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Gap Junctions or Hemichannel-Dependent and Independent Roles of Connexins in Cataractogenesis and Lens Development

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Abstract

In the last decade or so, increasing evidences suggest that the mutations of two connexin genes, *GJA3* and *GJA8*, are directly linked to human congenital cataracts in North and Central America, Europe and Asia. *GIA3* and *GIA8* genes encode gap junction-forming proteins, connexin (Cx) 46 and Cx50, respectively. These two connexins are predominantly expressed in lens fiber cells. Majority of identified mutations are missense, and the mutated sites are scattered across various domains of connexin molecules. Genetic deletion of either of these two genes leads to the development of cataracts; however, the types of cataracts developed are distinctive. More interestingly, microphthalmia is only developed in Cx50, but not Cx46 deficient mice, suggesting the unique role of Cx50 in lens cell growth and development. Knockin studies with the replacement of Cx46 or Cx50 at their respective gene locus further demonstrate the unique properties of these two connexins. Furthermore, the function of Cx50 in epithelial-fiber differentiation appears to be independent of its conventional role in forming gap junction junction channels. Due to their specific functions in maintaining lens clarity and development, and their malfunctions resulting in lens cataractogenesis and developmental impairment, connexin molecules could be developed as potential drug targets for therapeutic intervention for treatment of cataracts and other eye disorders. Recent advances in basic research of lens connexins and the discoveries of clinical disorders as a result of lens connexin dysfunctions are summarized and discussed here.

Keywords

Connexin; gap junctions; hemichannel; Cx46; Cx50; cataract formation; mutation; gene knockout; lens development

1. INTRODUCTION OF CONNEXIN BIOLOGY

Gap junctions formed by connexin molecules are transmembrane channels connecting the cytoplasm of neighboring cells and allow passage of small molecules ($M_r \leq 1000$ Da), such as metabolites, ions, and second messengers [1]. This type of intercellular channel is widely expressed in all types of tissues and organs. Cell-cell communication by gap junctions is

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critical in maintaining normal cell and tissue functions. Gap junctions are formed by a family of proteins named connexins and approximately 20 different human connexins have been identified [2]. Six connexin molecules oligomerize into a membrane channel known as connexon also called hemichannel. Connexons in adjacent cells join head-to-head across the extracellular “gap” to form intercellular channels. Thousands of these channels are clustered in the plane of the plasma membrane to form gap junction plaques. Each connexin protein traverses the lipid bilayer four times, with both the N- and C-termini facing the cytoplasm [3]. Structure-function studies have defined the unique roles of functional domains of connexin molecule (reviewed by Yeager and Harris (2007) [4]). M3, the third transmembrane domain was determined as the major pore-lining helix along with the second half of M1, the first transmembrane domain, NT, N-terminus, and E1, the first extracellular loop domain. The two extracellular loop domains (E1 and E2) are believed to be involved in the interaction between connexons in adjacent cells [5] and E2 is believed to play an role in selection of pairing partner of lens connexons [6]. The cytosolic loop domain (CL) as well as the C-terminal domain (CT) of the connexins are highly variable among the different connexins and are thought to be regulatory domains which contain multiple post-translational modification and protein binding sites [1,7]. The properties of gap junctions are defined by the connexins, with the channel formed by different connexins having different permeability that will permit the selective passage of metabolites and signaling molecules in the cell [8].

In addition to being the major components of gap junction channels, connexins have recently been shown to exist and function in the form of un-apposed halves of gap junction channels called hemichannels. These channels are localized at the cell surface, independent of physical contact with adjacent cells [9]. These hemichannels, like gap junction channels, display certain substrate selectivity and permit molecules with molecular weight less than 1 kDa to pass through [10]. However, the function of hemichannels is different from gap junctions; the former mediate communication between cells and extracellular matrix with the flux of ions and other small regulatory factors, while the latter is involved in the communication between adjacent cells. The existence of functional hemichannels has been reported in neural progenitors and neurons [11,12], astrocytes [13,14], heart [15], and bone cells [16,17]. Regulated hemichannels appear to provide a mechanism for ATP and NAD^+ release, which raises intracellular Ca^{2+} levels and promotes Ca^{2+} wave propagation in astrocytes, bone cells, epithelial cells, and outer retina cells [16,18–22]. These hemichannels are regulated by extracellular Ca^{2+} [23–25] and the Ca^{2+}-regulated hemichannels control isomotic volume of the cell [26]. We have also shown that hemichannels formed by Cx43 serve as the pathway for the exit of elevated intracellular PGE_2 in osteocytes induced by mechanical stimulation [27,28].

Mutations of multiple connexin genes have been identified directly linking to human diseases (reviewed by Laird (2006) and Lai-Cheong *et al.*, (2007) [29,30]). Cx32 mutation results in defective myelination diseases like Charcot-Marie Tooth. Mutations of Cx47 lead to the Pelizaeus-Merzbacher-like diseases. Cx26 mutation causes congenital sensorineural hearing loss identified in all major continents. Oculodentodigital Dysplasia (ODDD) is linked to the mutation of Cx43. Mutations of lens fiber connexins have been reported in

causing human congenital cataracts [31]. Most of these mutations result in the loss of the ability of connexins to form functional gap junctional channels.

