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Review

Syndromic and non-syndromic disease-linked Cx43 mutations

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ABSTRACT

There are now at least 14 distinct diseases linked to germ line mutations in the 21 genes that encode the connexin (Cx) family of gap junction proteins. This review focuses on the links between germline mutations in the gene encoding Cx43 (*GJA1*) and the human disease termed oculodentodigital dysplasia (ODDD). This disease is clinically characterized by soft tissue fusion of the digits, abnormal craniofacial bone development, small eyes and loss of tooth enamel. However, the disease is considerably more complex and somewhat degenerative as patients often suffer from other syndromic effects that include incontinence, glaucoma, skin diseases and neuropathies that become more pronounced during aging. The challenge continues to be understanding how distinct Cx43 gene mutations cause such a diverse range of tissue phenotypes and pathophysiological changes while other Cx43-rich organs are relatively unaffected. This review will provide an overview of many of these studies and distill some themes and outstanding questions that need to be addressed in the coming years.

1. Overview

Gap junctions are intercellular channels that directly link adjacent cells to allow for the exchange of ions, secondary messengers and other small molecules in a process known as gap junctional intercellular communication (GJIC) [1]. The protein constituents of gap junctions, connexins, oligomerize to form connexons that traffic to the cell surface where they may function as hemichannels. Connexons proceed to dock with connexons from the adjacent cell to form gap junction channels [2,3]. A few to thousands of gap junction channels cluster to form gap junctions that vary considerably in size [4,5] (Fig. 1). There are 21 unique connexin genes in the human genome [6,7] and two or more of these family membranes are routinely co-expressed within the same cell type [3]. Co-expressed connexin isoforms frequently and selectively combine to form both homomeric and heteromeric hemichannels as well as homotypic and heterotypic intercellular gap junction channels [3]. Most importantly, these diverse combinations of connexins create different types of channels with unique properties: channels can differ in ionic conductance, permeability to various molecules and sensitivity to voltage or pH [5,8]. These unique properties likely reflect distinct physiological roles for diverse gap junction channel types in different tissue types.

2. Germ line mutations in connexin encoding genes and disease

Connexin mutations underlie several human diseases [9–12]. For example, numerous mutations in the gene encoding Cx32 (*GJB1*) are associated with X-linked Charcot–Marie–Tooth (CMTX) disease [13]. Mutations in the gene encoding Cx26 (*GJB2*) account for roughly half of all cases of inherited non-syndromic deafness [12,14] while a subclass of autosomal dominant mutations are associated with skin diseases [15,16]. In another example, mutations in both Cx46 (*GJA3*) and Cx50 (*GJA8*) are linked with congenital lens cataracts [17,18]. In 2003, oculodentodigital dysplasia (ODDD) became known as the first human disease to be linked to germ line Cx43 gene (*GJA1*) mutations [19]. Consistent with location of the Cx43 gene, the ODDD locus was mapped to chromosome 6q22-q24 by linkage analysis [20] and location of the disease gene was further refined between D6S266 and D6S1639 markers [21]. ODDD is primarily an autosomal-dominant human disorder where patients display symptoms of congenital craniofacial deformities, anomalies of the teeth and eyes and limb deformities [21–23]. Digital malformations include syndactyly involving the third, fourth and fifth fingers and camptodactyly of the second to fourth toe [21–23]. Most ODDD patients also have mandibular overgrowth, abnormal dentition, enamel hypoplasia and early tooth loss [19]. In addition to these clinical symptoms, patients may exhibit syndromic disease symptoms that include a variety of neuropathies (reviewed in [24]) skin disease, bladder incontinence, thermosensitivity, lymphedema [25] and other conditions [19].

