant mutations resulting in 10 amino acid substitutions or deletion sites in the GJB2 gene encoding Cx26 linked to conditions of moderate to severe hearing loss and skin diseases that include Vohwinkel syndrome, keratitis-ichthyosis- deafness syndrome, hystrix-like ichthyosis-deafness syndrome, Bart-Pumphrey syndrome, and...
FIGURE 1. Cell, organotypic, and mouse reference models used to characterize Cx43 (GJA1) gene mutations linked to ODDD. A, the schematic model of Cx43 highlights the locations of the amino acid substitutions, deletions, duplications, and frameshift sites associated with ODDD. The location of the mouse mutation in the Gja1 gene resulting in a glycine-to-serine substitution at position 60 is also noted. B, Cx43 mutants often transport to the cell surface and form gap junction-like structures (red cylinders), whereas in other cases, the mutants are retained in intracellular compartments, including the endoplasmic reticulum (red lace) and the Golgi apparatus. C, when expressed in rat epidermal keratinocytes, green fluorescent protein-tagged Cx43 was localized to the site of cell-cell contact (arrow), whereas the frameshift Cx43 ODDD-linked mutant (Cx43fs260) was restricted primarily to a reticular network reminiscent of the endoplasmic reticulum. D and E, to assess the impact of an ODDD-linked mutant on organotypic epidermis differentiation, the Cx43G21R mutant was stably expressed in rat epidermal keratinocytes, and cells were grown into a complete organotypic epidermis with three to four vital layers (above the dotted line) and a cornified layer (between the dashed lines). In the vital layer, this Cx43 mutant was capable of assembling into gap junction-like structures (arrows), and there was no obvious alteration of epidermal differentiation. F, to further assess the role of autosomal dominant Cx43 mutants in disease, a G60S mutant mouse model designated Gja1G60S/H11001 was generated (41). This mouse model exhibited many of the hallmark features of ODDD, including the fused toes (arrow). G, immunolocalization of Cx43 (red) in the epidermis of thick skin obtained from these mutant mice.
palmoplantar keratodermas (12). Only seven autosomal dominant mutations have been associated with hearing loss without the added skin disease burden. Thus, the autosomal dominant nature of these mutations tends to be syndromic, whereas >100 recessive mutations in the same gene typically manifest as sensorineural hearing loss only (12). Thus, one could argue that a mixed background of wild-type and mutant proteins increases the spectrum of disease load if not the severity of the disease. There are 39 autosomal dominant mutations in the GJA1 gene encoding Cx43 (Fig. 1A) (16) with the resulting disease outcome being classified as ODDD highlighted by developmental defects in the craniofacial bones around the eyes and nose, loss of enamel resulting in early destruction of the teeth, and lack of soft tissue separation of two or three digits (14). In addition to these commonly found developmental disorders, patients often exhibit an assortment of conditions that range broadly from neurological to cardiac disorders (14, 21–24). It is possible that epigenetic effects may also contribute to these disease states. Interestingly, in cases in which the patient harbors a frameshift mutation (fs230 (24) or fs260 (25)) resulting in a gross deletion of the C terminus of Cx43, these individuals have an increased disease load of palmoplantar keratodermas or palmar hyperkeratosis not unlike what is found in some patients harboring dominant GJB2 mutations. The fact that one point mutation in Cx43 (L11P) (26) has been associated with hyperkeratosis suggests that the added disease load in the skin is not restricted solely to the events linked to the C terminus of the molecule.

Expression of Cx26 and Cx43 Mutants in Reference Cell Lines

A common approach to assess any new family or class of disease-linked mutants is to express cDNA constructs that encode the mutant protein in reference cell models that lack endogenous connexins and compare their localization and functional properties with their wild-type counterparts. Over half of the autosomal dominant Cx26 mutants (27–29) and ~10 Cx43 mutants (30–33) have been assessed using this basic approach and found to exhibit two distinct subcellular localization profiles. First, the vast majority of mutants reach the cell surface and assemble into gap junction-like structures as assessed by light microscopy. Based on combinations of dye transfer and electrophysiological measurements, these same mutants either are incapable of forming functional gap junction channels in gap junctional intercellular communication-deficient reference cells or form channels that function poorly in comparison with their wild-type counterparts. The general interpretation of these types of mutants is that their ability to traffic to the cell surface is retained, but they suffer from improper folding or oligomerization resulting in the permanent closing or near complete closure of the gap junction pore. Although channel malformation is the most common feature of Cx26 and Cx43 mutants, a second class of mutants (e.g. Cx26D66H (27) and Cx43fs260 (25)) exhibit protein trafficking defects. In the case of the D66H mutant, it may still retain the capacity to reach the cell surface but fail to stabilize and return to the trans-Golgi network, where it is most prevalent (29). On the other hand, the fs260 mutant appears to have difficulty in passing quality control mechanisms associated with the endoplasmic reticulum (Fig. 1, B and C) (30). Not surprisingly, there are also a few mutants that exhibit intermediate properties of being able to reach the cell surface and assemble into gap junction-like structures with relatively poor efficiency (32).