2. GAP JUNCTION, HEMICHANNEL AND CONNEXIN IN THE LENS

The crystalline eye lens forms the second refracting unit of the eye. The lens suspended from the ciliary body retains a stem cell population that continues to proliferate and differentiate throughout the life of the organism [32,33]. Fibers (comprised of more than 90% of the total lens volume) are coupled with cells at the lens surface through a highly developed gap junction-mediated intercellular communication network. This extensive network is vital since it facilitates ion and metabolite exchange throughout the avascular lens, maintaining osmotic and metabolic homeostasis as well as lens transparency [34]. It has been reported that between a third and a half of the cell surface of nature fiber cells is occupied by gap junctions, the largest fraction in many tissues of the body [35]. There are at least three different junctional interactions documented in vertebrate eye lens: epithelium/epithelium, fiber/fiber and epithelium/fiber [36], although there may be species variability [37,38]. Lens epithelial gap junctions contain Cx43 [39], which joins epithelial cells both to each other and to differentiating and elongating lens fibers. Two lens fiber connexins have been cloned and characterized from rodent (Cx46 and Cx50) [40,41], ovine (Cx44 and Cx49) [42,43], and avian (Cx56 and Cx45.6) [44,45] systems. Based on the naming of human and rodent homologues, we refer two lens fiber connexins as Cx46 and Cx50 across animal species in this review to avoid the confusion of nomenclatures. Heteromeric connexons have been identified which are comprised of two lens connexins, Cx46 and Cx50 [46] and chick Cx43 and Cx46 [47]. Electrophysiological studies further demonstrate the unique channel properties displayed by these heteromeric connexons formed by lens connexins, respectively [47–49]. Coexistence of heteromeric connexon adds another level of diversity of the gap junction composition in the lens.

The first evidence for hemichannels was actually demonstrated from a lens connexin, Cx46, exogenously expressed in *Xenopus* oocytes [40]. Cx46-forming hemichannels are gated by calcium and voltage, and are mechanosensitive [50–52]. Similarly, exogenously expressed Cx50 also form functional hemichannels in *Xenopus* oocytes and transfected cells, and these channels have different electrophysiological properties and sensitivities from Cx46-forming channels [25,53–56]. Additionally, hemichannels formed by Cx50 are sensitive to monovalent cations [57]. The density of Cx50 hemichannels is directly proportional to the magnitude of the Cx50 Ca^{2+}-sensitive current [58]. Structural studies indicated that Cx50-forming gap junction channels are much more than Cx50-hemichannels in the lens [59]. Since hemichannels formed by Cx46 are mechanosensitive, it is proposed that Cx46 hemichannels may assist in accommodation of the lens by providing transit path for volume flow as the lens changes shape [60]. However, the precise functions and regulatory mechanism of these hemichannels in the lens remain obscure.

3. GAP JUNCTION AND HEMICHANNELS IN CATARACTOGENESIS

i. Connexin 46 and 50 Mutations Identified in Congenital Cataracts

Lens homeostasis and the maintenance of transparency depend on an internal circulatory system composed of continuously circulating current that flow around and through the avascular lens [61]. The mammalian lens is proposed to generate an internal microcirculation with a standing flow of ionic current that is directed inward at the poles and outward at the equator [33,62]. In this model, the circulating current creates a net flux of metabolites, such as glucose and antioxidants, into lens fibers along the extracellular spaces between cells, whereas the intercellular flow mediated by gap junctional network removes metabolic wastes toward surface cells at lens equators. The physiological importance of lens connexins and their forming channels has been reinforced in the last decade through the identification of connexin mutations and their linkage to lens congenital cataracts in humans and rodents. Like mutations identified in multiple cystallin genes [63], multiple congenital mutations in lens connexins are also associated with lens obstruction called cataracts. Cataracts in general are lens opacities caused by precipitation of lens fiber proteins, which accumulate and cannot be cleared. Precipitation changes refractive index of proteins which subsequently interferes with light passage through the lens. The general accepted hypothesis is that damaged and insoluble proteins are cleared from fiber cells through a lens outflow network. Obstruction of this network allows accumulation of metabolic waste associated with increased oxidative stress, which result in protein cross-linking and precipitation. Mutations of the two lens fiber connexin genes that would affect the lens circulation and outflow pathway by reducing intercellular communication have been known to be responsible for the development of cataracts in human and rodent lenses. Multiple mutations of Cx46 and Cx50 are directly linked to autosomal congenital cataracts in human and rodent as summarized in Tables 1–3. The first lens connexin mutation was identified in a British family of multiple generations with zonular pulverulent “dust-like” cataracts, which is linked to a missense autosomal dominant mutation in the *GJA8* gene encoding Cx50 at codon 88, P88S on chromosome 1q [64]. Subsequently, the mutation of Cx50 gene at its amino acid codon 48, E48K, was mapped from linkage analysis of a three generation family of Pakistani origin [32]. This autosomal dominant mutation leads to the formation of autosomal dominant cataract “zonular nuclear” pulverulent type. Separately, mutations in human gene *GJA3* encoding Cx46 have also been identified in two families with inherited congenital cataracts [65]. One of the families has a missense mutation at position 63, N63S; the other has a mutation with an insertion of C at nucleotide 1137 causing a frameshift at codon 380, S380Qfs, that shifts the translational reading frame at amino acid residue 380. This frameshift results in the mutated protein with 31 amino acids longer than Cx46 and 87 aberrant amino acids in its C-terminus.

