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GENETIC DISEASES AND GENE KNOCKOUTS REVEAL DIVERSE CONNEXIN FUNCTIONS

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ABSTRACT

Intercellular channels present in gap junctions allow cells to share small molecules and thus coordinate a wide range of behaviors. Remarkably, although junctions provide similar functions in all multicellular organisms, vertebrates and invertebrates use unrelated gene families to encode these channels. The recent identification of the invertebrate innexin family opens up powerful genetic systems to studies of intercellular communication. At the same time, new information on the physiological roles of vertebrate connexins has emerged from genetic studies. Mutations in connexin genes underlie a variety of human diseases, including deafness, demyelinating neuropathies, and lens cataracts. In addition, gene targeting of connexins in mice has provided new insights into connexin function and the significance of connexin diversity.

INTRODUCTION

Multicellular organisms require the coordinated response of groups of cells to environmental stimuli. Intercellular channels present in gap junctions provide a simple method of synchronizing response through the direct exchange of ions, metabolites, and other messenger molecules between adjacent cells. These signaling pathways permit rapidly coordinated activities such as contraction of cardiac and smooth muscle (1, 2) and transmission of neuronal signals at electrical synapses (3–5). In addition, gap junctional communication plays a

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role in slower physiological processes, such as cell growth and development (6–8).

Vertebrate Intercellular Channels

In vertebrates, the structural proteins comprising intercellular channels are encoded by a multigene family called the connexins (9, 10). Currently, 14 mouse connexin genes have been cloned. In addition, at least six connexin genes have been isolated from other vertebrate species (11–14) for which rodent orthologs have not yet been identified. Thus, the total number of vertebrate connexin genes is likely to exceed 20. Initially, vertebrate connexins genes were separated into two groups, α and β, on the basis of overall sequence similarities, although there are no specific protein domains that reliably define either group. A common feature of both groups of connexins is a simple gene structure in which the entire coding region is contained within one exon whereas one or two introns are located in the 5' untranslated region. Analysis of the more recently characterized connexin sequences suggests that this simple categorization scheme may need refinement. For example, rodent Cx36, skate Cx35, and perch Cx34.7 may form an entirely new subfamily. These connexins (*a*) are expressed predominantly in central nervous system neurons, (*b*) contain an intron within the coding region, and (*c*) on the basis of primary sequence do not readily fit into either the α or β groups (11, 12, 15). Similarly, mouse Cx45, initially assigned to the α group (16), forms together with human Cx46.6 and zebrafish Cx43.4 a cohort more distantly related to α connexins than β connexins are (11). It is not yet clear whether there are functional correlates to these sequence differences.

Intercellular channels are unusually complex in that they span two plasma membranes. Connexins oligomerize to form single-membrane channels (hemichannels) called connexons (17), which align in the extracellular space between two cells to complete the intercellular channel. Each connexon is comprised of six connexins arranged radially around a central pore (18, 19) and can contain either a single type of connexin (homomeric) or multiple connexins (heteromeric). Theoretically, many types of heteromeric connexons can be generated, differing in either the number or the spatial organization of the different connexin proteins. Because an intercellular channel spans two plasma membranes, adjacent cells can contribute different types of connexons, giving rise to either homotypic, heterotypic, or heteromeric intercellular channels (Figure 1). Given the existence of more than 20 connexin genes, the number of structurally and thus physiologically distinct channel types may be very large. In vivo, clear evidence for the coassembly of multiple connexin proteins into single connexons and intercellular channels has been obtained (20–25).

One consequence of the molecular diversity in the connexin family is that intercellular channels assembled from different connexin proteins have unique

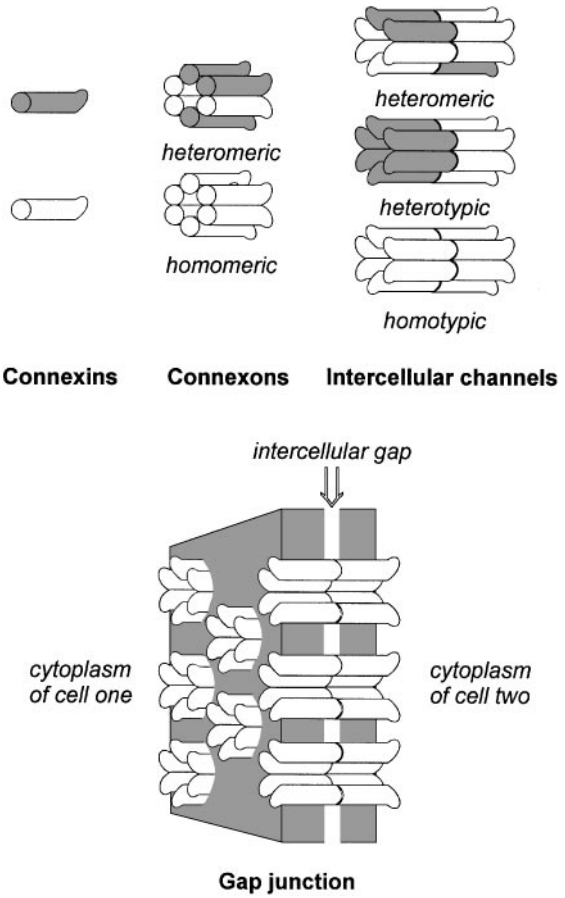


Figure 1 Organization of connexins into connexons, intercellular channels, and gap junctions. Connexin proteins oligomerize into connexons that are homomeric if they have one type of connexin or heteromeric if they contain multiple connexins. Connexons from adjacent cells align to form complete intercellular channels that span two plasma membranes. Each cell can contribute different types of connexons—giving rise to either homotypic, heterotypic, or heteromeric intercellular channels—that cluster in specialized membrane regions called gap junctions.

molecular permeabilities. Gap junctions have been historically described as relatively nonselective, permeable to a wide variety of molecules smaller than ~1200 Da (26). However, experiments carefully examining the movement of ions and dyes between cells expressing different connexins have revealed that there are connexin-dependent differences in the permeation of intercellular channels (27–29). Recently, this type of analysis has been extended to signaling molecules (30). Homomeric connexons made of Cx32 are permeable

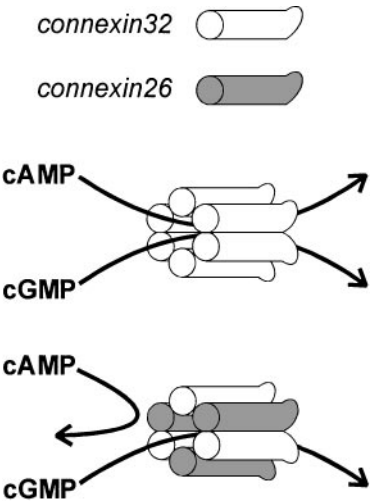


Figure 2 Isoform composition of connexin channels determines selectivity among cyclic nucleotides. Homomeric connexons made of Cx32 are permeable to both cAMP and cGMP. Heteromeric connexons containing both Cx26 and Cx32 are not permeable to cAMP but allow passage of cGMP. This is the first example of connexin-specific selectivity among second messengers (for details, see 30).

to both cAMP and cGMP, whereas heteromeric connexons composed of Cx32 and Cx26 lose permeability to cAMP but not to cGMP (Figure 2). It remains a technically daunting task to determine which molecules actually permeate intercellular channels in vivo. One recent report demonstrated that in cells metabolically labeled with [^{14}C]glucose, a significant amount of radioactive carbon exchanged through gap junctions was incorporated into ADP and/or ATP (31).

