Cx (connexin) proteins are components of gap junctions which are aqueous pores that allow intercellular exchange of ions and small molecules. Mutations in Cx genes are linked to a range of human disorders. In the present review we discuss mutations in β-Cx genes encoding Cx26, Cx30, Cx30.3 and Cx31 which lead to skin disease and deafness. Functional studies with Cx proteins have given insights into disease-associated mechanisms and non-gap junctional roles for Cx proteins.

Key words: connexin, gap junction, hearing, keratinocyte, skin.

INTRODUCTION

Intercellular communication is important in controlling homoeostasis in organisms and in permitting responses to external stimuli. GJ (gap junction) proteins mediate GJIC (GJ intercellular communication) by enabling the transfer of ions such as Ca$^{2+}$ and Mg$^{2+}$, and small molecules less than 1 kDa, such as inositol 1,4,5-trisphosphate, cAMP, cGMP and ATP, between cells. This is important in a range of physiological processes including cell growth, cell differentiation and metabolic co-ordination (reviewed in [1,2]). In chordates, the main component of GJs are Cx (connexin) proteins, 21 members of which are known to exist in humans and 20 in mouse. Cx genes are classified into groups (connexin) proteins, 21 members of which are known to exist in humans and 20 in mouse. Cx genes are classified into groups (reviewed in [8,9]). In chondrodonts, the main component of GJs are Cx (connexin) proteins, 21 members of which are known to exist in humans and 20 in mouse. Cx genes are classified into groups (reviewed in [8,9]). In chondrodonts, the main component of GJs are Cx (connexin) proteins, 21 members of which are known to exist in humans and 20 in mouse. Cx genes are classified into groups (reviewed in [8,9]). In chondrodonts, the main component of GJs are Cx (connexin) proteins, 21 members of which are known to exist in humans and 20 in mouse. Cx genes are classified into groups (reviewed in [8,9]). In chondrodonts, the main component of GJs are Cx (connexin) proteins, 21 members of which are known to exist in humans and 20 in mouse. Cx genes are classified into groups (reviewed in [8,9]). In chondrodonts, the main component of GJs are Cx (connexin) proteins, 21 members of which are known to exist in humans and 20 in mouse. Cx genes are classified into groups (reviewed in [8,9]).

Cx STRUCTURE AND LIFE-CYCLE

Prior to the discovery of Cx genes, hexagonal arrays of proteins were observed in plasma membranes of goldfish [5] and rat liver [6]. Electron microscopy and X-ray diffraction showed GJ plaques composed of multiple channels [7]. Cx proteins are transmembrane proteins, the structure of which is shown in Figure 1. The CT (C-terminus), which shows the highest variability in length between different Cx types, and the CL (cytoplasmic loop) can undergo post-translational modifications such as phosphorylation, which is implicated in regulating the life-cycle of certain Cx proteins (reviewed in [8,9]).

The Cx life-cycle has been reviewed in detail previously [10]. Briefly, six Cx proteins oligomerize to form a hemichannel in the ER (endoplasmic reticulum) or Golgi apparatus [11–13]. After exiting the ER, Cxs are believed to enter the Golgi apparatus [11,12]; however, Cx26 is able to reach the cell surface membrane independently [14,15]. The six Cx proteins in the hemichannel may be the same Cx protein, giving a homomeric hemichannel, and selected Cx types may form a hemichannel composed of different Cx proteins, termed a heteromeric hemichannel. For example, Cx26 can form hemomeric channels with Cx30 [16,17], but not Cx43 [18].

Microtubules are believed to facilitate the trafficking of hemichannels to the cell surface membrane in vesicles [19]. At the cell surface, hemichannels may exist unapposed to another channel, or dock with a hemichannel on an adjacent cell to form a GJ connecting the cytoplasms of the respective cells. A homotypic channel is composed of two identical hemichannels (which can be either homomeric or heteromeric), whereas a heterotypic channel is composed of different hemichannels. These channels can cluster together to form GJs, which can coalesce to form bigger GJ plaques [7]. GJs composed of different Cx types can have different properties, for example, differing permeabilities to molecules and ions (reviewed in [20]), therefore the expression of multiple Cx proteins in a cell type is likely to confer different properties for GJIC in tissue.