In the last few years, more mutations of these two lens fiber mutations have been identified in families around globe. For Cx46 gene, a four-generation family of Caucasian decent was identified with autosomal dominant congenital zonular pulverulent cataracts linked to chromosome 13q11 and a C→T mutation at position 560 (P187L) of Cx46 [66]. Another mutation at codon 32 of Cx46, F32L, was identified in a six-generation Chinese family with morphologically homogeneous “nuclear pulverulent” cataracts [67]. The punctuate opacities

are associated with the F32L as well as S380Qfs mutation [65,67]. Another missense mutation of Cx46 gene, P59L was mapped to be associated with autosomal dominant "nuclear punctuate" cataracts segregating in a six generation Caucasian pedigree [68]. A Cx46 mutation, N188T, was reported in a large Chinese family with autosomal dominant congenital nuclear pulverulent cataract [69]. This mutation is located in the second extracellular loop of Cx46 right next to the earlier reported P187L substitution [66]. Linkage analysis also identified another Cx46 mutation, R76H, from a large pedigree from Victoria, Australia with a faint lamellar opacity surrounding a nuclear pulverulent cataract [70]. However, this mutation was also identified in unaffected carriers, implying incomplete penetrance of the mutation. Using PCR based Single Stranded Conformational Polymorphism (SSCP) analysis, two missense mutations of Cx46, R76G and V28M, were identified with autosomal dominant congenital cataract in two families with Indian origin [71]. The R76G mutation has a total cataract phenotype, but cataract phenotype with the V28M mutation varied in its severity and the age of onset. The latter mutation was also identified in unaffected family members, suggesting the possible effect of a gene modifier(s) or environmental factors involved. This study also estimates that Cx46 mutations may account for 3.3% of the hereditary congenital cataract in the Indian population. In a Chinese family affected with autosomal dominant congenital nuclear cataracts, missense mutation of Cx46, W45S, was identified [72] and this amino acid residue located at the first extracellular loop is conserved across species for Cx50 and Cx46. Later, the same missense mutation of Cx50, W45S, was mapped to an Indian family [73]. Two N-terminus missense mutations of Cx46 have been reported recently. The congenital "ant-egg" cataract, an extremely rare phenotype that was reported in a large Danish family in 1967, is caused by a L11S mutation in Cx46 [74]. In a five-generation Hispanic family from Honduras, a D3Y missense mutation of Cx46 segregated with autosomal dominant zonular pulverulent cataract [75]. In addition, a heterozygous mutation of Cx46, T87M was reported which gave the appearance of "pearl box" cataracts in a family from India with two affected members [76]. This mutation is located at the second transmembrane domain of Cx46. Another Cx46 mutation in first transmembrane domain, R33L was reported in four generations of an Indian family associated with autosomal dominant congenital cataract with finely granular phenotype [77].

Similar to Cx46, multiple mutations have been reported for Cx50 genes. In contrast to most of the mutations occurring in extracellular and transmembrane domains, a C-terminus mutation of Cx50, I247M, was reported in a Russian family with zonular pulverulent cataract [78]. A novel heterozygous R23T mutation in Cx50 gene was reported in an Iranian family affected by a progressive autosomal dominant congenital nuclear cataract [79]. A missense mutation of Cx50, V64G, was identified in a Chinese family and this residue is conserved across species for Cx50 and Cx46 [72]. In a family of Indian origin with autosomal dominant congenital cataract a mutation at conserved codon at 79, V79L in the second transmembrane domain of Cx50 was reported. Affected members in three generations have bilateral cataracts that give the "full moon" with Y-sutural opacities [80]. Another Cx50 mutation at the same location of P88S, P88Q, was reported in a four-generating of British family associated with autosomal dominant lamellar pulverulent cataract [81]. Two missense mutations of Cx50, V44E and R198Q, were reported in two family of Indian origin associated with cataracts and microcornea, respectively [82]. In

addition, this report estimates that mutations in Cx50 cause 3.3% of congenital cataract in India. Another Cx50 mutation, P189L at the second transmembrane domain was mapped in Danish families with microcornea-cataract [83]. In contrast to most of the mutations identified with dominant traits, a frameshift insertion mutation of Cx50 at codon 203, 203fs, was found to co-segregate with a family of southern Indian origin with autosomal recessive cataract [84]. This mutation results in the loss of the second extracellular domain, fourth transmembrane domain and C-terminus. One affected family member also had microcornea and microphthalmia, whereas the affected siblings were normal with these parameters. A recent study identifies a Cx50 mutation, D47N in a family with congenital nuclear pulverulent cataracts [85]. Another cytoplasmic C-terminus mutation of Cx50, S276F, has been linked to a five-generation Chinese family with a dominant congenital nuclear pulverulent cataract [86]. In a three generation Indian family with five members affected with bilateral congenital “jellyfish-like” cataract and microcornea, a highly conserved amino acid codon tryptophan at 45 was substituted by serine, W45S [73], and this mutation co-segregated completely with the disease phenotype. Interestingly a similar substitution has been reported in Cx46 resulting in antosomal dominant nuclear cataract, but without microcornea in Chinese families [87]. Recently, an insertion mutation of Cx50, ins776G, causes a recessive triangular cataract in a consanguineous family in Germany [88]. This is the second recessive mutation of human Cx50 thus far reported.

Apart from humans, four Cx50 mutants are reported in mice: D47A, V64A, G22R, and S50P. D47A mutation was identified in No2 cataractous mouse exhibiting a bilateral, congenital hereditary nuclear opacity of the ocular lens [89]. In an ethylnitrosourea (ENU) mutagenesis screen, a mouse mutation called *Aey5,* with an autosomal dominant congenital cataract was identified, resulting in a mutation at codon 64 of Cx50, V64A [90]. Mice homozygous for the *Lop10* mutation [lens opacity 10 (Lop10)] developed microphthalmia with dense cataracts, and this autosomal semi-dominant cataract mutation was mapped to mouse chromosome 3 associated with G22R mutation of Cx50 gene [91]. By ENU mutagenesis, another Cx50 mutation, S50P was identified associated with phenotypes of whole cataracts and small eye [92]. In addition to mouse, connexin mutations have been linked to cataracts in rats. Cx50 was identified as a strong candidate gene with R340W mutation for formation of cataracts in the Upjohn Pharmaceuticals Limited (UPL) rat model [93]. Cx46 mutation, E42K, is identified in a rat strain with congenital nuclear cataracts [94]. This site is located on the border between the first extracellular loop and first transmembrane domain. L7Q mutation of rat Cx50 causes an autosomal semi-dominant cataract and microphthalmia [95]. In contrast to mutations of Cx46 found in humans, rat mutation is either recessive or semidominant in inheritary traits. Cx46 phosphorylation was found to be reduced associated with the development of selenite-induced cataracts in rats, although the amounts and proteolysis of Cx46 and Cx50 were not altered [96]. Recently, a mutation called Aey 12 has been identified in a new connexin-like gene (Gjf1) in the mice that develop variable microphthalmia [97]. Only a few of the mutations in mouse and rat, however, have been extensively characterized for gap junction and even less for hemichannel activity.