Invertebrate Gap Junction Channels

Invertebrate organisms also use gap junctional communication to coordinate activity, although connexins have never been found in invertebrate genomes. These organisms are believed to assemble intercellular channels from proteins belonging to a different gene family, originally designated OPUS, an acronym derived from the founding members ogre, passover, unc-7, and shaking-B (32). Recently, this family was renamed innexin for invertebrate analogs of the connexins (33). Because passover and shaking-B represent the same gene, there are thus far two cloned innexin genes in *Drosophila* [shaking-B/passover and lethal (1) ogre (34–36)] and at least 24 innexins in the *Caenorhabditis* genome (37–39). No vertebrate orthologs of any innexin gene have been isolated.

Innexins are predicted to encode membrane proteins with four transmembrane domains, analogous to the vertebrate connexins. However, their primary sequences show no homology with members of the connexin family (38, 39).

Several lines of evidence suggest that innexins are the channel-forming proteins comprising invertebrate gap junctions. First, some mutant phenotypes are well reconciled with the known functions of intercellular channels. For example, coordination of smooth and cardiac muscle contraction requires junctional communication, and eat-5 mutants lose synchrony of pharyngeal muscle contraction. In addition, eat-5 mutants lose detectable dye coupling, a reliable indicator of junctional communication, between anterior and posterior pharyngeal muscle groups (38). Similarly, nonlethal mutants of shaking-B fail to establish stereotypical electrical synapses in the giant fiber system. Normal appositions between interneurons and tergotrochanteral motor neurons are maintained, but normal dye coupling between these cells is not observed in the mutants (35, 40, 41).

Not all innexin mutant phenotypes are easily understood in the context of intercellular channel activity. For example, lethal alleles of ogre exhibit a significant reduction in the numbers of postembryonic neuroblasts and their offspring whereas viable alleles produce defective vision due to structural abnormalities restricted to the optic lobe (42). In addition to the lack of a clear causal relationship between these phenotypes and intercellular channel activity, the localization of the ogre gene product appears to be largely cytoplasmic, which is not consistent with its presumed role as an intercellular channel (43).

Recently, strong support for the hypothesis that innexins comprise invertebrate intercellular channels was provided by in vitro expression studies demonstrating that the lethal allele of shaking-B can form intercellular channels. Pairs of *Xenopus* oocytes injected with RNA encoding shaking-B(lethal) developed robust intercellular conductances three orders of magnitude above background (44). Another innexin, *Caenorhabditis* wxn-1, also makes intercellular channels when expressed in paired *Xenopus* oocytes (Y Landesman, TW White, TA Starich, JE Shaw, DL Paul, unpublished data). However, as seen with certain connexins (45), not all innexins are capable of inducing intercellular conductance in oocyte pairs. Shaking-B(neural), an alternatively spliced form of shaking-B expressed largely in the nervous system, failed to form active intercellular channels (44).

Surprisingly, the physiological properties of invertebrate and vertebrate intercellular channels are similar, despite the complete lack of sequence identity between connexins and innexins. One striking similarity is in the gating of these channels by voltage. Imposition of a potential difference between coupled cells results in junctional currents that generally decrease over time, reflecting a voltage-dependent closure of the intercellular channels. Two characteristic

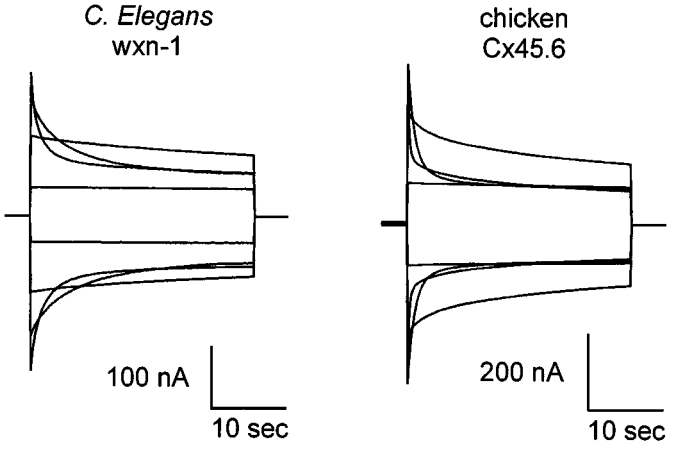


Figure 3 Similarities in the macroscopic voltage gating of innexin and connexin channels. The effect of transjunctional voltage on the junctional currents developed by oocyte pairs expressing a connexin (chicken Cx45.6) or an innexin (*Caenorhabditis elegans* wxn-1) is shown. Depolarizing voltage steps of 10, 30, 50, and 70 mV were applied to one cell of each pair. At transjunctional voltages greater than 20 mV, innexin and connexin junctional currents slowly decreased over the time of the voltage step (30 s), with similar kinetics. In both cases, the voltage-dependent closure was not complete and a substantial residual current remained. The voltage gating of the innexin channel fell well within the range of behaviors exhibited by members of the connexin family (reviewed in 140).

features of connexin voltage gating are also observed with innexins. First, the kinetics of voltage gating is unusually slow, taking several seconds for the decay in junctional currents to reach equilibrium. Second, in both cases, the voltage-dependent closure is not complete and a substantial residual current remains (Figure 3). When the activity of single channels was examined, a third similarity in gating behavior was observed. Like most ion channels, connexin and innexin channels gate between fully open states and substates on a fast time scale (1–2 ms) (46–49). However, both channel types also exhibit an unorthodox, slower gating step between the fully closed state and a substate (10–60 ms) (48, 50–52; but also see 53). Finally, connexin and innexin channels appear to be sensitive to the same classes of pharmacological agents. For example, both are closed by decreases in intracellular pH and millimolar concentrations of alkanols or halothane. Together, these data illustrate a remarkable similarity in channel properties obtained in the absence of any significant primary sequence identity.