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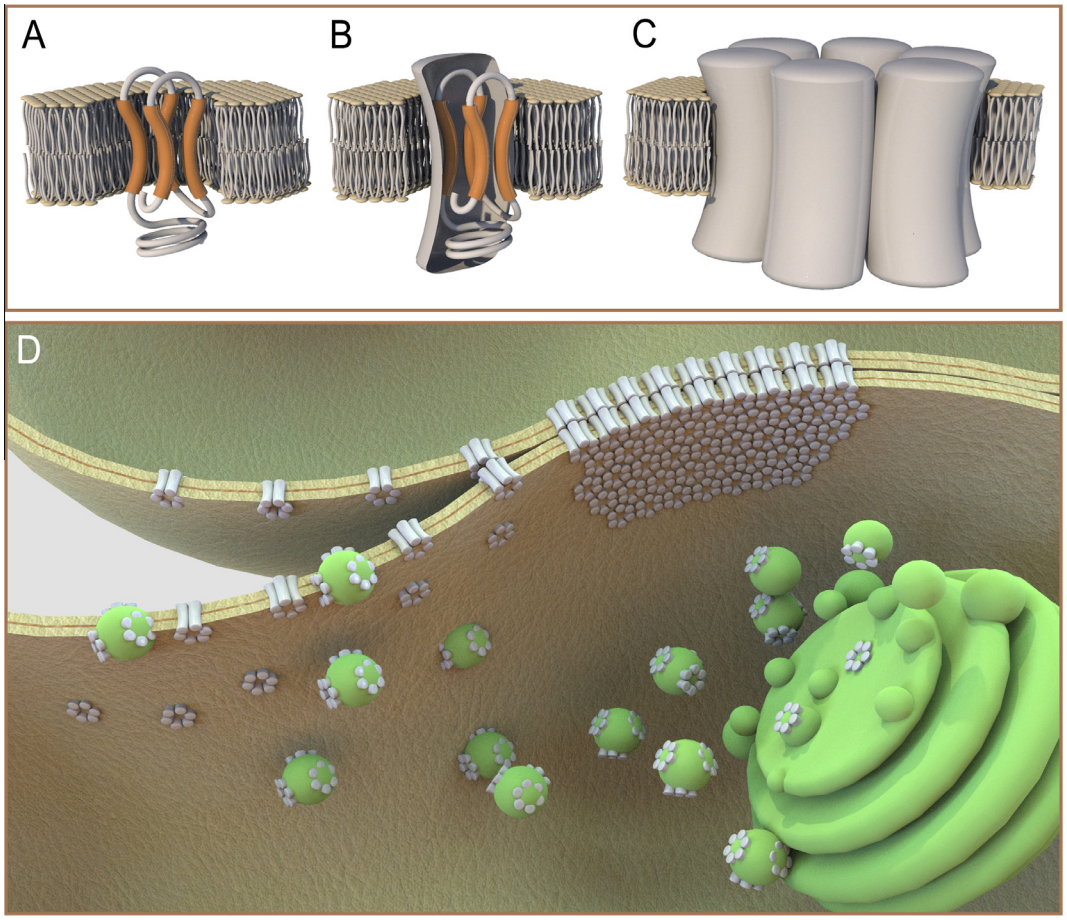

Fig. 1. Schematic model of Cx43 assembly into a gap junction. (A) The polytopic connexin is depicted spanning the lipid bilayer four times. (B) While a resident of the endoplasmic reticulum Cx43 is expected to fold properly and avoid endoplasmic reticulum associated degradation. (C) Cx43 oligomerizes into hexamers or connexons a process that completes in the Golgi apparatus. (D) Upon vesicular transport from the Golgi apparatus, connexons arrive at the cell surface where they may function as hemichannels. Connexons dock with connexons from a neighboring cell to form gap junction channels that proceed to cluster into semi-crystalline gap junctions that may consist of hundreds of channels.

3. Salient features of the Cx43 molecule

It is has long been known that Cx43 is a polytopic membrane protein that passes through the lipid bilayer 4 times yielding the amino and carboxy termini exposed to the cytoplasm [26–28]. The highly conserved two extracellular loops form disulfide bonds that provide structural determinants critical in connexin folding and connexon/connexon interactions as two hexamers dock to form a complete gap junction channel [29–31]. Upon channel clustering into crystalline-like arrays known as gap junction plaques the assembly of a gap junction is complete [32–34] (Fig. 1). When considering connexin biology, there has been a remarkable and purposeful focus on Cx43 for a number of valid reasons. First, it is by far the most broadly and ubiquitously expressed member of the connexin family being expressed in nearly all vital organs that include the brain, heart, lungs, bone, stomach and intestine [3]. At the cellular level, Cx43 has been documented in well over 40 distinct primary cell types making it the most diversely found connexin of the human anatomy [3]. Second, Cx43 is probably the most extensively phosphorylated connexin and distinct phosphorylation processes regulate events related to connexin assembly, disassembly, turnover and channel function [35–37]. Third, Cx43 has an extraordinarily rapid half-life of only 2–4 h [38–40]. While this feature is shared by other connexins, it is particularly intriguing in the case of Cx43 as this connexin plays critical roles in vital organs such as the heart where one might predict it would be long lived. To put this into perspective, it is likely that humans turnover and remodel all of their Cx43 gap junctions in the heart every day [40]. Finally, Cx43 has a particularly long C-terminal tail which encompasses the domains that bind to an extensive interactome [41,42] that governs and regulates pH gating, channel assembly, plaques size and likely numerous other events that we are only beginning to be realize [43,44].

4. The Cx43 mutation composite

To date, there are 73 known Cx43 mutations linked to ODDD that result in the introduction of a mutational change in the first two-thirds of the Cx43 amino acid sequence [19,22,25,45–66] (Fig. 2). It is notable that all but 9 of the 73 mutations are autosomal dominant missense changes where a single amino acid is substituted for another. Of these substitutions, the amino acid residue changes can be: (a) a non-polar residue substituted for another -polar residue (e.g., G2V); (b) an acidic residue replaced by another acid residue (e.g., E110D); (c) a basic residue swapped for another basic residue (e.g., R202H); (d) an acidic residue switched to a basic residue (e.g., E48K) or vice versa (e.g., K134E); or (e) the introduction of prolines into the polypeptide to induce "kinks" into the sequence (e.g., L113P). In essence virtually all categories of conserved and non-conserved amino acid substitutions in the Cx43 polypeptide sequence results in ODDD no matter where the mutation occurred in the first 2/3rd segment of the gene sequence. Only three autosomal dominant frame-shift mutants have been identified to date and both the fs230 (2 variants with different sequence extensions) and fs260 mutations lead to a series of non-sense amino acids before