ii. Roles of Gap Junction Channels or Hemichannel in Cataract Formation

Examining naturally occurring mutations has provided great insight into lens connexin function and structure. Using *Xenopus* oocyte pairs expressing exogenous connexin cRNA and electrophysiological measurements, Cx46 mutant containing a missense mutation, N63S or a frame shift mutation, S380Qfs of Cx46 fails to form functional gap junction channels [98]. Moreover, both mutations acted like “loss of function” rather than “dominant negative” mutations, because they did not affect the gap junctional conductance induced by either wild-type human Cx46 or wild-type Cx50 [98]. Cx46 S380Qfs mutant does not localize to gap junction plaques in transfected Hela cells and fails to induce gap junctional or hemichannel currents when expressed in *Xenopus* oocytes [99]. Interestingly, mutation of a diphenyalanine restored membrane localization and gap junction function, implying the mechanism of disease-causing mutation by containing an intracellular retention domain in prevention of Cx46 trafficking to plasma membrane.

In concert with Cx46 mutation, D47A mutant of Cx50 associated with No2 mouse cataract acts as in loss-of-function manner without strong dominant inhibition [100] when analyzed in the *Xenopus* oocyte expression system. Cx50 mutant P88S at the second transmembrane domain was reported to not only fail to form functional gap junctions, but also act in a “dominant negative” manner as an inhibitor of human Cx50 when co-expressing with wild type Cx50 in *Xenopus* oocytes and in transfected cells [101,102]. Moreover when this mutant was expressed exogenously in connexin-deficient Hela cells, it tends to accumulate in the cytoplasm associated with multilamellar membrane sack-like structures, but does not appear to be localized in the endoplasmic reticulum, Golgi apparatus, lysosomes, endosomes or vimentin filament [102]. Similarly, Cx50 mutation at the same site, P88G, did not induce intercellular conductance and also significantly inhibited gap junction current when co-expressed with wild type Cx50 in paired *Xenopus* oocytes [81]. In transfected Hela cells, this mutant is primarily retained in cytoplasm, but intriguingly, the expression at appositional membranes was increased when incubated at 30°C instead of 37°C. Analogous to P88S or G mutation, D47N mutation of Cx50, cannot induce intercellular conductance in paired Xenopus oocytes and its expression is confined to cytoplasm when exogenously expressed in Hela cells [85]. Decreased temperature to 27°C resulted in formation of gap junctional plagues. However, in contrast to P88G, co-expression of this mutant did not impair gap junctional conductance. Intriguingly, although mutations of Cx46 and 50 primarily lead to impairment of gap junctional communication, the types of cataracts formed are highly heterogeneous, which implies the involvement of more complicated mechanisms in cataract development as a consequence of these mutations.

Wild type Cx46 forms functional hemichannels in the nonjunctional membranes [98,103]. When single oocytes injected with connexin cRNA were analyzed using a two-electrode voltage clamp technique, large outward currents were developed that activated at potentials positive to −20 mV [98]. N63S mutant of Cx46 reduces its ability to form functional hemichannels when expressed in *Xenopus* oocytes and the amplitudes of hemichannel currents are much smaller than those formed by wild type Cx46 [98,104]. Hemichannel opening is known to be highly responsive to extracellular divalent cations and removal of calcium caused opening of these channels. N63S mutation results in hemichannels with

increased sensitivity to magnesium blockade with increased binding affinity as compared to wild type Cx46 [104]. Surprisingly, conserved mutation, N63Q failed to produce functional hemichannels, but this mutation was able to induce gap junctional conductance when paired with wild type Cx50, implying the potential role of asparagine at position 63 in regulating the action of divalent cations. On the other hand, no hemichannel currents can be detected when expressing S380Qft mutant in *Xenopus* oocytes [98]. Additionally, hemichannels formed by N63S mutant have increased sensitivity to magnesium blockade. This is the first report showing the potential involvement of reduced hemichannel activities in cataractogenesis.

iii What has been Learnt from Lens Connexin-Deficient and Transgenic Mouse Models?