HEMICHANNELS

The existence of functional hemichannels at the cell surface membrane, indicating that hemichannels may have a function beyond being simply half of a GJ, was first revealed when Cx46 was overexpressed in Xenopus oocytes and displayed conductance under low, but not high, levels of extracellular Ca$^{2+}$ [21].

Hemichannels are involved in paracrine signalling by enabling the transfer of molecules between the cell and the extracellular environment [22,23]. Cx hemichannels have a role in Ca$^{2+}$ signalling and can propagate Ca$^{2+}$ waves [24], for example, between astrocytes [25] and osteocytes [26]. Stimuli such as stress causes PLC (phospholipase C) activation, mobilization of intracellular Ca$^{2+}$ stores and opening of hemichannels leading to ATP release from the cell (reviewed in [27]). ATP binds to purinergic receptor
2Y on other cells, causing an intracellular increase in Ca\(^{2+}\) and inositol 1,4,5-trisphosphate concentrations and hemichannel opening, allowing propagation of Ca\(^{2+}\) waves [27].

Hemichannels are believed to be closed at physiological levels of extracellular Ca\(^{2+}\) or higher, but can open in response to stimuli such as low Ca\(^{2+}\) [22,24,28,29]. Cx43 hemichannels imaged in buffer containing 1.8 mM Ca\(^{2+}\) were closed, but had an increased probability of opening as the Ca\(^{2+}\) levels were decreased from 1.4 to 0 mM [30].

**Cx MUTATIONS AND HUMAN DISEASE**

The important physiological role of Cx proteins is illustrated by the range of disorders associated with mutations in Cx genes including syndromic disease (affecting multiple tissue types), skin disease only, NSHL (non-syndromic hearing loss) (deafness only), neuropathy and cataracts. Mutations in different Cxs can cause the same disease, indicating that certain Cx types have similar functions in a selected tissue type. For example, Cx26 and Cx30 are expressed in the cochlea [31], and mutations in both genes can cause deafness. Different mutations within the same Cx gene can also give rise to completely different disorders, for example p.66delD in Cx31 is associated with neuropathy and hearing loss [32], whereas other mutations cause EKV (erythrokeratodermia variabilis) only or NSHL. This demonstrates how Cx mutations may cause pathogenesis in a tissue-specific manner, the reasons for which are largely unclear.

*In vitro* functional assays with Cx mutants, and mouse models of Cx gene knockout or Cx gene mutations linked to skin disease or NSHL, have been used to investigate the properties of wild-type Cxs and their mutants. Investigations involving the overexpression of reporter protein-tagged Cxs have demonstrated that specific Cx mutants may display trafficking defects, be associated with a cell death phenotype or form non-functional GJs, as indicated by dye transfer studies. Expression studies in *Xenopus* oocytes have indicated that specific Cx mutants decrease oocyte viability and affect GJ conductance and/or lead to outward membrane currents [33–35].

Mouse models of Cx gene knockout, and three skin disease models, have been made which provide some insights into Cx-mediated disease (Table 1); however, important differences exist between the human phenotype and that of mice. Double-allele knockout for *mGjb2* (Cx26) is embryonically lethal in mice [36]; however, humans who are homozygous for loss-of-function *GJB2* mutations such as c.35delG, which leads to a severely truncated Cx26 protein, are deaf. Only 5% of the skin of mice heterozygous for the EKV Cx31 mutant had any phenotype, which was some hyperproliferation in the granular layer [37], and mice expressing the KID (keratitis-ichthyosis-deafness) syndrome Cx26 mutant p.S17F lacked keratitis, unlike patients harbouring this mutant [38]. These differences may be because of alternative functions for Cx types in mice compared with humans and/or due to differences in the processing of Cx mutants.
Table 1 Phenotype of β-Cx gene knockout and disease model mice