Dominant and trans-Dominant Cx26 and Cx43 Mutants

When both mutant and wild-type connexin counterparts are coexpressed within the same cell, the obvious question raised is whether these species will intermix and, if so, does the mutant kill the function of the wild-type connexin or does the wild-type connexin rescue the function of the mutant. Clear evidence for mutant and wild-type Cx43 co-mixing in some state of oligomerization was recently provided by co-immunoprecipitation experiments in which differentially tagged wild-type and mutant (G21R and G138R) Cx43 proteins were found to interact (34). Although this relationship is likely to occur among many if not most of the autosomal dominant Cx43 and Cx26 mutants, this principle has yet to be widely demonstrated. However, coexpression studies in which wild-type Cx26 or Cx43 was coexpressed with various mutant counterparts have repeatedly demonstrated that the mutants typically inhibit the function of their wild-type counterparts (28, 29). However, this interpretation needs to be carefully assessed, as mutant connexins are often overexpressed in relationship to their wild-type connexin proteins. Attempts to overcome this limitation have involved coexpressing equal quantities of cDNAs encoding differentially fluorescent protein-tagged wild-type and mutant counterparts and assessing the intensity of red and green fluorescent proteins found at gap junction plaques (34). Using this more elaborate approach, both the G21R and G138R mutants were found to be dominant-negative to Cx43 even when predicted to be expressed at a 1:1 molar ratio. In addition, these two mutants exhibited different potency levels, suggesting that not all mutants are likely to be equally effective in inhibiting the function of wild-type Cx43 (34).

Given that many autosomal dominant Cx26 mutants and a couple of Cx43 mutants are syndromic, it was speculated that one or more of these mutants may have trans-dominant effects on other members of the connexin family. The rationale for this possibility is built on the fact that connexins can co-oligomerize with other members of the connexin family in vivo to form a variety of heteromeric connexon arrangements. For example, it is reasonable to predict that Cx26 mutants might directly interact with coexpressed Cx32 because both wild-type Cx32 and Cx26 form heteromeric arrangements (35). Functional analysis in which Cx26 mutants were coexpressed with Cx32 revealed that the mutants were dominant to this connexin (28, 29). The mechanism of this effect still remains speculative, but because keratinocytes may express upwards of nine different connexin family members, selective mutant-connexin interactions may result in a broader shutdown of overall gap junctional intercellular function, causing skin abnormalities.
MINIREVIEW: Connexin-linked Diseases

Organotypic Reference Models
Although basic two-dimensional reference culture cellular systems can provide insights into how mutants are expressed and managed within a defined cellular system, they do not recapitulate the complexity of cell-cell interactions and signaling that occur in three-dimensional organoids or organotypic cultures. Because autosomal dominant Cx26 and Cx43 mutants cause clinically detectable skin phenotypes, expressing these mutants in undifferentiated keratinocytes and inducing the differentiation and stratification of the skin offer a potential advantage. To that end, both Cx26 and Cx43 mutants have been expressed in rat keratinocytes, and their differentiation into an organotypic epidermis has been assessed for architectural defects in generating normal vital and cornified cell layers as well as for the ability to express molecular markers indicative of keratinocyte differentiation (36–38). Intriguing studies have revealed that several Cx26 and Cx43 mutants that are known to cause hyperkeratosis and other skin defects failed to cause any notable changes in the architecture or differentiation of organotypic epidermis (Fig. 1, D and E). It is notable that these mutants typically manifest their effects in the thick skin of the palms and soles of patients, raising the possibility that an epidermal phenotype will not recapitulate in organoid cultures unless exposed to mechanical friction. In addition, it is possible that organotypic cultures fail to satisfactorily recapitulate human epidermis and that more sophisticated approaches involving primary human keratinocytes are required. Thus, the generation of mouse models of human diseases may prove to be necessary to examine Cx26 and Cx43 mutant effects on organ development, differentiation, and susceptibility to disease.

Mouse Models of Connexin Disease
In 2003, Hodgins and co-workers (39) reported on the first mouse model of a human connexin disease. In this seminal study, the authors engineered a mouse that expressed the Cx26D66H mutant driven by the keratin-10 promoter and that recapitulated the genotype of this autosomal dominant disease (Fig. 1, D and E). It is notable that these mutants typically manifest their effects in the thick skin of the palms and soles of patients, raising the possibility that an epidermal phenotype will not recapitulate in organoid cultures unless exposed to mechanical friction. In addition, it is possible that organotypic cultures fail to satisfactorily recapitulate human epidermis and that more sophisticated approaches involving primary human keratinocytes are required. Thus, the generation of mouse models of human diseases may prove to be necessary to examine Cx26 and Cx43 mutant effects on organ development, differentiation, and susceptibility to disease.