Mouse models with genetic deletion of lens connexins offer invaluable means in the understanding of the roles of gap junctions/hemichannels in lens development and cataractogenesis (reviewed in Gong *et al.*, (2007) [31]). Mice deficient of either of the two lens fiber connexin genes give rise to distinct phenotypes, although Cx46 and Cx50 have been shown to co-localize at similar regions in fiber cell membranes as well as form heteromeric connexons [46]. Disruption of Cx46 gene using knockout mouse model results in the development of nuclear cataracts, and lens opacity appears to be associated with accumulation of -cystallin cleavage products, leading to the formation of an insoluble complex of disulfide-associated polypeptides [105]. Moreover, the coupling conductance in mature fibers was totally abolished, whereas in differentiated fibers the conductance was greatly reduced [106]. In addition, by using mosaic expression of green fluorescence protein (GFP) as a marker, the uniform GFP distribution is abolished in Cx46/Cx50 double knockout mouse lens, [107], indicating the importance of these two connexins in the regulation of intercellular protein distribution in the lens. Intriguingly, the level of Cx50 remains unchanged, which implies Cx46, not Cx50, is the major connexin in forming gap junction channels in mature fibers. Loss of Cx46 causes calcium accumulation as a result of increased influx and decreased outflux at the lens center core and subsequent activation of the Ca^{2+}-dependent protease, Lp82, which cleaves γ-crystallin [108]. In addition to Lp82, calpain 3 is also found to be involved in age-related cataract formation in Cx46 knockout mice [109]. Comparing to Cx46-deficient mice, cataract formation is delayed until at least 5 weeks of age vs. 2 weeks in double knockout mice without Cx46 and calpain 3, and cleavage of γ-crystallin was also not detected. The proper distribution of calcium in lens fiber cells through Cx46 appears to be a crucial mechanism that regulates Ca^{2+}-dependent proteases and prevents proteolysis. Calcium measurement further substantiates this hypothesis and demonstrates that loss of intercellular coupling in Cx46 knockout results in the blockage of the efflux path that leads to accumulation of Ca^{2+} [110]. Furthermore, the distribution patterns of calcium in wild type, knockout and knockin lenses offer additional experimental evidences in support of “microcirculation” model.

Two groups have reported studies on Cx50 knockout mice [111,112]. In contrast to Cx46 knockout, both the studies have shown that Cx50 ablation in mice results in less severe cataracts as well as reduced eye size defined as microphthalmia. In a comparative study of Cx46 and Cx50 knockout, protein levels of these two connexins appeared to be independently regulated and no compensatory effect was observed when either of connexin

was disrupted [112], whereas the presence of cleaved Cx50 in the lens nucleus is dependent upon Cx46. In addition, a delayed denucleation process was observed in the interior lens fiber of Cx50 knockout [112]. Measurement of junctional conductance in Cx50-deficent lenses shows the loss of pH sensitivity in the response to acidification in differentiating lens fiber region [113]; however, junctional conductance induced by exogenous chick Cx50 expressed in paired oocytes is sensitive to pH [114]. Genetic background has been reported to have certain impact on cataractogenesis with different cataracts formed in the 129S6 and C57BL/6J mice of Cx50 knockout, but not on lens growth [115]. This study suggests that these two properties of Cx50 are independent of each other and a genetic modifier only influences cataractogenesis. Combined with the data using Cx46 knockout, these studies suggest that Cx46 provides junctional couplings in mature fibers, whereas Cx50 is required for pH-sensitive gating of gap junctions in differentiating fibers [113]. Unlike Cx46 knockout, the deletion of Cx50 does not provoke detectable proteolysis of γ-crystallin proteins, although their conversion to insoluble form may contribute to cataractogenesis [116]. All these results suggest that Cx50 and Cx46 play important, but distinctive roles in lens homeostasis and development. These findings reveal that Cx50-mediated physiology is essential for multiple aspects of normal lens growth and function, and that maintenance of lens development and homeostasis requires the channel diversity resulting from the expression of two connexins in lens fibers. Genetically incongruous mixing of lens fiber connexin genes in mice by mating knockin and knockout mice has shown that heterozygous replacement of Cx50 with Cx46 in the Cx50 locus has normal lens growth, but exhibited cataracts with disruption of lens fibers [116]. This study suggests that one allele of Cx50 provides sufficient genetic background for the lens development regardless of whether the native Cx46 gene is present. By analyzing gap junctional conductance in lenses from Cx46 “knockin” Cx50 locus, Cx50 knockout and wild type, knockout of Cx50 reduced conductance to 44% of wild type in differentiating fibers and knockin of Cx46 partially restored conductance [117], whereas knockin increased conductance in mature fibers. Moreover, knockout of Cx50 makes channel pH sensitive in both differentiating and mature fibers. The paper concludes that channel function not only depends on the sequence of connexin, but also on the gene locus it expresses, although the difference in the levels of connexin expression at different gene locus might be another possible factor attributing to this phenotype. Recently, transgenic mice overexpressing Cx50 driven by βB1-crystallin was reported [118]. Surprisingly, the lenses from these transgenic mice are smaller and developed cataracts even at postnatal day 1. The phenotypes observed could be caused by a dramatic increase of Cx50 protein and its predominant intracellular retention with cytoplasmic vesicles, while the distribution of other membrane or membrane-associated proteins appeared not to be altered.

Despite their distinctive functions and regulations demonstrated in these knockout models, several evidences also suggest the close functional relationship between Cx46 and Cx50. G22R mutation of Cx50 in *Lop10* mice acts in a dominant negative manner for reducing the phosphorylated form of Cx46 [91]. Interestingly, double homozygous mutant mice with *Lop10* and Cx46 knockout (*Lop10/Lop10,* $\alpha 3^{-/-}$) shows relatively normal lens fibers compared to the *Lop10* mice. These authors postulated that Cx46 is partially responsible for cellular impairment in the *Lop10* mice. A later study by the same group showed that a

knockin Cx46 on the locus of Cx50 rescued the severe cataract by the G22R mutation of Cx50 [92]. Although Cx50(G22R) mutant failed to mediate gap junctional communication, co-expression with Cx46 partially restored the junctional conductance, which supports the hypothesis that G22R mutant interacts with wild type Cx46 to form functional channels that may rescue the defects caused by G22R mutation. However, it is intriguing that knockin Cx46 appears to behave quite differently from the endogenous Cx46 present in *Lop 10* mice. A mutation of Cx50, S550P, was identified in ENU-mutagenized mice [119]. Primary lens fibers failed to fully elongate in heterozygous lenses, but not in homozygous mice. However, this defect was corrected in $Cx50^{S50P/-}Cx46^{+/+}$ mice that lacks wild type Cx50. Unlike G22R mutation, S50P mutant appears to interact with endogenous Cx50, but not Cx46, in prevention of primary fiber cell elongation. A following up study shows that the mixed expression of S50P mutant with wild type of either Cx50 or Cx46 resulted in functional heteromeric gap junctional channels and hemichannels [120]. Interaction of this mutant with wild type Cx46 appeared to rescue S50P mutant of Cx50, which by itself is retained inside the cell, to localize at cell-cell appositions. These studies further illustrate the close functional relationship between Cx46 and Cx50 in the lens.