<table>
<thead>
<tr>
<th>Cx</th>
<th>Modifications</th>
<th>Phenotype</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCx26 (mGjb2)</td>
<td>Double knockout</td>
<td>Lethal at ED6, probably due to aberrant placental glucose uptake</td>
<td>[36]</td>
</tr>
<tr>
<td></td>
<td>Targeted disruption/conditional knockout in inner ear</td>
<td>Cell death in the inner ear and hearing loss</td>
<td>[83,84]</td>
</tr>
<tr>
<td></td>
<td>p.R75W (NSHL mutant)</td>
<td>Sensory hair cell and organ of Corti degeneration in the inner ear, hearing loss</td>
<td>[86]</td>
</tr>
<tr>
<td></td>
<td>p.D66H (VS mutant) expressed under the keratin 10 promoter</td>
<td>Hyperkeratosis and increased TUNEL staining</td>
<td>[98]</td>
</tr>
<tr>
<td>mCx30 (mGjb6)</td>
<td>Double knockout</td>
<td>Hyperkeratosis and deafness, keratitis absent</td>
<td>[38]</td>
</tr>
<tr>
<td>mCx30.3 (mGjb4)</td>
<td>Double knockout</td>
<td>Hearing impairment</td>
<td>[135]</td>
</tr>
<tr>
<td>mCx31 (mGjb3)</td>
<td>p.F137L (EKV mutant)</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Double knockout</td>
<td>60% of mice died between ED10.5 and 13.5, surviving mice had no apparent abnormalities</td>
<td>[136]</td>
</tr>
<tr>
<td></td>
<td>p.F137L (EKV mutant)</td>
<td>Lethal at ED7.5 for homozygous mice, only 5% of analysed skin of heterozygous mice was affected</td>
<td>[37]</td>
</tr>
</tbody>
</table>

Previous studies have revealed roles for Cx hemichannels in the invasion of specific bacterial pathogens, which are able to manipulate Cx hemichannels to increase the efficacy of infection, and in inducing the immune system in response to bacterial components [39–42]. Cx26 and Cx43 hemichannels have been found to facilitate cell invasion by type III bacterial gastrointestinal pathogens [39–41]. For example, the causative bacterium of shigellosis dysentery, Shigella flexneri, induced the opening of Cx26 hemichannels leading to increased bacterial invasion [39], and overexpression of Cx26 was found to increase S. flexneri invasion [43]. Cx43 hemichannels are implicated in enhancing infection of cells by the gastrointestinal pathogens Yersinia enterocolitica [40] and Citrobacter rodentium in mice [41], and mice heterozygous for Cx43 knockout showed increased resistance to diarrhoeal disease caused by C. rodentium compared with wild-type mice [41].

Peptidoglycan, derived from the cell wall of Gram-positive bacteria including Staphylococci species, was found to induce the expression of IL-6 (interleukin-6), TLR2 (Toll-like receptor 2) and Cx43, as well as increasing Cx43 hemichannel activity [42]. Inhibition of Cx43 hemichannel activity prevented IL-6 and TLR2 up-regulation, indicating a functional role for Cx43 hemichannel activity in inducing immune system signalling [42].

Cx EXPRESSION IN THE EPIDERMIS AND INNER EAR

The epidermis is the outermost layer of the skin composed of four main layers: the basal (deepest) layer, spinous and granular layers, and the outer stratum corneum. Keratinocytes migrate from the basal layer up through the epidermal layers where they undergo terminal differentiation to ultimately create the stratum corneum formed of dead corneocytes. GJs are found in the basal, spinous and granular layers of human and rat epidermis, but not in the stratum corneum [44,45], and different Cx types are expressed at distinct locations in the epidermis. This may be because certain Cxs have functional roles at different stages of keratinocyte differentiation, for example, by altering GJ permeability and/or by unknown non-gap junction functions. Cxs expressed in both human and rodent epidermis, but with differences in their localizations, include Cx26, Cx30, Cx30.3, Cx31, Cx31.1 and Cx43 [3,4,46–49].

Cx expression patterns in both mice and humans are altered after injury, indicative of significant roles for Cx proteins in co-ordinating wound healing. Initially, Cx31.1 and Cx43 were down-regulated, which was preceded by an up-regulation of Cx26 and Cx30 expression at the wound site [50–52] that may mediate keratinocyte proliferation and/or migration into the wound. However, continuous Cx26 expression in murine epidermis led to hyperproliferation and inhibited wound closure, indicating that deregulated Cx26 expression can disrupt epidermal homoeostasis [53]. Cx43 expression remained low throughout the entire wound-healing process in humans, indicating that Cx43 down-regulation is necessary for wound re-epithelialization [52]. This is supported by transient Cx43 knockdown and GJ inhibition studies, which increased the rate of wound closure in mouse models, mouse and human three-dimensional organotypic cultures and ex vivo total skin wound-healing models [54–59].