The clarification of the identity of invertebrate intercellular channel proteins is a major advance in gap junction biology and opens up powerful genetic

systems to studies of intercellular communication. Recently, a great deal of information on the physiological roles of connexins has also become available from genetic studies in vertebrates. The remainder of this review focuses on the functional roles of intercellular communication that have been elucidated from studies of connexin mutations in human hereditary disease and in mice with targeted deletion of connexin genes.

CONNEXIN MUTATIONS IN HUMAN DISEASE

Although intercellular channels have been well characterized in terms of structure and biochemistry, information regarding their biological roles is still limited. However, the association of connexin mutations with an increasing number of human pathologies, including X-linked Charcot-Marie-Tooth disease (54), sensorineural hearing loss (55), and congenital cataract (56), provides an unequivocal demonstration that gap junctional communication is crucial for diverse physiological processes. Moreover, analysis of the functional consequences of the specific connexin mutations underlying these human disorders is providing new insights into the roles of connexin diversity in vivo.

Mutations in Cx26 Cause Nonsyndromic Deafness

Genetic deafness is one of the most prevalent inherited sensory disorders, affecting about 1 in 2000 children. Both autosomal recessive (DFNB1, OMIM 220290) and autosomal dominant (DFNA3, OMIM 601544) forms of deafness were mapped near the chromosomal location of human Cx26 (57, 58). These forms of deafness are described as nonsyndromic because no other organ system exhibits defects. Because Cx26 was known to be highly expressed in cochlear cells (59), it became a likely candidate gene for these disorders. Subsequently, Kelsell and colleagues identified Cx26 coding region mutations in affected members of three families with recessive nonsyndromic deafness. In the same study, a different Cx26 mutation was identified in a pedigree with a dominant pattern of inheritance (55).

Multiple studies have confirmed that mutations in Cx26 are associated with recessive deafness (60–65). The most frequent mutation identified is a single base deletion (35delG) resulting in a frameshift and consequent premature termination of the Cx26 protein. This deletion comprises 50–70% of all mutant alleles in various ethnic populations. The prevalence of a single mutation has not been observed in other connexin-related disorders and may reflect a mutational hot spot within the Cx26 gene. Other Cx26 defects include nonsense mutations and small deletions/insertions, most of which also lead to premature termination of protein translation and the probable loss of function. In addition, recessive deafness in compound heterozygotes (individuals where each

allele contains a different Cx26 mutation) has been reported. Taken together, these data reveal a tight association between mutations in Cx26 and autosomal recessive nonsyndromic deafness.

In contrast, the role of Cx26 mutations in dominant forms of deafness has been more controversial. Kelsell et al originally reported a heterozygous point mutation (M34T) in a family with dominant deafness and proposed that the mutant allele inhibited the activity of the wild-type Cx26 allele (55). This hypothesis was supported by functional expression studies using pairs of *Xenopus* oocytes programmed with connexin RNAs. The M34T variant, which does not form functional channels when expressed alone, acted as a dominant inhibitor of wild-type Cx26 channel activity when coexpressed (66). However, the ability of the M34T variant to cause dominant deafness was brought into question by the finding of heterozygous M34T mutations in individuals with normal hearing (62, 67). In light of the functional data demonstrating dominant inhibition, it is difficult to explain the normal hearing in carriers of the M34T allele. Possible mechanisms include second-site mutations that silence the allele or compensatory changes in the expression of other genes. Analysis of M34T allelic expression in carriers with normal hearing may help to resolve this issue. Regardless of its role in dominant deafness, the M34T variant can contribute to deafness in compound heterozygotes (62), which is consistent with the data demonstrating loss of channel activity.

Recently, additional Cx26 mutations linked to dominant deafness have been found. In one study, dominant deafness segregated with a heterozygous missense mutation of Cx26, causing a nonconservative amino acid substitution (R75W). The dominant inhibitory effect of the R75W variant on channel function was also demonstrated using the paired oocyte expression system (68). As in the case of M34T, the R75W mutant was unable to induce electrical conductance between adjacent cells and completely suppressed the activity of coexpressed wild-type Cx26. For comparison, a neighboring mutation (W77R) observed in a family with autosomal recessive deafness (63) was also tested. The W77R mutant also failed to induce intercellular channel activity, but it did not inhibit the ability of wild-type Cx26 to form functional channels when coexpressed, which is consistent with its recessive pattern of inheritance. An additional Cx26 point mutation (W44C) has been found in the original pedigree used to map autosomal dominant deafness (57). All 10 of the deaf individuals, but none of the 17 normal hearing individuals, of a kindred were heterozygous for the W44C allele (69). Collectively, these results indicate that heterozygous Cx26 mutations can have serious functional consequences, and they strongly support the identification of Cx26 as both the DFNB1 and DFNA3 genes.

Although the precise role of Cx26 in the etiology of nonsyndromic deafness is not known, it is likely that junctional communication influences the ionic environment of inner ear sensory epithelia. In mammals, there are no clear

reports of gap junctions involving vestibular hair cells, but gap junctions and junctional communication have been well documented in other cochlear cells (70). Cells interconnected by gap junctions fall into two groups: nonsensory epithelial cells among which hair cells are dispersed, and connective tissue cells at more distal locations to the hair cells. Immunocytochemical analysis indicates that Cx26 is present in nearly every location where cochlear gap junctions have been found (59), and it is believed to be the major connexin expressed by supporting cells.

It has been proposed that serially arranged gap junctions of epithelial and connective tissue cells serve as a mechanism for recycling endolymphatic K^+ ions that pass through sensory cells during auditory transduction. The mammalian cochlea has an unusual arrangement of extracellular fluid-filled spaces, which provides a unique environment for the sensory cells. Basolateral hair cell surfaces are bathed in perilymph, which has an ionic composition similar to that of other extracellular fluids. The apices of hair cells, however, are bathed in endolymph, which has high K^+ and low Na^+ concentrations, similar to intracellular fluids. When hair cells are activated by sound, receptor potentials are generated from the flow of K^+ ions from endolymph into the hair cell. The K^+ ions are then released from hair cells into interstitial space within the organ of Corti (71), where K^+ channels in cochlear supporting cells permit uptake of these ions (72). It was proposed that junctional communication between the supporting cells facilitates recirculation of K^+ ions back to the endolymph by providing an intercellular pathway for conveyance and distal release of K^+ ions. A conceptually similar role for junctional communication has been proposed in the spatial buffering of K^+ by astrocytes in the central nervous system (73). Appropriate evaluation of this model in terms of nonsyndromic deafness awaits the development of transgenic animals with Cx26 mutations.