Apart from Cx46 and Cx50, ubiquitously distributed Cx43 is expressed in lens epithelium and differentiating equator region. Mouse model deficient of Cx43 was established through homologous recombination more than a decade ago [121]. Cx43 knockout mice are embryonic lethal and died at birth due to a blockage of the right ventricular outflow tract. Abnormality of neonatal lenses of Cx43 knockout was reported showing grossly dilated extracellular spaces with loose apposition of epithelial cells and underlying fiber cells, and the appearance of intracellular vacuoles [122], whereas the expression and distribution of Cx46 and Cx50 are normal. In contrast, a separate study by another group shows the normal embryonic lens and eye development of Cx43 knockout through embryonic day 19 [123]. In double knockout mice without both Cx43 and Cx50, a substantive reduction in epithelial-epithelial and epithelial-fiber gap junction was observed, although differentiated fiber-fiber communication was unaltered [123]. Despite these alternations in intercellular communication, these embryonic lenses from double knockout were structurally normal. Because of embryonic lethality of Cx43 knockout, only embryonic lenses were mainly studied; thereby the possible role of Cx43 in postnatal lens development remains unresolved.

4. CHANNEL-DEPENDENT AND INDEPENDENT FUNCTION OF CONNEXINS IN CELL GROWTH AND DIFFERENTIATION, AND LENS DEVELOPMENT

The lens is derived early in embryonic development by inductive interactions between the optic vesicle and the ectoderm resulting in invagenation of a segment of ectoderm to form a hollow epithelial structure called the lens vesicle [124]. Epithelial cells in the anterior lens vesicle form a monolayer on the anterior surface. Subsequently, posterior cells exit the cell cycle and form primary fiber cells [125]. Special anterior epithelial cells migrate from the anterior surface to the equatorial aqueous/vitreous humor interface called the bow region. Here, these epithelial cells at the lens equatorial, bow region continuously differentiate into secondary fiber cells which exit the cell cycle, pushing their way between the anterior

epithelium/posterior capsule and the older layers of differentiated fiber cells in the center to form the bulk of lens fibers [126]. As such the relatively simple yet highly ordered structure renders the lens an excellent model for studying basic features of cell function and differentiation.

During lens differentiation, the newly-formed fiber cells go through a complex maturation process with the accumulation of high concentrations of proteins known as crystallins and cytoskeletal proteins [127,128], as well as multiple major plasma membrane proteins such as connexins, major intrinsic protein (MIP), also called aquaporin-0 (AQP0), and key regulatory enzymes in lipid and glucose metabolism [129]. In addition, there is a gradual loss of essentially all intracellular organelles, including the nucleus, which would otherwise interfere with the transparency of the lens [130]. These cellular properties, together with an elastic capsule and zonular fibers, result in the optical transparency, high refractive index and elasticity. With the loss of cellular organelles, the fibers lose the ability to support an active metabolism. Since there is no blood supply inside the lens, the center of the lens depend fully on an extensive network of gap junction-mediated intercellular communication with cells on the lens surface to maintain their metabolic activities and homeostasis [1]. This extensive system is vital because lens is avascular and therefore must facilitate homeostasis and metabolic exchange through gap junction to maintain lens transparency without a continuing supply of newly synthesized transport proteins.

In Cx50-knockout mice, the eyes and the lenses are 32% and 46% reduced in masses, respectively, in comparison with control littermates [115]. Targeted replacement of Cx50 with Cx46 by genetic knock-in restores lens transparency, but does not restore normal growth, implying that intrinsic properties of Cx50 are required for normal lens growth and development [131]. Incongruous mixing of lens fiber connexin genes in mice further suggests that normal lens size is evident regardless of whether the native Cx46 gene is present, implying that one allele of Cx50 provides sufficient genetic background for the lens development and the addition of Cx46 allele(s) would not contribute to the overall growth of the lens [116]. Several studies suggest that the role of Cx50 in regulating lens growth is through the stimulation of fiber differentiation. A delay of fiber cell denucleation is observed in Cx50 deficient mice [112]. In addition, *Aey5* mutation of Cx50 (V64A) exhibits reduced lens size and abnormal, distributed nucleation in the anterior region [90]. Our group has shown that only chick Cx50, not other two lens connexins Cx43 and Cx46, stimulates epithelial to fiber cell differentiation and subsequent expression of major differentiation markers [132]. This stimulatory effect appears to be independent of lens cell proliferation. However, in other two reports, the process of epithelial cell proliferation is reported to be compromised in Cx50 knockout mice. Mitotic activities determined by 5’-bromo-2’-deoxyuridine (BrdU) labeling in lenses of Cx50 knockout and Cx50 with Cx46 “knockin” and wild type mice showed an increase in mitotic index in wild type, but it is not evident in Cx50 knockout mice, and this growth deficit was partially rescued in knockin lenses [133]. A recent study by the same group shows that Cx50, but not Cx43, exerts a major impact on epithelial cell proliferation [134]. They show the peak of Cx50 gap junctional activity closely coincided with an increase in the number of mitotic epithelial cells. It is speculated that channels formed by Cx50, but not Cx46, mediates propagation of the growth signals in lens epithelial cells. Immunocytochemical and ultrastructural analyses of knockout mice