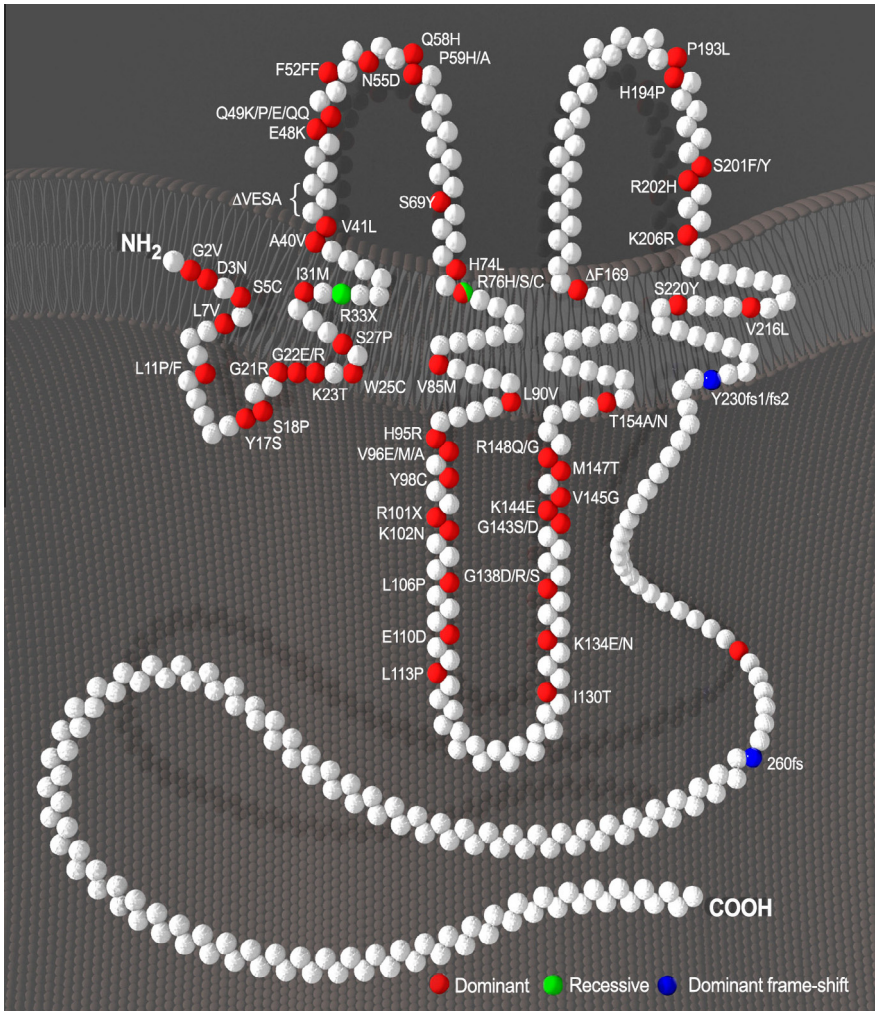

Fig. 2. A composite map of the Cx43 polypeptide depicting the sites of ODDD-linked mutations. Note that all but 9 of the 73 mutations shown are autosomal dominant where a single amino acid is substituted for another. Only two autosomal recessive mutations in the gene encoding Cx43 have been linked to ODDD.

resulting in a stop codon that eliminates the C-terminal of Cx43. Two deletion mutants have been reported where the VESA sequence is eliminated from the 1st extracellular loop domain and the phenylalanine at 169 is lost from within or near the 3rd transmembrane domain. Two further mutants have been reported where an amino acid is duplicated (e.g., Q49 and F52) within the 1st extracellular loop region. In addition, a truncation mutation at residue 101 (e.g., 101X) located at the beginning of the cytoplasmic loop results in the loss of over two/thirds of the connexin molecule. In addition to the overwhelming predominance of autosomal dominant mutations, two autosomal recessive mutations (R76H and R33X) have been reported [22,67]. Patients that are homozygous for the R76H mutant not only exhibit symptoms of ODDD but also Hallermann–Streiff syndrome characterized by a small stature, congenital cataracts, hypotrichosis, beaked nose, skeletal anomalies and teeth defects. Remarkably, not only are subjects that are heterozygous for the R76H amino acid substitution normal but if a patient is heterozygous for a similar R76S mutant they exhibit classical ODDD only. Curiously, the autosomal recessive R33X mutation would result in the premature truncation of Cx43 at residue 33 leading to patients that have no Cx43 hemichannel or channel function [67]. The fact that a Cx43 knockout is not lethal to the patient during development is astounding but may speak to the redundancy and compensatory mechanisms that exist in vital organs.

One mutation in the *GJA1* gene has been linked to a developmental disorder other than ODDD [68]. In this unique study involving six individuals from three families, an autosomal recessive missense mutation causing a R239Q change in the amino acid sequence was found to cause craniometaphyseal dysplasia [68] which is characterized by hyperostosis of craniofacial bones but a complete absence of classical ODDD characteristics involving the eyes, teeth and digits. In another case, the E42K mutation in Cx43 was found to be associated with sudden infant death syndrome which is thought to be linked to cardiac arrhythmias [69]. Finally, in a few very rare cases, reports have shown that *GJA1* mutations can be associated with hearing loss and skin disorders with little or no evidence of ODDD [70–72].