The mammalian inner ear contains the cochlea, which consists of three tubular compartments: the scala media, which contains endolymph, and the scala tympani and scala vestibule, which contain perilymph. In response to sound, endolymphatic K+ penetrates and activates organ of Corti hair cells in the scala media before passing into the perilymph and recirculating back to the endolymph [60]. Resulting nerve impulses to the brain are interpreted as sound. Cx26, Cx30, Cx31, Cx32 and Cx43 are expressed in the cochlea, and GJs, which are present in the organ of Corti, have been predicted to facilitate K+ circulation [61,62].

Mutations in GJB2 (Cx26), GJB3 (Cx31), GJB4 (Cx30.3) and GJB6 (Cx30) are associated with syndromic disease, skin disease only or NSHL (Table 2). Certain GJA1 (Cx43) mutations are associated with ODDD (oculodentodigital dysplasia) with the additional feature of PPK (palmoplantar keratoderma) [63–65].

MECHANISM OF Cx MUTATIONS IN DISEASE

The mechanisms by which Cx mutants cause disease in vivo are largely unknown and are likely to differ depending on the Cx type and the mutation. Figure 2 summarizes potential mechanisms of disease, which are (i) accumulation of a Cx mutant in the cytoplasm, (ii) a Cx mutant exerting a dominant-negative effect, (iii) loss of GJ function and (iv) aberrant hemichannels.

When overexpressed in vitro, certain Cx mutants display trafficking abnormalities, localizing to the cytoplasm in contrast with wild-type protein which forms GJ-like aggregates at the cell surface (Figures 1B and 1C). For example, specific CMTX (X-linked Charcot–Marie–Tooth disease) Cx32 mutants accumulate in the ER and Golgi apparatus [66–69]. EKV Cx31 mutants have a cytoplasmic localization, and ER stress is implicated as the mechanism of the in vitro cell death phenotype caused by overexpressing them [70].
Table 2  Diseases associated with mutations in Cx26, Cx30, Cx30.3 and Cx31 genes in humans

<table>
<thead>
<tr>
<th>Cx protein/gene</th>
<th>Disease</th>
<th>Inheritance</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cx26/GJB2</td>
<td>BPS</td>
<td>D</td>
<td>[137]</td>
</tr>
<tr>
<td></td>
<td>PPK and deafness</td>
<td>D</td>
<td>[138–140]</td>
</tr>
<tr>
<td></td>
<td>KID syndrome</td>
<td>D</td>
<td>[141,142]</td>
</tr>
<tr>
<td></td>
<td>HID syndrome</td>
<td>D</td>
<td>[143]</td>
</tr>
<tr>
<td></td>
<td>VS</td>
<td>D</td>
<td>[144–146]</td>
</tr>
<tr>
<td></td>
<td>Clouston-like syndrome with deafness</td>
<td>D</td>
<td>[147]</td>
</tr>
<tr>
<td></td>
<td>Mucocutaneous abnormalities with deafness</td>
<td>D</td>
<td>[148]</td>
</tr>
<tr>
<td>Cx30/GJB6</td>
<td>HED</td>
<td>D</td>
<td>[106,107]</td>
</tr>
<tr>
<td>Cx30.3/ GJB4</td>
<td>EKV</td>
<td>D</td>
<td>[109,149]</td>
</tr>
<tr>
<td>Cx31/GJB3</td>
<td>EKV</td>
<td>D</td>
<td>[108,150,151]</td>
</tr>
<tr>
<td></td>
<td>NSHL</td>
<td>D/R</td>
<td>[110]</td>
</tr>
<tr>
<td></td>
<td>Peripheral neuropathy and deafness</td>
<td>D</td>
<td>[32]</td>
</tr>
</tbody>
</table>