show that in Cx46 knockout where Cx50 is alone, postnatal differentiation of secondary fiber proceeds faster with an increased numbers of smaller fiber cells; in contrast, in Cx50 knockout, the lens mass and number of fiber cells are reduced [135]. In Cx50 deficient lens, terminal differentiation is impaired and clearance of nucleus and other organelles are delayed. In addition, this study suggests that decreased proliferation of the epithelium is not the sole feature that causes the reduction of lens size. In the germinative and transitional zones a reduced number of abnormally large fiber cells and the prolonged existence of cytoplasmic organelles and nucleus were observed. Very interestingly, the large junction domains consisting of Cx50 have topographic interaction with MIP in differentiating lens fiber cells of Cx46 knockout mice, similar as wild type; conversely the small junctional plaques containing Cx46 have no obvious topographic interaction with MIP. This results further support the findings of the direct physical interaction of Cx50, but not Cx46, and MIP in the differentiating young fibers [136,137].

Interestingly, several lines of evidences suggest that the effect of Cx50 on promoting lens epithelial-fiber differentiation is likely to be independent of gap junction channel function. We have shown that overexpression of chick Cx50 does not enhances intercellular coupling [132]. More importantly, Cx50 mutants that fail to form functional gap junctions exert similar stimulatory effects on lens epithelial-fiber differentiation as wild type Cx50 [138]. Similar studies by Le and Musil [139] show that the inhibition of intercellular coupling by a gap junction blocker, 18-glycyrrhetinic acid, does not affect epithelial-fiber cell differentiation and formation of fiber-like structures in lens primary cell cultures. These studies support the hypothesis that Cx50 is unique among the lens connexins with its extra-channel role in epithelial-fiber differentiation and lens development. This connexin molecule appears not to be a determining factor for cell differentiation since mature fibers are still formed in knockout mice; instead it has a positive impact on the rate and extent of the formation of lens fibers. However, the molecular mechanism by which Cx50 influences differentiating lens cells remains largely unknown.

Our group has shown that C-terminus is required for the stimulatory effect of Cx50 [138]. Chimeras of chick Cx50 and Cx46 constructs were developed in which the Cx50 C-terminus was exchanged for Cx46 (Cx50*46C) and the Cx46 C-terminus was exchanged for Cx50 (Cx46*50C). Only the Cx46*50C stimulated lens differentiation similar to wild type Cx50. Expression of cytosolic C-terminal domain alone has no such effect, implying the importance of properly expressed gap junction proteins on the plasma membrane in epithelial-fiber differentiation. There are several reports related to gap junction-independent roles of connexins in the control of cell growth and the suppression of tumorigenicity (reviewed by Jiang and Gu (2005) [140]). Cx50 and Cx46 display differential physiological and gating properties attributed to their respective C-termini. Cx50 displays a high sensitivity to pH gating [141] and appears to regulate Cx46 expression in differentiating fibers [113]. In contrast, Cx46 exhibits less pH sensitivity and is responsible for coupling mature lens fibers [113,141]. These physiological differences suggest that certain regulatory factor(s) may associate and modulate the Cx50 C-terminus through undefined mechanisms to promote lens cell differentiation and fiber formation.

5. REGULATORY MECHANISM OF LENS CONNEXINS

The cytosolic loop regions as well as the C-termini of the connexins are highly variable among the different connexins and all thought to be regulatory domains which contain multiple post-translational modification and protein binding sites [1,7]. Specifically for lens fiber connexins, several consensus sequences for known kinases have been localized in the C-termini which control various physiological properties of the gap junctions. The regulatory mechanisms of connexin proteins through phosphorylation, proteolysis and binding partners have been reviewed previously [7,142,143]. We have shown that chick Cx50 is *in vivo* phosphorylated by casein kinase II and this phosphorylation provides signals for protein turnover likely facilitated through proteasomal pathway [144,145]. Moreover, Cx50 is cleaved at C-terminus by caspase 3 in mature fibers. Phosphorylation by casein kinase II prevents the proteolytic cleavage by caspase 3, suggesting this specific phosphorylation and cleavage is alternately regulated [145]. Studies by Dunia *et al.* [146] and by our laboratory [136] demonstrate that the localization of MIP is closely correlated with newly formed gap junctional plaques at the narrow zone of the lens bow region where younger lens fiber cells are actively differentiating. Freeze fracture labeling showed the co-existence of MIP and gap junctions in the same micro-domain [147]. These experimental evidences imply that the interaction between MIP and Cx50 may facilitate the assembly of Cx50 into nascent gap junctional plaques. A recent freeze-fracture and fracture labeling study shows that the junctional assembly and packing organization between connexons and MIP in Cx50 knockout mouse lens differ from wild type, of which junctional plaques containing Cx46 has no obvious interaction with MIP [135]. The close relationship between MIP and lens gap junctions has been supported by observation in MIP-deficient mice [148] that the size range of fiber gap junctions is significantly altered. The percentage of membrane areas in gap junctional plaques is almost halved as compared with normal lens. Earlier studies by Tanaka *et al.* [149] also show a correlation between the decrease and final absence of MIP and a decrease in gap junction structures during cataract development in the Nakano mouse lens. MIP appears to associate transiently with ovine lens fiber gap junctions [150,151]. We have observed that MIP uniformly co-localizes and interacts with gap junctions in the differentiating chick lens fibers, an association which disappears in mature fibers, possibly due to the cleavage of intracellular loop of Cx50 [136,137]. There is a direct physical interaction between the intracellular loop domain of Cx50 and the C terminal domain of MIP [137]. However, this interaction appears not be related to the water transporting activity of MIP, because Duchesne *et al.* [152] report that the MIP mutant without C-terminal domain does not change the transport activity of the protein. Furthermore, in the lens of Cx50 knockout mice, MIP level appears to be normal by Western blots and electron microscopy with immuno-gold labeling [112,153]. Thus, these findings suggest a potential role for MIP in assisting assembly and formation of nascent gap junctions, whereas connexins appear to play lesser roles in MIP function. In addition to the interaction with MIP, both connexins of rodent lens fibers have been observed to interact with the PSD95/Dlg/ZO-1 (PDZ) domain of the tight junction protein Zonular Occluden-1, implying its function in facilitating the targeting of connexons to sites where adjacent cell membranes are closely opposed [154]. However the precise regulatory functions of these interactions with lens fiber connexins remain to be elucidated.