Human Peripheral Neuropathy and Mutations in Cx32

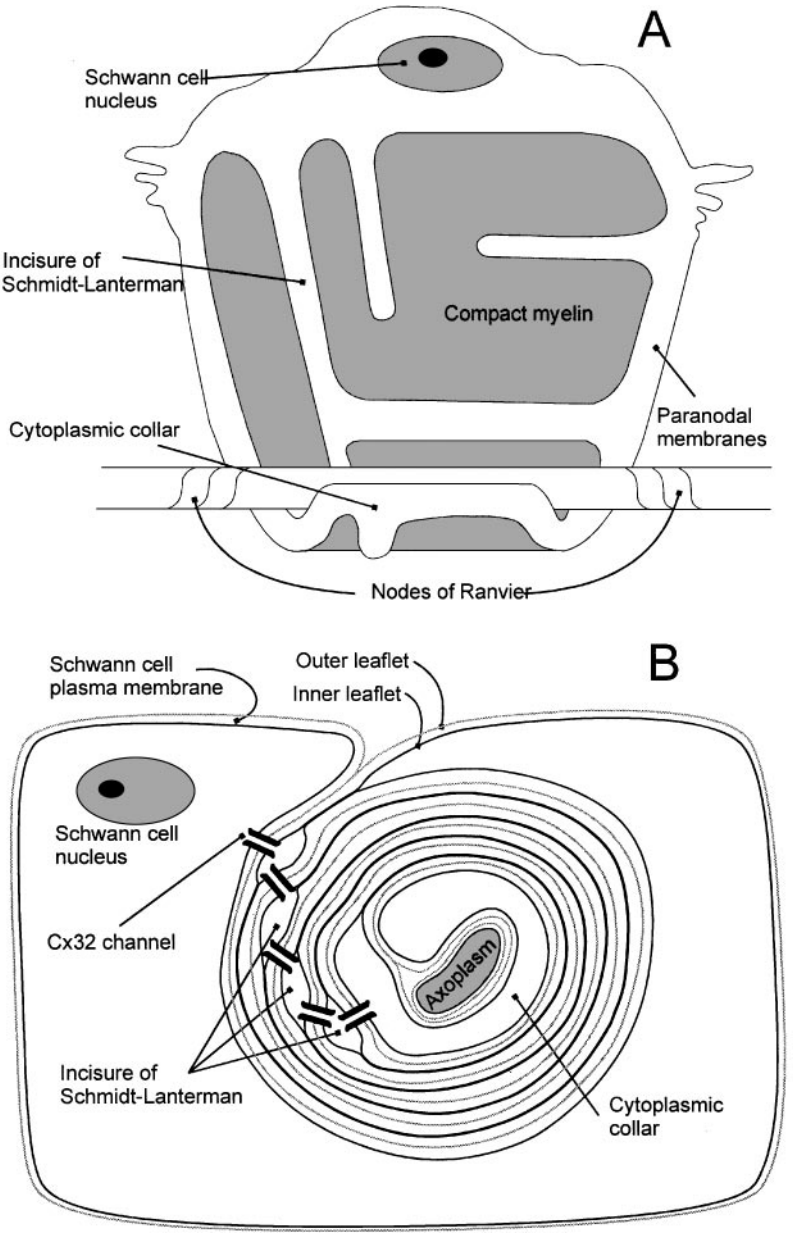
The first disease shown to result from mutations in a connexin is a form of Charcot-Marie-Tooth disease (CMT). CMT is a genetically heterogeneous group of neuropathies resulting in progressive degeneration of peripheral nerve, and it is characterized by distal muscle weakness and atrophy as well as impairment of sensation and deep tendon reflexes. Most forms of CMT, which affect 1 out of every 2500 people, are demyelinating syndromes associated with Schwann cell defects. Linkage studies placed the locus of an X-linked form (CMTX) near the map location assigned to Cx32 on the X chromosome. Subsequently, direct sequence analysis of the Cx32 coding region in CMTX patients revealed numerous mutations (54, 74).

The association of genetic abnormalities in Cx32 with a demyelinating disease of the peripheral nervous system was unexpected because gap junctions are extremely rare, if present at all, between adjacent myelinating Schwann

cells (75). Nonetheless, immunolocalization of Cx32 reveals that Schwann cells express Cx32 and concentrate it in the uncompacted membranes adjacent to the nodes of Ranvier and at the incisures of Schmidt-Lanterman (54, 76). This distribution suggests a unique functional role for Cx32 in Schwann cells, as illustrated in Figure 4. Unlike compact myelin, the paranodal membranes and incisures enclose cytoplasm, providing continuity between the Schwann cell body and the cytoplasmic collar of the myelin sheath adjacent to the axon (Figure 4*A*). Presumably, this continuity permits diffusion of nutrients and ions between perinuclear and periaxonal Schwann cell cytoplasm and is likely to play a critical role in the transduction of signals between Schwann cell and axon. As the myelinating Schwann cell wraps the axon, the cytoplasmic continuity assumes a spiraling shape, forming the incisures and paranodes. In a large motor nerve, a Schwann cell may elaborate so many turns that the length of the spiraling pathway is too great for molecular diffusion to support the metabolic needs of the cell (77). To solve the problem, Bergoffen et al (54) proposed that a shorter, radial pathway for diffusion is generated by intracellular [reflexive (78)] gap junctions directly connecting adjacent wraps of myelin at incisures and paranodal membranes (Figure 4*B*). Cx32 mutations disrupting the normal functioning of these gap junctions could affect the diffusion of nutrients or signaling molecules between Schwann cell body and distal processes. Support for this hypothesis was obtained from recent studies using intracellular dye injection and video microscopy showing that only molecules small enough to permeate gap junctions can rapidly diffuse between adaxonal and perinuclear Schwann cell cytoplasm (79).

At least 90 different Cx32 mutations are associated with CMTX (80), which supports the identification of Cx32 as the disease-causing gene. The mutations are mostly coding region mutations but they do not cluster in any particular sequence domain. To determine the effects on channel activity, 21 different mutations have been analyzed using the paired oocyte system or transfected tissue culture cells (81–84). In about half the cases, active intercellular channels

Figure 4 Cx32 may form reflexive gap junctions within, rather than between, Schwann cells. (*A*) Schematic diagram of a myelinating Schwann cell unwrapped from the axon it invests. In compact myelin, Schwann cell cytoplasm is extruded and the cytoplasmic leaflets of the plasma membrane come into close apposition (*grey areas*). In contrast, cytoplasm is retained at incisures of Schmidt-Lanterman and paranodal membranes to maintain continuity between Schwann cell body and the cytoplasmic collar of the myelin sheath adjacent to the axon [adapted from Doyle & Colman (141)]. (*B*) Cross-sectional view of the connections formed by Cx32 at the incisures of Schmidt-Lanterman in a single Schwann cell. Cx32 is specifically localized to incisures and paranodal membranes, where intracellular (as opposed to intercellular) channels dramatically reduce the path length between Schwann cell body and periaxonal cytoplasm (adapted from 142).