Figure 2  Potential disease-associated mechanisms of mutant Cx proteins

A Cx gene mutation may cause disease because: 1. a Cx mutant mistraffics and accumulates in the cytoplasm, organelles and/or leads to ER stress; 2. a dominant mutant has a dominant-negative effect on the functioning of the wild-type Cx or on other Cx types, affecting hemichannel formation and/or GJ function; 3. the mutant is unable to form GJs at the cell surface membrane and thus are loss-of-function mutants (this may be because the mutant cannot traffic to the cell surface membrane or form the ‘correct’ homotypic/heterotypic GJs required for its normal function in tissue); or 4. a mutant Cx results in the formation of ‘leaky’ hemichannels at the cell surface membrane, leading to deregulated exchange of molecules with the extracellular environment.

Specific syndromic Cx26 mutants appear to have a dominant-negative effect on the wild-type Cx26 protein and/or on other Cx types. For example, p.del42E has been found to exert a dominant-negative effect on wild-type Cx26, Cx37 and Cx43, thus potentially impairing GJs which incorporate these Cx types [71]. Several of the most common NSHL GJB2 mutations are insertion, deletion or nonsense mutations, and thus are considered to be loss-of-function mutations [72]. For example, c.35delG is unable to form GJs in vitro, and thus impaired GJ function could cause NSHL [73]. Certain syndromic Cx26 and Cx30 mutants linked to an in vitro cell death phenotype have been implicated in forming aberrant hemichannels at the cell surface, which are predicted to lead to deregulated exchange of molecules with the extracellular environment and cause cell death [33–35,74].

Cx26 MUTATIONS LINKED TO NSHL

GJB2 mutations cause between one third and 50% of prelingual genetic NSHL in many ethnic groups, the majority of which are recessive mutations [75–80] (http://davinci.crg.es/deafness/). It has been hypothesized that the most common recessive loss-of-function Cx26 mutants linked to NSHL cannot form functional GJs in the cochlea, thus disrupting K⁺ transport [62,81]. However,
these mutants only cause deafness, not skin disease, indicating that a simple loss of Cx26 GJ function is not sufficient to cause skin pathology.

Degeneration of the organ of Corti hair cells was discovered in a human temporal bone donor who had had congenital deafness and was compound heterozygous for c.35delG and p.E101G [82]. In the inner ear of mice, targeted disruption of Cx26 [83] or conditional Cx26 knockout [84] resulted in the death of different cell types in the inner ear soon after onset of hearing. These studies indicate that loss-of-function Cx26 mutants could decrease cell viability, leading to cochlear damage and hearing impairment in humans.

Functional studies with Cx26 mutants p.W44C, p.R75W and p.M163L, which are associated with dominant NSHL, have indicated that they have a dominant-negative effect on wild-type or other Cxs, and/or their expression is associated with increased cell death [71,85–87]. In vitro studies demonstrated that p.W44C did not form GJs and had a dominant-negative effect on wild-type Cx26 and Cx37 [71]. p.R75W, which segregated with PPK and deafness in a family but was also found in a control (the hearing ability of whom was unknown), failed to form functional channels and acted in a dominant-negative fashion to inhibit wild-type Cx26 in Xenopus oocytes [85]. Transgenic mice expressing this mutant were deaf and exhibited hair cell degeneration and organ of Corti abnormalities [86]. The overexpression of the dominant NSHL Cx26 mutant p.M163L in cell lines was associated with a cell death phenotype which did not appear to be caused by aberrant hemichannel activity [87]. These studies indicate that dominant NSHL Cx26 mutants may lead to decreased Cx26 and Cx30 GJ function in the cochlea through a dominant-negative effect and can reduce cell viability, both of which could cause NSHL.

RECESSIVE NSHL Cx26 MUTANTS: POTENTIAL HETEROZYGOTE ADVANTAGE?

The carrier frequency of specific recessive NSHL Cx26 mutations is relatively high, and specific mutations are more prevalent in certain ethnic groups. c.35delG causes 20% of childhood hereditary NSHL in those of European ancestry [88], with a carrier frequency of 2–4% in Mediterranean populations [76,89,90] and an overall frequency of 1.3–2.5% in Northern European populations [76,91]. c.235delC has a prevalence of 1% in the Japanese population [92] and c.167delT has a carrier frequency of 4% in Ashkenazi Jews [93].