6. FUTURE PROSPECTIVE AND CHALLENGES

Significant progress and advances have been made in the last decade in our understanding of the function and regulation of lens gap junctions and connexins in cataractogenesis and lens development. More connexin mutations are being identified and linked to various types of human congenital cataracts in many regions around the world. Mouse models with various genetic manipulations are established and characterized, obtaining invaluable knowledge of the *in vivo* roles of connexins in the lens. Despite all these great achievements, one of the future challenges remains to be the mechanistic elucidation concerning the regulation of gap junction and/or hemichannels in accommodating the metabolic and homeostatic needs of the lens and the regulatory role of connexin play in cell differentiation and lens development. Furthermore, the most crucial challenge is related to the aspect of translational research. Up to now, the clinical application and drug development based on connexin biology are rather limited. The next phase of the investigation ought to utilize the current knowledge aiming for the development of effective therapeutic means for the intervention of cataracts, and other lens and eye disorders.

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Table 1.

Cx46 Mutation Identified in Human Congenital Cataracts

Mutation	Location	Cataract Types	Inheritary	Family Origin	References
Human Cx46					
D3Y	NT	Zonular Pulverulent	Autosomal dominant	Hispanic	[75]
L11S	NT	“Ant-egg”	Autosomal dominant	Danish	[74]
V28M	M1	Variable	Autosomal dominant (incomplete penetrance)	Indian	[71]
F32L	M1	Nuclear pulverulent & punutate	Autosomal dominant	Chinese	[67]
R33L	M1	Finely granular	Autosomal dominant	Indian	[77]
W45S	E1	Nuclear	Autosomal dominant	Chinese	[73]
P59L	E1	Nuclear punctuate	Autosomal dominant	Caucasian	[68]
N63S	E1	Zonular pulverulent	Autosomal dominant	British	[65]
R76G	E1/M2	Total	Autosomal dominant	Indian	[71]
R76H	E1/M2	Lamellar nuclear pulverulent	Autosomal dominant (incomplete penetrance)	Australian	[70]
T87M	M2	Pearl box	Autosomal dominant	Indian	[76]
P187L	E2	Zonular pulverulent	Autosomal dominant	Caucasian	[66]
N188T	E2	Nuclear pulverulent	Autosomal dominant	Chinese	[69]
S380Qfs	CT	Zonular pulverulent & punctuate	Autosomal dominant	British	[65]

Table 2.

Cx50 Mutation Identified in Human Congenital Cataracts

Mutation	Location	Cataract Types	Inheritary	Family Origin	References
Human Cx50					
R23T	NT	Nuclear	Autosomal dominant	Iranian	[79]
V44E	M1	Cataract & microcornea	Autosomal dominant	Indian	[82]
W45S	E1	Jellyfish-like bilateral & microcornea	Autosomal dominant	Inidian	[73]
D47N	E1	Nuclear pulverulent	Autosomal dominant	British	[85]
E48K	E1	Zonular nuclear pulverulent	Autosomal dominant	Pakistani	[32]
V64G	E1/M2	Nuclear	Autosomal dominant	Chinese	[72]
V79L	M2	“Full mone” Y-sutural opacity	Autosomal dominant	Indian	[80]
P88S	M2	Zonular pulverulent	Autosomal dominant	British	[64]
P88Q	M2	Lamellar pulverulent	Autosomal dominant	British	[81]
Ins776G	CL	Triangular	Autosomal recessive	Germany	[88]
P189L	E2	Cataract & microcornea	Autosomal dominant	Danish	[83]
R198Q	E2	Cataract & microcornea	Autosomal dominant	Indian	[82]
203fs	E2	Cataract	Autosomal recessive	Indian	[84]
I247M	CT	Zonular pulverulent	Autosomal dominant	Russian	[78]
S276F	CT	Nuclear pulverulent	Autosomal dominant	Chinese	[86]

Table 3.

Connexin Mutation Identified in Cataracts of Mouse and Rat

Mutation	Location	Cataract Types	Inheritary	Origin	References
Mouse Cx50					
G22R	M1	Microphthalmia & dense cataract	Autosomal semidominant	Lop10 mutation cataract mouse	[91]
D47A	E1	Nuclear	Autosomal dominant	No2 cataract mouse	[89]
S50P	E1	Whole cataract & small eye	Autosomal dominant	ENU mutagenesis	[92]
V64A	E1/M2	Nuclear and zonular cataract & microphthalmia	Autosomal dominant	Mouse Aey5 generated by ENU mutagenesis	[90]
Rat Cx50					
R340W	CT	Cataract	Autosomal semidominant	UPL rat strain	[93]
L7Q	NT	Cataract & microphthalmia	Autosomal semidominant		[95]
Rat Cx46					
E42K	E1/M1	Nuclear	Autosomal recessive	Cataract rat strain	[94]