A
Schwann cell nucleus
Incisure of Schmidt-Lanterman
Compact myelin
Paranodal membranes
Cytoplasmic collar
Nodes of Ranvier
B
Schwann cell plasma membrane
Outer leaflet
Inner leaflet
Schwann cell nucleus
Cx32 channel
Axoplasm
Incisure of Schmidt-Lanterman
Cytoplasmic collar

were not observed, which is consistent with a model in which complete loss of function causes the disease. However, channel activity was readily detected in 11 cases. In 9, only subtle alterations in macroscopic properties such as sensitivity to gating by pH and voltage were observed. The physiological consequence of these alterations is currently not clear. However, marked changes in functional properties in two active variants were revealed by a more detailed single-channel analysis (83). A S26L mutation exhibited a significant reduction in permeability to small solutes, whereas a M34T mutation showed a decrease in the channel open probability. These changes can be clearly interpreted as partial loss of function and are more easily reconciled with a disease phenotype.

Human Cataracts and Mutations in Cx50

Congenital cataracts are a leading cause of visual impairment or blindness, often following an autosomal dominant pattern of inheritance (85–87). A form of congenital cataract exhibiting zonular pulverulent opacities (CZP1, OMIM 116200) in an extensively studied eight-generation kindred (56, 88, 89) was recently mapped near the human Cx50 gene (90, 91). Cx50 was strongly implicated as a candidate gene for CZP1 because it is predominantly expressed in the lens (92). Sequence analysis of the coding region of Cx50 in affected members of this large pedigree revealed a C to T transition in codon 88 resulting in the nonconservative substitution of serine for proline in the second transmembrane domain (P88S). This mutation segregated with the cataract phenotype and was not detected in unaffected family members or in a control population (56).

Although the effect of this mutation on the activity of Cx50 has not yet been tested, several independent observations suggest that it is likely to result in a change in channel activity. First, the mutated proline is strictly conserved among all known connexins. Second, the same mutation in human Cx32 has been found to segregate with the peripheral neuropathy CMTX in two independent pedigrees (93, 94). Finally, in vitro mutagenesis of the equivalent proline residue in Cx26 altered channel activity. Cx26 proline mutants were no longer able to form functional homotypic channels, although they could form heterotypic channels with wild-type Cx26 or Cx32. Moreover, the heterotypic channels containing mutated Cx26 exhibited dramatically aberrant voltage gating (95). These data raise the possibility that the P88S mutation may have dominant effects on wild-type channel activity, a possibility that can be directly tested in vitro.

Mutations in Cx50 may perturb the intercellular communication that joins the cells of the lens into a functional syncytium (96). Because the lens is an avascular cyst, intercellular channels allow cells in the interior of the organ to gain access to metabolites absorbed at the surface from the aqueous humor (97).

This metabolite exchange has been proposed to maintain the precise intracellular ionic conditions necessary to prevent precipitation of the crystallins and subsequent cataract formation (98–100). Supporting this idea is the observation that mice with targeted deletions of lens connexins develop cataracts (100, 101) (see below).

Why Are the Disease Phenotypes So Restricted?

The confinement of phenotype to the lens for Cx50 mutations is reasonably consistent with its expression pattern. Cx50 is largely, although not exclusively (102–104), restricted to this organ. However, a surprising aspect of both CMTX and nonsyndromic deafness is that the connexin mutations do not cause gross functional abnormalities in multiple organ systems. Both Cx32 and Cx26 are major components of gap junctions in many cell types [for review, see Bruzzone et al (9)]. Why, then, do most cell types expressing mutated versions of these connexins appear to function normally? The explanation may be that most cells express multiple connexins. Other connexins, either normally present or up-regulated in response to the mutation, could compensate. In this scenario, myelinating Schwann cells or cochlear supporting cells may fail because either they express only one connexin or the other connexins they can produce are unable to duplicate a Cx32- or Cx26-specific function. It remains to be determined whether multiple connexins are simultaneously expressed in these cell types.

DIRECTED MUTATIONS IN CONNEXIN GENES

The analysis of connexin mutations associated with human genetic diseases has provided critical insights into their biological function. However, detailed study of disease etiology would be greatly facilitated by animal models. Potentially, such models could derive from the generation of mice with targeted disruptions of specific connexin genes. In addition, this approach could be used to examine the biological roles of connexins not yet implicated in human genetic disease. To date, seven connexin gene knockouts have been reported. In some cases the knockout phenotypes have confirmed existing hypotheses of gap junction function, and in others they have identified new questions about the role of connexin diversity in vivo.

Deletion of Cx26 Results in Embryonic Lethality

Unlike loss of function mutations of human Cx26, targeted disruption of murine Cx26 results in lethality at embryonic day 11. The failure of Cx26 knockouts to thrive was explained by postulating a role for junctional communication in transplacental movement of nutrients. It was reported that transfer of a nonmetabolizable glucose analogue across the chorioallantoic placenta from maternal

to fetal blood was reduced in the knockout embryos (105). In mice, glucose must cross two adjacent cell layers, syncytiotrophoblast I and II, to be transported from maternal to fetal blood. Entry of maternal glucose into the cytoplasm of type I cells is facilitated by GLUT1 transporters in its plasma membrane. Subsequently, glucose diffuses between the two syncytiotrophoblast cell layers, presumably through intercellular channels made of Cx26. Glucose release into the fetal blood from syncytiotrophoblast II cytoplasm again utilizes the GLUT1 transporter (106). As described above, mutations in the human Cx26 gene result in hereditary deafness, not embryonic lethality. However, the human placenta contains only one syncytiotrophoblast cell layer rather than two layers connected by Cx26, as in mice. Thus, the loss of functional Cx26 presumably does not result in placental transport defects in humans.

Hepatic Abnormalities in Cx32 Knockout Mice

Although Cx32 mutations are associated with a human neuropathy, Cx32-deficient mice exhibit only subtle defects in the peripheral nervous system. However, dramatic abnormalities are present in the liver, an organ in which Cx32 is abundantly expressed. First, mobilization of glucose from glycogen stores is severely affected in these animals. Following electrical stimulation of postganglionic sympathetic neurons entering the liver at the porta hepatis, glucose release was decreased 78% in knockout animals. This lowered response was not due to decreased noradrenaline receptors or glycogen stores because normal glucose release was observed from knockout hepatocytes after vascular perfusion of either noradrenaline or glucagon (107). These data provide an example of a coordinated tissue response to external stimuli mediated by intercellular channels. Hepatocytes at the portal end of the hepatic lobule receive stimulation by sympathetic fibers that terminate at the edge of the lobule, whereas hepatocytes at the venous end of the lobule are not directly innervated. Stimulation results in the generation of intracellular 1, 4, 5-trisphosphate and release of Ca^{2+} from intracellular stores. Because both of these second messengers can pass between cells via intercellular channels (108, 109), junctional communication most likely propagates signals for glucose mobilization in hepatocytes far removed from the local noradrenaline stimulus.