The high prevalence of recessive NSHL Cx26 mutations may be because of a founder effect, and/or because these mutations confer a benefit to the carrier in one or more tissues which outweighs the detrimental effect of hearing loss, and thus a heterozygote advantage [43,76,94–96]. The recessive NSHL Cx26 mutant p.R143W is present at a high frequency in deaf individuals in Ghana [97] and, in vitro, it was found to have a predominantly cytoplasmic localization and demonstrated impaired intercellular dye transfer [43].

The skin of homo- or heterozygote carriers of p.R143W appears normal; however, they have a thicker epidermis and a higher sweat salinity compared with wild-type individuals [94]. A thicker epidermis may give increased resistance to injury, and a higher sweat salinity on the skin could offer increased protection against microbial colonization, giving advantages to carriers of this mutation [94]. c.35delG carriers also have a thicker epidermis than wild-type individuals, which may confer similar benefits [96]. Keratinocytes overexpressing p.R143W showed increased cell viability [95], increased migration and, when grown on DED (de-epidermalized dermis), gave thicker three-dimensional organotypic cultures, possibly due to an extended terminal differentiation programme [43]. As Cx26 is up-regulated at the wound site after injury, this could have implications for swifter wound healing [43]. HeLa cells overexpressing wild-type Cx26, but not p.R143W, showed increased invasion of S. flexneri, thus potentially being a carrier of this mutant may give increased resistance to invasion by gastrointestinal bacterial pathogens such as S. flexneri [43].

These investigations indicate that carrying a Cx26 recessive NSHL mutant may actually confer an advantage to that of wild-type, which may explain their surprisingly high prevalence in the human population. Future studies with other common NSHL Cx26 mutants could indicate whether they display similar properties.

MUTATIONS IN Cx26 ASSOCIATED WITH SYNDROMIC DISEASE

Specific Cx26 mutations are linked to syndromic disease (skin disease with deafness) including BPS (Bart–Pumphrey syndrome), PPK with deafness, VS (Vohwinkel syndrome), KID syndrome and HID (hystrix-like ichthyosis-deafness) syndrome (Table 2). All of the known Cx26 mutations associated with these diseases are dominant single missense mutations, the majority of which are located in the NT (N-terminus) or EL (extracellular loop) 1 of Cx26.

Functional studies have indicated that specific syndromic mutants may exert a dominant-negative effect on wild-type or other Cxs, and/or form aberrant hemichannels at the cell surface membrane [33–35,71,74,85,98]. The PPK and deafness Cx26 mutant p.del42E and the VS mutant p.D66H had a dominant-negative effect on wild-type Cx26 and Cx43 [71]. In transgenic mice expressing p.D66H, the mutant appeared to have a dominant-negative effect on wild-type Cx26 and Cx30, but not on Cx43 [98]. These data indicate that certain syndromic Cx mutants may contribute to disease by their effect on multiple Cx types in the epidermis.

The overexpression of certain syndromic Cx26 and Cx30 mutants in Xenopus oocytes and/or human cell lines has been associated with a cell death phenotype which has been attributed to the presence of ‘leaky’ hemichannels at the cell surface membrane [33–35,74]. The expression of the KID syndrome Cx26 mutants p.A40V, p.G45E, p.G12R, p.N14K and p.D50N [34,35,99] in Xenopus oocytes bathed in low Ca2+ caused cell death and large outward currents which could be prevented by raising the extracellular Ca2+ levels, indicating that these mutants form leaky hemichannels [34,35]. However, high Ca2+ levels did not rescue the cell death phenotype of a human-derived cell line, HEK (human embryonic kidney)-293 cells, expressing p.D50N [87], possibly due to differences in the processing of the mutant in the two expression systems.

ATP is a paracrine messenger involved in keratinocyte proliferation and differentiation [100,101] and it has been suggested that if aberrant Cx hemichannels exist in vivo, they could cause deregulated release of ATP from keratinocytes, disrupting keratinocyte proliferation and/or differentiation [33]. A future challenge is to ascertain whether expression of these KID syndrome mutants cause aberrant hemichannels to exist in the skin of patients and if so what the implications are.