To summate, the fact that many changes, no matter how minimal, in the amino acid sequence of Cx43 have physiological consequences highlights the conservation of the molecule and the importance of all polypeptide motifs and domains. In keeping with the intolerance of the molecular structure to changes introduced by mutations we now know that each segment of Cx43 has unique and sometimes overlapping functions. The N-terminal domain is important in channel gating and oligomerization [73–76]. The transmembrane domains align precisely to establish a gap junction pore that can be gated by voltage, pH, phosphorylation and calcium as well as regulate heteromeric channel compatibility [75,77,78]. The extracellular loops require essential and precise disulfide bonds to ensure that proper channel docking is orchestrated across the gap junction gap [75]. The cytoplasmic loop contains interactive motifs for binding the C-tail during pH gating [79] and also a few putative binding sites for members of the connexin interactome [41].

Interestingly, the paucity of mutations effecting the C-terminal tail suggests that the *GJA1* gene is resistant to mutation or such mutations may be lethal during development.

5. Mechanisms of disease linked to Cx43 mutations

Five potential major classes of connexin mutants are possible and four of these classes are now known to occur in Cx43 mutants linked to ODDD. The first includes, on first pass, those mutations that have no discernible effect on the normal operation of the gap junction channel, and could effectively be considered benign polymorphisms. The Cx30 T5M mutant might be considered a member of this class as it has been reported to retain significant channel function but yet causes hearing loss (Berger et al. 2014, resubmitted). In another experimental case, while the disease-linked G2V mutant exhibits a complete loss of channel function, the G2S mutant, which has not been found to be disease causing, has full or even enhanced channel function [80]. It has yet to be discovered if a fully channel competent mutant can be linked to ODDD. If such a situation does arise, the fact that the mutant is disease causing would likely reflect our lack of understanding of more subtle changes in the regulation of such a channel or hemichannel that is formed by the mutant either alone or in combination with co-expressed wild type Cx43. The second mutant class includes mutants that continue to assemble into gap junction channels, but clearly have documented reductions in channel function. For example, several mutant forms of Cx32 associated with CMTX have been shown to have altered conductance, permeability, or gating properties [81]. In Cx43, the I130T mutant would be an example of a mutant that has documented attenuation of channel function or altered channel regulation [82–85]. The third class of mutants includes those with altered intracellular transport and assembly as demonstrated for some Cx32 mutants associated with CMTX [86] or Cx26 mutations linked to deafness and skin disease [87]. The fs230 and fs260 Cx43 mutants are excellent examples of where the mutants take up residence in the endoplasmic reticulum or Golgi apparatus, respectively and/or fail to pass the necessary quality control mechanisms that surveys mis-folded or aberrant proteins [88,89]. These mutants may in fact be functional if successfully transported to the cell surface in association with wild type proteins but this has yet to be clearly established. The fourth class could represent gain-of-function mutants where hemichannel or channel function is enhanced beyond what is observed for wild-type Cx43. The G138R mutant is one such mutant where it has been documented to have increased hemichannel function [90,91], albeit a complete loss of gap junction channel function. Finally, in the fifth class, Cx43 mutants may efficiently transport to the cell surface and assemble into gap junction plaques but they are functionally dead. The G21R mutant is an excellent example of such a mutant [92]. Caution needs to be exercised in defining this class of mutants as being fully competent in their ability to assemble into gap junctions as this is routinely defined by evidence of anti-Cx43 antibody or GFP labeled plaque-like structures and resistance of the gap junctions to detergent solubilization. Both of these criteria could be considered consistent with complete gap junction plaque assembly but are not definitive as confirmation of the prototypical gap junction structure at the electron microcopy has not been demonstrated. Nevertheless, this class of Cx43 mutants would be transported to the cell surface but result in functionally inactive channels, possibly reflecting improper oligomerization during transit or defects in hemichannel docking.

6. Structure and function lessons learned from examining Cx43 mutants

For many years investigators have been performing mutational and chimeric analysis of Cx43 in order to elucidate the structural and functional relationships within the Cx43 polypeptide. While this has yielded many novel insights, nature has provided an extensive mutational toolkit that can, in turn, be used to assess the importance of individual amino acids or motifs for the proper Cx43 folding, trafficking and function. Of the 73 mutants that have been linked to ODDD, over 20 have been engineered and expressed in reference cell systems, organotypic cultures or mice in order to assess how each mutation alters the structure or function of Cx43 [24,80,84,85,88,90,92–99] (Fig. 3). While this approach yields important insights into the underlying molecular and mechanistic causes of ODDD, other basic properties governing Cx43 channel assembly and function can be ascertained. For example, using the knowledge that the G2V, D3N, L7V, L11P, Y17S and S18P mutants were all loss-of-function mutants [80] together with the published 3.5 A resolution crystal structure of Cx26 [100] one could predict that that N-terminal of Cx43 may in fact be positioning itself in the pore of the Cx43 channel [80]. Such a prediction was supported by the kinked α-helical NMR structure of a 23mer polypeptide encoding G2 or G2V. The disease-causing valine at position 2 would establish an interaction with W4, an amino acid predicted to be important in intramolecular interactions with key residues within the 1st transmembrane domain (I34, L35). Upon engineering the W4A mutant it was found to generate a "kinkless" polypeptide and also was non-functional. Finally, NMR analysis suggested that a G2S substitution may promote a channel open state due to the small side chain. Not only was the G2S mutant fully functional but it exhibited gain-of-function properties [80]. Collectively, these studies supported the notion that Cx43, like what has been proposed for Cx26 [100], may have the N-terminal position at or in the channel where it could interact with the 1st transmembrane domain actively participating in the open and close state of the channel. While this model remains to be confirmed and fully tested it is an example of where naturally occurring disease-causing mutations can be strategically combined with artificial mutants to assess the structural architecture of a Cx43 motif.