Cx32 knockout mice also exhibit a 25-fold increased rate of spontaneous hepatic tumor formation. The susceptibility to tumors induced by intraperitoneal injection of the carcinogens is also markedly increased (8). These data are consistent with the hypothesis that inhibitory signals passing through intercellular channels contribute to growth control (110). Cx26, which is also present in normal hepatocyte gap junctions, is markedly reduced in the Cx32 knockout, although some Cx26 expression and channel activity persists (8, 105). It is currently unclear why the residual communication provided by Cx26 is unable

to prevent the increased hepatocarcinogenesis in Cx32-deficient mice. Interestingly, no abnormalities in hepatic function, or increased incidence of cancers of any type, have been reported in humans with Cx32 mutations (CMTX, see above).

Unexpectedly, peripheral myelination in Cx32 knockout animals is only slightly affected compared with humans with essentially null mutations in Cx32 (see above). Although morphological signs of demyelination can be detected by electron microscopy, the number of affected fibers is relatively small and nerve conductance properties are altered only slightly (112). These findings are consistent with the observation that communication between the abaxonal and adaxonal aspects of Schwann cell cytoplasm in Cx32 knockouts is not qualitatively different from wild-type mice (79). Although a direct demonstration has not been made, one explanation is that murine Schwann cells express additional connexins not present in human cells. In support of this notion, Cx46 has been detected in adult rodent sciatic nerve and cultured Schwann cells (113).

Deletion of Cx37 Leads to Female Infertility

Ovarian follicular development requires complex intercellular signaling to orchestrate growth. At birth, the ovary contains primordial follicles consisting of meiotically arrested oocytes surrounded by a single layer of granulosa cells. Periodically, subsets of primordial follicles undergo further development during which oocytes increase in size while the granulosa cells proliferate, stratify, and develop a fluid-filled antrum. During this period, the innermost layer of granulosa cells extend processes through the zona pellucida, a thick extracellular matrix, to make gap junctions with the oocyte (Figure 5). Granulosa cells also make gap junctions with each other. Around the time of antrum formation, the oocyte matures enough to resume meiosis but is inhibited from doing so, presumably by surrounding granulosa cells. It has been hypothesized that gap junctions between the oocyte and granulosa cells transduce inhibitory signals, one of which is likely to be cAMP (114–117). After ovulation, meiosis resumes and the granulosa cells remaining in the follicle proliferate and differentiate into progesterone-secreting cells to form a corpus luteum. One model for control of luteinization postulates that the oocyte actively represses granulosa differentiation by releasing inhibitory factors. However, the nature of these signals and their mechanism of transduction have not been defined.

Female mice lacking Cx37 are infertile because ovulation does not occur (7). Cx37 knockouts show defects in at least three aspects of ovarian development. First, the follicle stops growing as the antrum begins to form. The extent of growth inhibition is variable, but mature follicules are never observed. Second, only a small percentage of oocytes mature sufficiently to resume meiosis. This finding suggests that growth-promoting signals from follicular cells

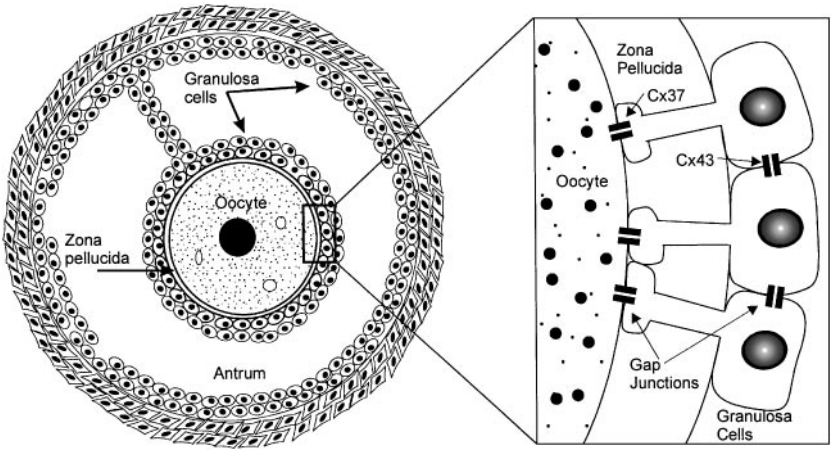


Figure 5 Cx37 is required for bidirectional signaling between oocytes and granulosa cells. In a mature ovarian follicle, the oocyte is surrounded by several layers of granulosa cells and this aggregate is suspended within a fluid-filled antrum. Granulosa cells adjacent to the oocyte extend processes through the zona pellucida and assemble intercellular junctions with the oocyte, including gap junctions that contain Cx37. In turn, granulosa cells are coupled to each other by intercellular channels containing Cx43. In Cx37 knockouts, oocyte-granulosa coupling is ablated, and granulosa-granulosa coupling persists. As a result, female mice lacking Cx37 show defects in follicular growth, oocyte maturation, and control of luteinization [adapted from Nicholson & Bruzzone (143)].

necessary for oocyte maturation permeate through gap junctions. Third, ovaries of sexually mature knockouts contain 10-fold more corpora lutea than do ovaries of wild-type. Because luteinization in normal animals occurs at ovulation when, by definition, oocyte-granulosa cell communication is lost, the premature luteinization observed in the knockout suggests that junctional communication is a major mechanism regulating corpus luteum formation.

Cx37 is readily detected in the junctions between oocyte and granulosa cell, whereas Cx43 appears to be the most abundant connexin in granulosa/granulosa junctions (7, 118). In the Cx37 knockout, gap junctions between oocyte and granulosa cells are lost and intercellular communication cannot be detected. In contrast, communication between granulosa cells is not affected. Thus, loss of only the oocyte-granulosa signaling pathway appears to account for the complex changes in ovarian development leading to infertility. One unresolved issue is the role of junctional communication in maintaining meiotic arrest. Previous models predict that meiotically competent oocytes lacking junctional communication with granulosa cells would undergo premature meiotic resumption. The fact that the majority of knockout oocytes did not mature

sufficiently to become meiotically competent precludes a simple test of this model.

Aberrant Cardiac Conduction in Cx40-Deficient Mice

In the mouse, knockout studies suggest that Cx40 has an important functional role in synchronizing contraction of cardiac muscle (119, 120). Cx40 is expressed at high levels in the His-Purkinje system (121), a network of cells specialized for rapid conduction of excitation to the apical ventricular myocardium. The conduction properties in the His-Purkinje system can be monitored by three-lead electrocardiography (ECG). As diagrammed in Figure 6, each cardiac cycle is initiated in the sinoatrial node. Depolarization then spreads through the atrial myocardium from right to left and superior to inferior, causing a wave of contraction to extend down toward the ventricles. The excitatory impulse is prevented from passing to the ventricular myocardium by a thick connective tissue septum. The wave of excitation impinges on the atrioventricular (AV) node, which introduces a delay as the action potential passes slowly through the AV node cells. Subsequently, the impulse is rapidly conducted along the His-Purkinje bundles, which are insulated by subendocardial connective tissue, to excite the ventricular myocardium at the apex of the heart. The result is a wave of contraction that travels upward back through the ventricles, expelling their contents into the pulmonary artery and aorta.