In another study, a mouse was generated by Fishman and co-workers (42) engineered a mutant mouse to express a known human ODDD-linked I130T mutant. Intriguingly, this mutant mouse exhibited syndactyly, as described for the Gja1130T mouse, as well as molecular characteristics that included aberrant Cx43 phosphorylation and trafficking, leading to reduced intercellular junctional conductance (42). Consequently, both mouse models appeared to reliably mimic the human ODDD disease phenotype at least with regard to morphological features. However, although the Cx43I130T mutant mice exhibited no obvious morphological defects of the heart, these animals showed a tendency toward spontaneous and inducible ventricular tachyarrhythmias (42), whereas incidents of bradycardia, bone weaknesses, and hematopoietic defects were seen in the Cx43G60S mice (41). Interestingly, these latter defects are rarely reported in the small cohort of human ODDD patients, raising some concerns that these conditions may be more mouse-specific. However, it will be interesting to determine whether these mouse models can be used to provide clues as to what subclinical disease may be present in the human ODDD patient population that typically remains undetected but may manifest during injury or additional synergistic disease burden. For instance, the Cx43G60S mouse became increasingly neutropenic with age, raising the question as to whether ODDD patients become increasingly immunosuppressed in their senior years. At the molecular level, the question remains as to whether the massive decrease in phosphorylated Cx43, seen in both reference mouse models, would also exist in ODDD patients. In mice, it appears that functional Cx43 can be reduced to a fraction of its normal complement in a wide array of tissues before abnormalities become apparent, arguing for Cx43 being produced at considerably higher levels than essential for most organ function. Moreover, given the critical role of Cx43 in cardiac function, it remains to be determined whether ODDD patients are more at risk for spontaneous heart failure, as observed in some mutant mice. This hypothesis will take some time to test owing in large part to the fact that the reported instances of ODDD in the human population to date are in the hundreds. It is possible that as a larger cohort of patients is identified, these types of studies will become more feasible.

In a more recent study, Fishman and co-workers (42) engineered a mutant mouse to express a known human ODDD-linked I130T mutant. Intriguingly, this mutant mouse exhibited syndactyly, as described for the Gja1130T mouse, as well as molecular characteristics that included aberrant Cx43 phosphorylation and trafficking, leading to reduced intercellular junctional conductance (42). Consequently, both mouse models appeared to reliably mimic the human ODDD disease phenotype at least with regard to morphological features. However, although the Cx43I130T mutant mice exhibited no obvious morphological defects of the heart, these animals showed a tendency toward spontaneous and inducible ventricular tachyarrhythmias (42), whereas incidents of bradycardia, bone weaknesses, and hematopoietic defects were seen in the Cx43G60S mice (41). Interestingly, these latter defects are rarely reported in the small cohort of human ODDD patients, raising some concerns that these conditions may be more mouse-specific. However, it will be interesting to determine whether these mouse models can be used to provide clues as to what subclinical disease may be present in the human ODDD patient population that typically remains undetected but may manifest during injury or additional synergistic disease burden. For instance, the Cx43G60S mouse became increasingly neutropenic with age, raising the question as to whether ODDD patients become increasingly immunosuppressed in their senior years. At the molecular level, the question remains as to whether the massive decrease in phosphorylated Cx43, seen in both reference mouse models, would also exist in ODDD patients. In mice, it appears that functional Cx43 can be reduced to a fraction of its normal complement in a wide array of tissues before abnormalities become apparent, arguing for Cx43 being produced at considerably higher levels than essential for most organ function. Moreover, given the critical role of Cx43 in cardiac function, it remains to be determined whether ODDD patients are more at risk for spontaneous heart failure, as observed in some mutant mice. This hypothesis will take some time to test owing in large part to the fact that the reported instances of ODDD in the human population to date are in the hundreds. It is possible that as a larger cohort of patients is identified, these types of studies will become more feasible.
Conclusions and Future Perspectives

The discovery of autosomal dominant connexin gene mutations linked to human diseases has further raised the profile of connexins in health and disease. The fact that single amino acid substitutions are potent inhibitors of Cx26 and Cx43 channel function strongly suggests that the majority of the polypeptide motifs of these connexins are essential and intolerant to change. Although it is intriguing to propose that mouse models in which specific mutations leading to coexpression of the mutant and wild-type connexins are engineered into the mouse genome represent the optimal reference model to examine the molecular, cellular, and organ phenotypes of the disease, this may be insufficient due to connexin expression pattern and regulational differences that exist between mice and humans. To that end, it will continue to be necessary and important to examine the localizational and functional characteristics of connexin mutants in defined and well understood two- and three-dimensional cellular systems. It would also be advantageous to establish cell lines (e.g., fibroblasts and keratinocytes) from ODDD patients in which the biochemical and functional properties of the connexin mutants can be examined in yet another reference model that offers further linkages to the human condition. In the end, meaningful insights into how connexin mutants cause developmental defects or organ failure will likely continue to require the summation of results from multiple reference in vitro and in vivo models.

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