7. Dominant and transdominant properties of Cx43 mutants

Since ODDD is primarily an autosomal dominant disease [45] it is assumed that wild-type Cx43 and mutant Cx43 are always co-expressed in any cell that is programmed to express Cx43. Such a condition raises three possibilities as to how the mutant and wild-type Cx43 may interact. First, the wild-type Cx43 may partially rescue the function of the Cx43 mutant and maintain Cx43-based coupling at a level above 50%. To date there is no evidence in the characterized library of Cx43 mutants that such a situation exists. This may not be surprising as we know that heterozygous Cx43 knockout mice are relatively disease-free [101] and do not exhibit any phenotypes that mimic the clinical presentation of ODDD in humans. Thus, disease may not present in patients if the overall based level of Cx43-GJIC is above 50%. Second, mutant Cx43 may retain some residual channel function and together with co-expressed wild-type Cx43 maintain Cx43-based GJIC coupling at or greater than 50%. The I130T mutant would fit in this classification as its overall channel function when express alone is estimated to be ~20% of that of wild-type Cx43 and overall Cx43-based coupling in cells engineered to express both the I130T mutant and wild-type Cx43 remains near 50% [82]. This situation is also observed in isolated cells from genetically-modified mice where both wild-type and the I130T mutant are expected to be co-expressed at equal ratios [82,83,94]. The third group of Cx43 mutants is likely the biggest class where the mutant acts as a dominant on co-expressed Cx43 resulting in total Cx43-based coupling to be well below 50%. The G21R, G138R and the frameshift mutants are excellent examples of this class of mutants [89,92,99,102]. This situation has been shown to also occur in

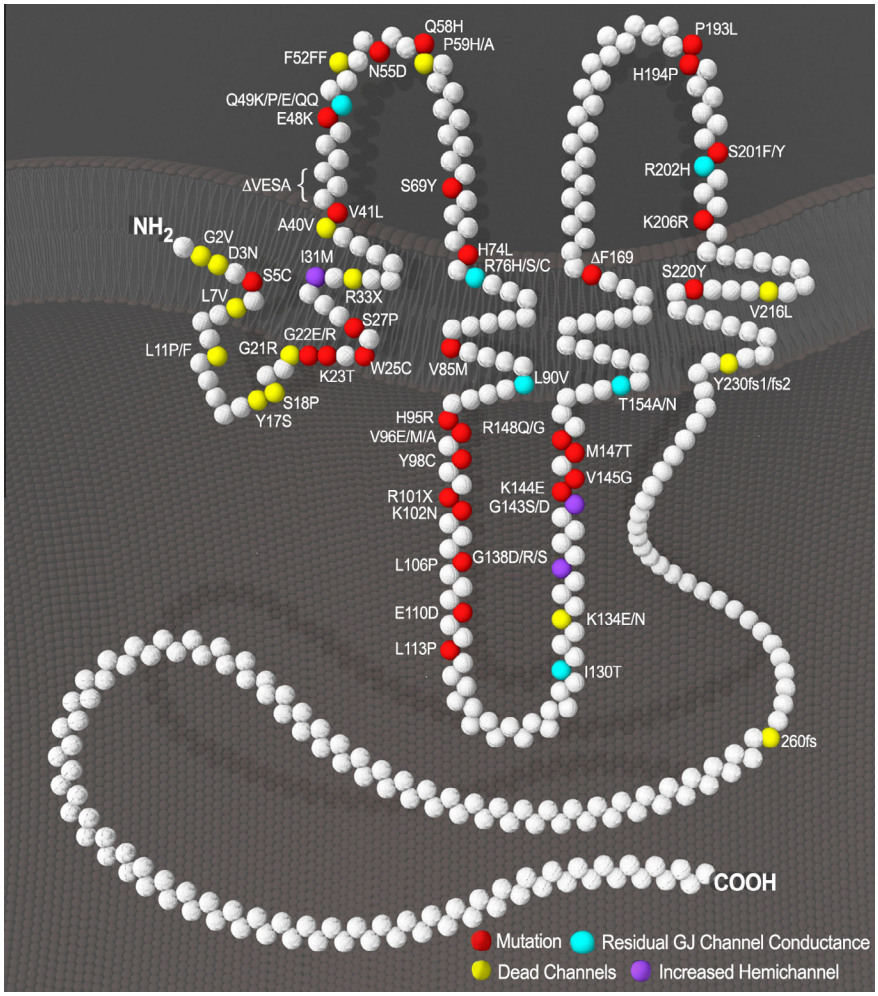

Fig. 3. A model depicting the ODDD-linked mutants that have been expressed and characterized in reference cells, organotypic constructs and/or in mice. Note that the vast majority of mutants studied did not assemble into functional gap junctions (yellow). Six mutants have been reported to have residual gap junction channel function (blue) while 3 mutants have been observed to have gain-of-hemichannel-function (purple). Note that the I130T and G138R mutations used to generate genetically-modified mice are both located in the cytoplasmic loop region of Cx43.

genetically-modified mice that are heterozygous for the G60S mutant and mimic clinical features of ODDD [82,97,103,104].