Two laboratories have knocked out the Cx40 gene (119, 120) with similarities and differences in the phenotype. A representative example of an ECG taken from a wild-type and a Cx40 knockout mouse is shown in Figure 6. In these recordings, the first deflection (P wave) results from atrial depolarization. This is followed by a long PR interval, which corresponds to the slow conduction through the AV node and rapid conduction through the His-Purkinje system. The second series of positive and negative deflections (QRS complex) results from ventricular depolarization, and the last deflection (T wave) is due to ventricular repolarization. In both studies, dramatic changes in atrioventricular and intraventricular conduction were observed.

First, atrioventricular conduction was slowed in Cx40 knockouts. The interval between the start of atrial depolarization and the start of ventricular depolarization (PR interval) was $\sim$20% longer in knockouts than in wild-type or heterozygote control animals. The PR interval reflects the time required for excitation to traverse the atrium, AV node, and His-Purkinje system. AV node conduction is typically slow and accounts for most of the interval. Because there is no Cx40 in the AV node, it is reasonable to propose that AV node conduction is unaffected in Cx40 knockouts. That being the case, the 20% difference in PR intervals most likely reflects a much greater difference in His-Purkinje conduction times between knockout and wild-type animals.

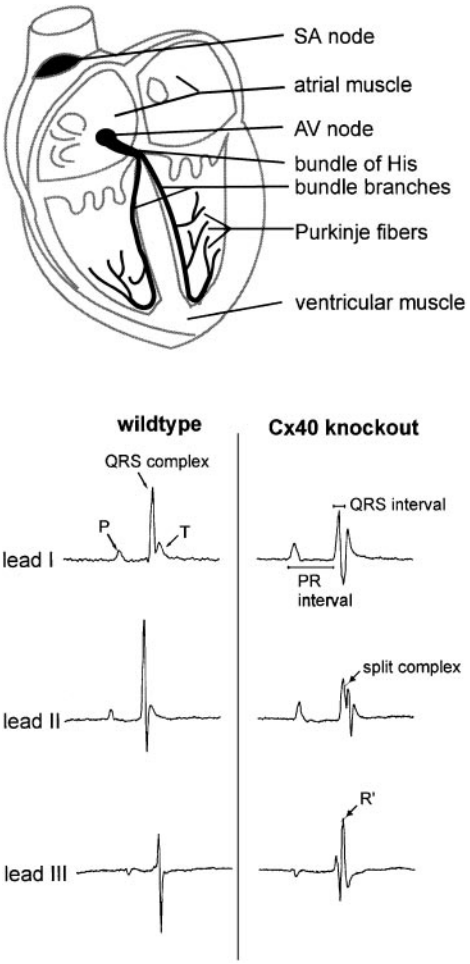


Figure 6 Slowed cardiac conduction in Cx40 knockouts. Each heartbeat is initiated in the sinoatrial node (SA) and propagates through the atrial muscle arriving at the atrioventricular (AV) node. The action potential then rapidly travels down the His bundle, its branches, and the Purkinje fibers to excite the ventricular myocardium at the apex of the heart. In an electrocardiogram (ECG), the P wave corresponds to depolarization of the atria. The PR interval is the sum of slow conduction through the AV node and rapid conduction through the His-Purkinje system. The QRS complex reflects the depolarization of the ventricles, and the T wave their repolarization. Cx40 knockout ECGs display prolonged PR intervals and QRS durations compared with wild-type ECGs, indicating that both AV and intraventricular conduction are delayed. Cx40 knockout ECGs also exhibit split QRS complexes and rSR' morphology, a possible indication of uncoordinated ventricular activation (adapted from 119).

Intraventricular conduction was also substantially delayed in Cx40 knockouts; QRS complexes were ~33% longer than wild-type control animals. The intraventricular delay was not likely to reflect slow propagation through the working myocardium because Cx40 is not expressed in ventricular muscle (122); it was more likely the result of slow conduction through Purkinje fibers, which could result in altered spatial or temporal activation of the ventricles. In support of this idea, lead II QRS complexes were also frequently split, whereas lead III complexes often exhibited rSR' morphology in which there is a second larger R wave following an S wave. Although these complex patterns can be difficult to precisely interpret, in humans they are generally indicative of asynchronous activation of right and left ventricles. Unlike the case of Cx43 (see below), elimination of only one copy of the Cx40 gene had no effect on conduction.

Significantly, the knockout phenotypes were not completely identical in the two studies. Both observed increases in P wave duration, indicating slower atrial depolarization, which is consistent with the expression of Cx40 in atrial myocytes (123). However, the difference between control and knockout was 9% in one case and 36% in the other. Another discrepancy between the studies was observed with regard to transient cardiac arrhythmia. In normal sinus rhythm, P waves are followed at a consistent interval by a QRS complex, indicating that ventricular depolarization is being triggered by impulses originating in the atrium and that conduction through the His-Purkinje system is not completely blocked. Simon et al consistently observed sinus rhythm (119), which could be explained by the presence of Cx45 in the His-Purkinje system (124). In contrast, Kirchhoff et al (120) reported a loss of sinus rhythm in 20% of the knockout animals. These differences have not been resolved but may be due to variations in the strain background or the anesthesia protocols used during the ECG measurements.

Abnormal Cardiac Development and Function in Cx43 Knockout Mice

Cx43 is widely distributed throughout vertebrate tissues. Originally characterized in myocardium (125), Cx43 is found in almost all organs in varying amounts. A targeted disruption of the Cx43 gene (126) resulted in a dramatic malformation at the origin of the pulmonary artery involving the development of labyrinthine, anomalous septae that partially or completely occlude the right ventricular outflow to the lungs. As a result, the animals are unable to oxygenate their blood independently of the placenta and they die postnatally of asphyxiation. The remainder of embryonic development is grossly normal, even though Cx43 is widely expressed throughout embryogenesis, beginning at the 8-cell stage (127). Embryonic survival is likely due to the normal coexpression of

other connexin genes. For example, morula stage embryos express at least five connexins in addition to Cx43 (128).