MUTATIONS IN Cx30 ASSOCIATED WITH NSHL AND HED (HIDROTIC ECTODERMAL DysPLASIA)

Mutations in the Cx30 gene GJB6 are linked to dominant and recessive NSHL and the syndromic skin disease HED. Unlike for
Cx26 in which hundreds of mutations are associated with NSHL, only one missense mutation in Cx30, p.T5M [102], and digenic inheritance of a large GJB6 deletion, is associated with NSHL [103,104]. Both GJB2 and GJB6 are located on 13q12.11, and immunohistochemical staining on skin from a patient who was compound heterozygous for c.35delG in GJB2 and the 342 kb deletion reported in GJB6, which was del(GJB6-D13S1830) [104], showed decreased expression of Cx26 in the stratified epithelial sweat gland [105]. This indicates that the GJB6-D13S1830 deletion may affect GJB2 regulatory elements, altering Cx26 expression, and thus NSHL may result from reduced Cx26 protein production in the cochlea [105].

Dominant missense mutations in Cx30 are associated with HED, also called Clouston syndrome [106,107]. This is characterized by skin and hair abnormalities which may lead to total alopecia, nail hypoplasia, palmoplantar keratoderma and skin hyperpigmentation [106,107].

Aberrant hemichannels have been implicated as the cause of the cell death phenotype associated with the expression of the HED mutants p.G11R and p.A88V in Xenopus oocytes and HeLa cells, and of the increased release of ATP from HeLa cells expressing these mutants [33].

DISEASES ASSOCIATED WITH Cx30.3 AND Cx31 MUTATIONS

Mutations in Cx30.3 and Cx31 are linked to dominant and recessive EKV [108–110]. The Cx31 mutations p.R180X and p.E183K are associated with dominant [111], and p.141delI and p.1141V with recessive [112] NSHL, and the Cx31 mutant p.66delD is associated with dominant peripheral neuropathy and sensorineural hearing loss [32].

When overexpressed in HeLa cells, p.R180X, p.E183K and p.141delI had a primarily cytoplasmic localization, whereas p.1141V appeared to traffic to the cell surface; however, their expression did not affect cell viability [113]. Potentially these mutants could cause a reduction in Cx31 GJ function, or have a dominant-negative effect on other Cxs in the cochlea, which could cause NSHL.

The erythrokeratodermas EKV and PSEK (progressive symmetric erythrokeratodermia) are both characterized by fixed hyperkeratotic plaques of the skin. In EKV, transient areas of erythema are also present which can move or alter in shape over time. The Cx30.3 mutation p.F137L has been found to be associated with EKV with [109] or without [114] erythema gyratum repens, and the Cx31 mutation p.G12D has been found to be associated with EKV [108] and PSEK [115].

Overexpression of wild-type and the dominant EKV Cx31 mutants p.R42P, p.C86S and p.G12D tagged to EGFP (enhanced green fluorescent protein) in HeLa and keratinocyte cell lines showed that the mutants had a cytoplasmic localization and caused elevated levels of cell death [70,113,116]. Raising the extracellular levels of Ca^{2+} did not rescue the cell death phenotype of these mutants, and cells expressing p.G12D and loaded with Calcium Orange did not exhibit dye loss until after the cell had died, indicating that aberrant hemichannels are not the main mechanism of cell death [70]. However, their overexpression was associated with an up-regulation of proteasomal markers and UPR (unfolded protein response) proteins, including the ER chaperone BiP (immunoglobulin heavy-chain-binding protein)/GRP78 (glucose-regulated protein of 78 kDa) and the ER-resident protein/transcription factor ATF6 (activating transcription factor 6), indicating that ER stress is the major cause of cell death for these mutants [70]. ER stress has been implicated in a range of diseases (reviewed in [117]) and the UPR is expressed during keratinocyte differentiation in vivo [118]. Decreased expression of UPR markers HRD1 [HMG-CoA (3-hydroxy-3-methylglutaryl-CoA) reductase degradation 1] and BiP/GRP78 was observed in squamous cell carcinoma and psoriasis, implicating a potential deregulation of the UPR in certain skin disorders [118].