Since most cells in the human anatomy express two or more connexins and since Cx43 is by far the mostly ubiquitously expressed connexin, the obvious question is whether Cx43 mutants can crosstalk and exhibit transdominant negative properties on other co-expressed connexin family members. On first pass, the co-expressed connexins to consider are ones that previously have been shown to co-oligomerize or interact with Cx43. These connexins would include Cx37, Cx40 and Cx45 although this list is probably incomplete due to the fact that only a limited number of connexins have been tested [105–108]. Evidence for this trans-dominant negative effect is sparse and this may explain why the hearts of ODDD patients are relatively unaffected even though cardiomyocytes co-express Cx40 or Cx45 together with Cx43. Here, co-expressed connexins may act to salvage the heart from exhibiting a disease phenotype in ODDD patients even in the absence of any increase in expression. On the other hand, bone development and remodeling anomalies are common in ODDD patients, and it is well known that osteoblasts and osteocytes express Cx43, Cx45 and Cx46 [91,109–111]. In this example it is likely that mutant Cx43 is involved in dominant and/or transdominant action on co-expressed wild-type connexins. In another case, the autosomal recessive Cx43 R33X mutant was found to exhibit trans-dominant effect on GJIC in reference cells engineered to express the mutant together with Cx40 [112]. A more remote option but yet still possible is that mutations in Cx43 expands their capacity to interact with a greater variety of connexin family members beyond what is possible for wild-type Cx43. While this has yet to be documented for Cx43, disease causing Cx26 mutants have been occasionally shown to be capable of acting as trans-dominant negatives on the function of wild-type Cx43 [87,113]. These later two scenarios may explain why frame-shift Cx43 mutants (e.g., fs230, fs260) cause skin disease where potential interactions with up to 8 other connexins are theoretically possible if the mutants acquire the gain-of-function capacity to interact with any of the keratinocyte connexins.

8. Lesson learned from mouse models of ODDD

Creation of mice that harbor ODDD-linked mutation was recognized in the last decade as a necessary strategy to elucidate the pathophysiological mechanisms of the disease and to uncover subclinical disease that may only be revealed upon injury or comorbidities. While cell reference models and organotypic cultures have been successfully used to assess the ability of Cx43 mutants to traffic, assemble and function the fact that mutant expression is not regulated by the endogenous Cx43 promoter nor is the dose

of the mutant adequately controlled limits the impact of these studies. Since ODDD is predominantly an autosomal dominant disease it is critical to assess the consequence of any of the mutants in native tissue where the Cx43 gene is under the control of its native promoter and where the ratio of the mutant to wild-type Cx43 is expected to be 1:1. The best means to solve both of these issues is to use genetically-modified mice.

The first generation of mice engineered to examine the relationship between Cx43 mutations and disease was acquired through an N-ethyl-N-nitrosourea screen where heterozygous mice were identified that harbored a missense mutation in the *Gja1* gene resulting in a glycine to serine amino acid substitution at position 60 (mice referred to here as $Cx43^{G60S}$ mice) [103]. Although this mutation has not been identified in patients with ODDD, the mutant mice exhibit many of the same phenotypic features as ODDD patients, including fusion of some of the toes (syndactyly), absence of the middle phalanx of the fifth digit of the hind limbs, small eyes, craniofacial bone defects and enamel hypoplasia [103]. Together with genetically matched littermate controls, $Cx43^{G60S}$ mice have proven to be extremely valuable in providing insights into organ development and function, tissue differentiation, sub-clinical disease, wound healing and several other pathophysiologies [82,93–95,103,104,114–120]. To that end, there has been numerous papers published using this novel mouse model of ODDD highlighting the versatility and importance of mouse models of human disease [82,93–95,103,104,114–120]. In many studies, cells were removed from vital organs and cultured to allow for cell and molecular assessment of GJIC and the potential dominant and/or transdominant effect the G60S mutant exhibited on co-expressed wild-type and other connexin family members. Intriguingly, when cultured granulosa cells from G60S mutant mice were assessed for GJIC, these cells that only express Cx43 were found to contain only 10–15% normal Cx43-based GJIC suggesting that the mutant was exhibiting a strong dominant-negative on the function of co-expressed Cx43 [103]. By extrapolation and through further analysis, it is likely that $Cx43^{G60S}$ mice have less than 20% Cx43 based-GJIC in any tissue or cell that is programmed to express Cx43. However, there may be exceptions to this observation as one study showed that Lucifer yellow and sulforhodamine-B transfer was not reduced in astrocytes where multiple connexins may be co-expressed to account for the loss-of-function G60S mutant [121].