The sensitivity of the conotruncal region of the developing heart to the lack of Cx43 has been attributed to changes in the migratory behavior of neural crest cells that express Cx43 and participate in the development of the outflow pathway (129). Neural crest cells derived from Cx43 knockouts displayed slower rates of migration in vitro whereas neural crest cells derived from transgenic lines constitutively overexpressing Cx43 showed accelerated migration. Consistent with this finding, the constitutive overexpressors also exhibited conotruncal abnormalities, although other structures that were normal in the knockout were affected (130). Finally, partial rescue of the cardiac defect was observed when the Cx43 overexpressors were bred into the Cx43 knockout line. The effect of changes in Cx43 levels in other neural crest derivatives remains to be determined.

Recently, a significant abnormality in cardiac conduction was reported for heterozygotes carrying one disrupted Cx43 allele (131, 132). Although these animals are grossly normal and otherwise healthy, ventricular conduction is 30% slower in them than in control animals. This is consistent with the fact that Cx43 protein levels are reduced by 50%, but it is nevertheless surprising because the magnitude of junctional communication was previously not believed to be a rate-limiting step. Indeed, the level of coupling necessary to entrain sinoatrial node pacemaker cells has been estimated to be only 3–5 active intercellular channels (133, 134).

Cx46 and Cx50 Provide Critical But Different Functions in Lens Physiology

The lens has two distinct types of cells: A simple cuboidal epithelium lines the anterior surface, and differentiated fibers constitute the rest of the lenticular mass. Mitotically active epithelial cells at the lens equator differentiate to give rise to the lens fibers, which lose intracellular organelles and accumulate high concentrations of soluble proteins known as crystallins (135). These cellular properties, together with an elastic capsule and zonular fibers, result in the optical transparency, high refractive index, and elasticity necessary for accommodation. With the loss of cellular organelles, the fibers lose the ability to support oxidative phosphorylation and an active metabolism. Gap junctional communication joins all the cells of the lens into a functional syncytium, and it is likely that this intercellular communication allows the metabolically active epithelium to maintain the precise intracellular ionic conditions necessary to prevent precipitation of the crystallins and cataract formation (98–100). In support of this hypothesis, both Cx46 and Cx50 knockout mice develop cataracts, although the nature of the defects is distinctly different in each case.

Cx46 knockout mice exhibit a progressive "senile-type" cataractogenesis. Lens growth and development are normal, but after the mice reach three weeks of age, lens opacities appear. In adult animals, aberrant proteolysis of crystallins was observed, which may have contributed to their conversion to insoluble forms associated with cataractogenesis (101). Because Cx46 and Cx50 have a similar, if not identical, distribution in lens fibers (25), presumably both connexins contribute to intercellular channels joining fibers. The ability of lens fibers to communicate in Cx46 knockouts was not directly tested, although it may persist because Cx50 distribution was unaffected in Cx46 knockout lenses (101). Thus, cataracts in the Cx46 knockout could develop either from a reduction in the absolute number of intercellular channels, or because the presence of Cx46 provided an activity not obtainable from Cx50 alone. The first possibility would be supported if targeted disruption of the Cx50 and Cx46 genes produced the same phenotype, whereas the second possibility could result in different phenotypes.

Deletion of the Cx50 gene by homologous recombination results in reduced growth of the lens and eye and the appearance of zonular pulverulent nuclear cataracts (100a). The microphthalmia most likely results from the retardation of lens growth, as studies of chick eye development following extirpation of the lens demonstrated that the growth of the eye is dependent on the lens (137). Similarly, ablation of the lens by transgenic expression of toxic genes results in reduced eye growth in mice (138, 139). In turn, the reduction in lens size resulted from a transient inhibition of growth rate during the first postnatal week. The mechanism whereby Cx50 intercellular channels stimulate fiber growth and differentiation remains to be determined.

Cx50 knockout mice develop cataracts in the first postnatal week, providing a model to study the early cellular events associated with human congenital cataracts that result from mutations in Cx50 (described above). Injection of gap junction permeable tracers into Cx50 knockout lenses before cataracts had formed demonstrated the persistence of intercellular communication between epithelial cells, between fiber cells, and between the epithelium and fibers. These results suggested that intercellular communication per se was not sufficient to prevent cataractogenesis, but that intercellular channels incorporating the unique properties of Cx50 were required. In contrast to the Cx46 knockout (101), the deletion of Cx50 did not provoke aberrant proteolysis of crystallins, although their conversion to insoluble forms led to lens opacity.

Comparison of the Cx46 and Cx50 knockout studies strongly suggests that the channel diversity resulting from the expression of two connexins in lens fibers is required for normal lens homeostasis. The restriction of the growth phenotype to loss of Cx50 but not Cx46 suggests that an early postnatal growth signal may propagate efficiently through Cx50 but not Cx46 channels. Alternatively, at the

critical time in development, there could be differences in the spatial/temporal patterns of expression for Cx50 and Cx46. If Cx50 expression preceded that of Cx46 during fiber development, then differentiation could be transiently delayed until the appearance of Cx46 and the restoration of appropriate communication. Because the levels of intercellular communication were not quantitatively measured in either case, it is possible that the distinct phenoptypes result from different decreases in the absolute numbers of intercellular channels in the two knockout models. A definitive answer will await "knock-in" studies, where one of these two proteins has been replaced by the other, so that lenses in the resulting animals lack only connexin diversity and not intercellular channel quantity.

CONCLUSION

Critical functions for gap junctions have been elucidated by the discovery of disease-causing mutations in human connexins and the targeted disruption of mouse connexin genes. In addition, the realization that innexin genes encode gap junction proteins opens new avenues for studies of their biological roles. Genetic studies of intercellular communication are tempered by questions of redundancy and compensatory expression, and it is often difficult to explain the restriction, or even absence, of a phenotype following mutation of a particular gap junction gene. For example, Cx37 and Cx40 are both abundantly expressed in vascular endothelial cells, but no vascular phenotype has yet been reported for either knockout. This could be a case of redundancy, and interbreeding the Cx37- and Cx40-deficient animals may result in a more pronounced vascular phenotype. The generation of animals with multiple connexin knockouts is ongoing and will no doubt answer some of these types of questions.

The identification of connexin genes that underlie human disease will, in the short term, allow definitive genetic counseling in families carrying the mutated alleles. So far, only the Cx50 knockout mice provide a model for research into the basic mechanisms of cataract formation, as the Cx26 and Cx32 knockouts do not closely replicate the human pathologies resulting from mutations in these genes. In the case of deafness, alternative transgenic animal models might be developed using human dominant-negative Cx26 mutations. Animals expressing the dominant alleles under promoters with temporally or spatially restricted expression could overcome the embryonic lethality of Cx26 knockouts.

Finally, one of the most difficult challenges remaining will be to uncover the mechanisms whereby gene disruption leads to pathological changes. For intercellular channels, this question ultimately comes down to finding the specific molecules that no longer can pass between cells in the affected tissue.

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