Cx-BINDING PARTNERS AND NON-GAP JUNCTIONAL FUNCTION OF Cx PROTEINS

Cxs were traditionally perceived as GJ proteins that had few interacting partners [119]; however, it is becoming increasingly apparent that Cx-binding partners have a role in GJ regulation and assembly (reviewed in [119–121]). In addition, evidence suggests that at least some Cxs have non-gap junction/hemichannel functions which are independent of their GJ function in cells, including altering cell growth, differentiation and resistance to injury (reviewed in [122,123]). This is significant because it means that Cxs do not mediate effects solely through their participation in GJ channels, and that Cx mutants may cause disease via their affect on partner proteins.

More than 40 Cx-interacting partners or components of the ‘GJ proteome’ [124] have been identified, including junctional proteins, enzymes and cytoskeletal proteins such as drebrin (reviewed in [120,121]). For example, Cx43 interacts with components of adherens junctions such as β-catenin [125] and tight junctions including ZO (zona occludens) proteins [126]. There are fewer known Cx26-, Cx30- and Cx31-binding partners compared with that of Cx43. In rat cochlear tissue, Cx26 interacts with OCP (organ of Corti protein)-1 [127]. Cx31 can form heterotypic interactions with Cx26, Cx30 and Cx32 [17,128] and yeast two-hybrid screening demonstrated that p11, a member of the S100 protein family, binds to Cx31 [129].

To decipher the significance of Cx-binding partner interactions, future studies are required to elucidate the full GJ proteome of wild-type and mutant Cx types, and to verify these interactions in the relevant tissue type in vivo. This could demonstrate whether a Cx mutant gains or loses the ability to interact with specific partners or participate in signalling pathways, which could be important in Cx-mediated disease.

Non-gap junctional roles for Cx types have been discovered by performing functional investigations with Cx mutants which do not form GJs, and by using GJ inhibitors. Overexpression of Cx32, Cx40 and Cx43 in cell lines gave increased resistance to injury, even when treated with GJ inhibitors and when cells were isolated from one another [130], and Cx43 overexpression led to a delay in the G_1 phase of the cell cycle, independent of GJ function [131]. Despite mistrafficking to the cell surface membrane, the EKV Cx31 mutants p.R42P and p.C86S induced differentiation of SH-SY5Y neuronal cells to the same level as that of wild-type Cx31 [132], Cx26 and Cx43 mutants rescued a neuronal migration phenotype independent of GJ function [133], and expression of both functional and non-functional Cx26 reduced the growth rate of a breast cancer cell line [134].

Collectively, these studies indicate that Cxs participate in intracellular signalling pathways, irrespective of their ability to traffic to the cell surface membrane and form GJs. This may be significant in both the functioning of wild-type Cx and in the ability of a Cx mutant protein to cause disease.

CONCLUSIONS AND FUTURE PERSPECTIVES

The important physiological role of Cxs is demonstrated by the diversity of diseases associated with Cx gene mutations in humans; however, there are important unresolved questions. Different Cx types are expressed in the cochlea and skin, but
the exact role of each Cx type in different tissues remains to be determined. Functional studies have indicated that Cx mutants may contribute to disease by different mechanisms, for example, loss of GJ function may account for certain NSHL cases. However, this is clearly not the case for skin disease mutants where the Cx protein appears to gain an aberrant function in cells. A limitation of functional in vitro investigations and mouse models is that the activity of a Cx mutant is not assessed in conjunction with the full complement of Cxs as would be present in human tissue, thus hindering correlations between the results of in vitro studies compared with the mechanism of pathogenesis in the patient. A future challenge is to discern whether the characteristics Cx mutants display in vitro are recapitulated in vivo, which the establishment of patient-derived cell lines and characterization of patient biopsies could help to overcome.

It is becoming increasingly obvious that Cxs have non-gap junctional roles in tissues, and thus the fact that Cx mutants may mediate pathogenesis indirectly via their effects on other proteins, and thus on cellular processes, cannot be eliminated. Thus characterizing the full proteome for wild-type and Cx mutants may provide insights into the downstream signalling events which cause disease. It is interesting to note that recessive NSHL Cx26 mutations may even confer a heterozygote advantage to carriers [43,94–96], and future work may ascertain what the protective mechanisms are that they are linked with.

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