To gain further insight into the role of Cx43 in development and disease, two naturally occurring Cx43 gene mutations were used to engineer genetically-modified mouse lines, $Cx43^{I130T}$ and $Cx43^{G138R}$ [83,91]. In both cases, many common features of ODDD were mirrored in these novel mouse models of human disease and these mice further highlighted subclinical disease that may also exist in the ODDD populous but remain less well documented. In $Cx43^{I130T}$ mice, GJIC coupling and the most phosphorylated forms of Cx43 were reduced and there was a slowing of the conduction velocity in the heart with an increase in sensitivity to ventricular tachyarrhythmias [83]. Further investigation revealed that these mice and the $Cx43^{G60S}$ mice had myogenic, and likely neurogenic, bladder defects but this only manifested in a bladder function change in the $Cx43^{I130T}$ mice [94]. Variations in the phenotype of genetically-modified mice that harbor different missense mutations in the Cx43 gene was also seen in the fact that the $Cx43^{G60S}$ mice are consistently smaller than age-matched littermate controls while the $Cx43^{I130T}$ mice are of similar size and weight (Fig. 4). In still another example, in the mammary gland where $Cx43^{G60S}$ mice had a clear and distinct defect in milk ejection during lactation, this defect was completely absent in $Cx43^{I130T}$ mice [122,123]. These differences highlight the need to include multiple mouse models of ODDD to fully understand the pleiotropic variations in phenotypes that exist in these mice that may also provide a deeper understanding as to why some ODDD patients have a greater disease burden than others. To that end, $Cx43^{G138R}$ mice were engineered to conditionally express the Cx43 mutant and found to also mimic the commonly found developmental characteristics of ODDD [91]. Furthermore, mice conditionally engineered to expressing the G138R mutant in the heart were found to be susceptible to spontaneous arrhythmias probably as a result of a reduction in properly phosphorylated Cx43, reduced GJIC and an increase in ATP-release in cardiomyocytes [91]. Later $Cx43^{G138R}$ mice were used to show that Cx43-based GJIC is required for proper morphogen expression in limb development [96]. Here the authors showed that mice harboring the G138R mutant with syndactylies had reduced levels of sonic hedgehog and bone morphogenic protein 2, that together with increase fibroblast growth factors lead to defective interdigital apoptosis [96]. These findings provided a rationale as to why ODDD patients nearly always exhibited syndactylies.

9. Human research models of ODDD

In more recent years the challenge has been to examine Cx43 function in the most relevant models. To that end, obtaining human research models of human disease remains a challenge when considering rare connexin-linked diseases. Genotyping children with hearing loss for Cx26 and/or Cx30 gene mutations has now become a standard level of care in many developed nations owing to the fact that 40–50% of children with congenital neurosensory deafness harbor these mutations. However, the consequence of a positive-test has little clinical implication as no specific treatments for patients harboring connexin gene mutations is currently employed or available. At the research level, several laboratories have obtained skin biopsies to examine the expression and localization of connexins from patients that exhibit skin diseases where Cx26, Cx30.3, Cx30, or Cx31 gene mutations are involved [16,124–127]. Typically, ethical approval for such biopsies are

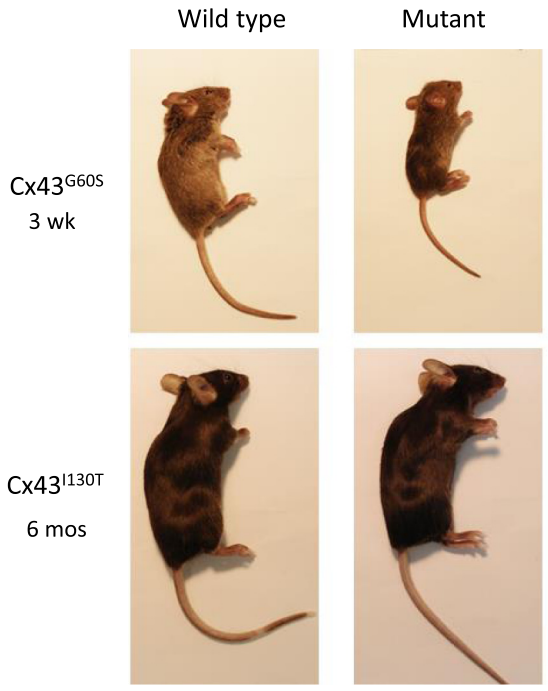

Fig. 4. Phenotypic differences between mouse models of ODDD. Low magnification images of female ODDD mice harboring the G60S or I130T mutants revealed that the $Cx43^{G60S}$ mice are substantial smaller with less body weight than their 3 week old matched littermate wild type controls. Conversely, 6 month old $Cx43^{I130T}$ mice and their matched littermate wild type controls are of similar size and weight. These findings suggest that each mouse model of ODDD may in fact reveal development differences that are unique to each ODDD causing mutation.

not particularly difficult to obtain given limited safety concerns and patient discomfort. However, three significant obstacles exist in attempts to obtain and examine tissues or cells from ODDD patients. First, ODDD is rare with only a few thousand cases clinically reported and with a population projection that less than 1 per 100,000 will present with ODDD. Thus, patient samples will always be difficult to obtain [45]. Second, given the rarity of the disease, it is unlikely that one would be able to identify more than a single family with a given mutation, making generic statements that transcends ethnic backgrounds, sex and age, difficult at best. Finally, once a patient is identified, resecting tissues where disease manifests is nearly impossible given that the common clinical development disorders of the hands, feet, eyes, bone and teeth are not readily accessible.

In one study, we were fortunate to have obtained skin dermal fibroblasts from two ODDD patients harboring the D3N and V216L mutations as well as unaffected close relatives which allowed for direct biochemical and molecular comparisons of cells that are on a similar genetic platform [95]. While neither of these patients exhibited any overt evidence of skin disease, previous wound healing studies in $Cx43^{G60S}$ mice suggested that there may be an underlying wound healing delay and this condition was deemed to be due in part to phenotypic changes in the dermal fibroblasts. In the human dermal fibroblasts, the V216L mutant exhibited strong dominant-negative properties on co-expressed Cx43 while only a modest effect was exhibited by the D3N mutant [95]. Both mutant expressing dermal fibroblasts grew slower and migrated less well than their family matched unaffected control cells, suggesting that these cells may not perform as well in wound healing. This hypothesis was supported by the restricted ability of mutant expressing cells to respond to TGFβ and increase the expressing of smooth muscle actin as this would be expected of cells undergoing differentiation into myofibroblasts during the wound healing response [95]. Collectively, these studies suggest that at least some ODDD patients would have compromised wound healing. Looking to the future it would be important to obtain dermal fibroblasts and other cell types from patients harboring a wide array of ODDD-linked mutations to explore other subclinical diseases that may exist in ODDD patients.

10. Summary and future perspectives

Examination of disease-causing Cx43 mutants using cell expression systems, organotypic cultures, genetically-modified mice and patient cells has led to a number of core discoveries that improves our understanding of how Cx43 functions and in what way any number of changes in Cx43 function can lead to disease. It is clear that one cannot simply assess whether a mutation is conserved or non-conserved nor assess the site of the mutation and predict patient disease load. Conversely, we can predict with some certainty that many changes in the Cx43 amino acid sequence will have disease consequences suggesting that Cx43 has a low tolerance for change if its full range of cellular function is to be maintained. We have also seen that in many cases, but not all, the mutation will cause a loss-of-channel function while a few mutations appear to provide a gain-of-function advantage (e.g., increased hemichannel function) that can be equally damaging to human development and physiology. While many mutants act as dominant-negatives, a few do not, raising questions as to why patients still present with the common clinical symptoms. Conversely, only a few mutants so far have been shown to have any transdominant-negative effects which may be surprising given the wealth of cells that co-express connexin family members that are capable of interacting with Cx43. Lastly, it appears likely that at least some patients have subclinical disease (e.g., delay in wound healing) that may only present upon injury or organ distress caused by other co-morbidities that are acquired during the aging process. Circumstantially, it is intriguing that more disease in ODDD patients becomes evident during aging which may be serendipitous or linked to organs that suffer from compromised Cx43 function for decades.

Looking forward there are many outstanding questions that remain. First, there is little explanation for organs like the heart not being more dramatically affected in ODDD patients particularly when Cx43 mutants are expressed that have a strong dominant-negative effect on co-expressed Cx43. If co-expression of other connexin members is sufficient to rescue the organ from disease one might predict that the bone and skin should also be protected from disease as multiple connexin family members are co-expressed in these organs, yet this is not the case. Second, it is remarkable that ODDD patients that may have as little as 15–20% normal Cx43-based GJIC do not have more developmental abnormalities or exhibit more disease susceptibility. Thus, it would seem much of the human physiology can tolerate and even thrive with minimal Cx43 function. This may be due to the notion that Cx43 is produced in excess or connexin family members act in a compensatory fashion even in the absence of increase expression. Third, the question remains as to whether ODDD can be treated. One option would be to use a pharmacology approach to increase the functional state of Cx43. Here one would need to argue that increased expression of Cx43 (both mutant and wild type) would drive cells into a state of having enhanced overall Cx43-based GJIC. Evidence for such a treatment option is essentially non-existent and the pharmacology is lacking. Only an antiarrhythmic peptide-based drug called rotigaptide and newer derivatives of this peptide (danegaptide) have reached clinical trials as an enhancer of Cx43 in cardiac pathologies [128–130]. Another theoretical option would be to design cell permeable drugs that could stimulate the expression of a complementary member of the connexin family (e.g., Cx40) that could serve many of the same roles of Cx43. Both of these strategies need to be coupled to tissue delivery systems that target the defective tissue and even then would only be appropriate for diseased organs that have not be subjected to permanent and irreversible developmental anomalies (e.g., incontinence, skin regeneration). Possibly a more realistic strategy would be to used targeted RNAi strategies to specifically knock down the mRNA that encodes mutant Cx43 but not wild type Cx43. Evidence exists that a 50% complement of normal Cx43 that is unimpeded by the co-expression of mutant Cx43 may sustain normal phenotype at least this appears to be the case in mice that are heterozygous for Cx43 ablation [101].

In summary, it has become apparent in both mice and humankind that survival and health depends on the expression and function of the most ubiquitously found member of the connexin family, Cx43. Studies into the pathophysiology of ODDD patients and mouse models of this disease will continue to shed insights into the mechanisms that surround the role of Cx43 in development and also during aging where health inevitability becomes compromised by comorbidities.

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