Unique and Redundant Connexin Contributions to Lens Development

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Connexin genes encode intercellular channels that help to coordinate development. In mice, the targeted deletion of different connexins produces disparate effects on ocular growth and differentiation in the lens, and the need for multiple channel subunits is poorly understood. Knockout of *Cx46* causes a loss of homeostasis and cataracts. Deletion of *Cx50* results in reduced ocular growth and cataracts. Targeted replacement of *Cx50* with *Cx46* by genetic knock-in corrected defects in cellular differentiation and prevented cataracts, but did not restore normal growth. These data show that intrinsic properties of *Cx50* were required for cellular growth, whereas nonspecific restoration of communication by *Cx46* maintained differentiation.

Gap junctions allow the intercellular diffusion of signaling molecules that regulate cellular growth and development (*1, 2*). Although junctional coupling is ubiquitous, multiple connexin (*Cx*) genes are required to provide direct intercellular communication. Connexin channels have unique permeabilities (*3*); consequently, intercellular communication mediated by distinct connexins could have diverse effects on growth and differentiation. Eye development is influenced by the lens (*4*), which principally expresses *Cx46* and *Cx50* (*5*). Knockout of *Cx46* in mice did not alter ocular growth but produced cataracts, which resulted from failed maintenance of differentiation and of crystallin solubility (*6*). Deletion of *Cx50* resulted in deficient lens and eye growth in addition to cataracts (*7*). These divergent phenotypes may result from different permeabilities of the two channel types, distinctive gating, or their differential contributions to the magnitude of intercellular communication (*8–10*). A reduction in connexin diversity, but not channel number, was engineered in mice by targeted substitution of *Cx46* for *Cx50* (Cx50KI46 mice) to address this issue of quantity versus quality of intercellular communication.

Replacement of the *Cx50* coding region with *Cx46* was achieved by homologous recombination (Fig. 1A). *Cx46* and *Cx50* lack introns within their coding regions, and the exchange resulted in minimal disruption of the native *Cx50* locus (*11*). Interbreeding of heterozygous parents produced offspring in a Mendelian ratio, as determined by polymerase chain reaction (PCR) analysis and Southern blotting (Fig. 1, B to D). Transcription of the knock-in allele was verified by Northern blotting (Fig. 2, A and B). Analysis of protein synthesis confirmed translation of the Cx50KI46 mRNA. A reduction in Cx50 protein corresponded to increased levels of Cx46 synthesis. Two other lens membrane proteins were unaffected by knock-in genotype (Fig. 2C). Immunocytochemical analysis showed that Cx50KI46 was properly targeted to lens fiber gap junctions (Fig. 2, D to F). Thus, targeted replacement of *Cx50* with *Cx46* resulted in a specific reduction of Cx50 protein linked to an increase in properly localized Cx46.

Normal lens growth results from the tightly regulated proliferation of epithelial cells that elongate into fibers, and eye growth is closely linked to lens size (*7, 12*). Overall growth of Cx50KI46 mice was identical to that of controls (Fig. 3A). Both eyes and lenses in the Cx50KI46 animals grew, but the growth rate lagged that of wild-type littermates. At postnatal day 0 (P0), eye and lens mass in the Cx50KI46 mice were not significantly different from controls ($P = 0.3$, Student's unpaired t test). By P14, Cx50KI46 eye mass was reduced 25% ($P = 8 \times 10^{-11}$) and lens mass declined 34% ($P = 10^{-12}$) relative to the wild type (Fig. 3, B and C). Thus, embryonic growth in Cx50KI46 mice was normal but postnatal ocular growth was severely impaired, and replacement of *Cx50* with *Cx46* did not repair the growth deficit resulting from *Cx50* knockout.

The ocular growth failure in *Cx50* knockout mice resulted from fewer, rather than smaller, lens fiber cells (*7*). Histological examination of P7 Cx50KI46 eyes and lenses revealed a similar proportional decrease in overall size despite normal cellular histology (Fig. 3, D to G). In addition, biochemical analysis of Cx50KI46 lenses produced yields of soluble and insoluble proteins (per unit weight) equal to those of wild-type lenses. *Cx50* (+/46) heterozygous animals have normal-sized lenses (*13*), hence one copy of *Cx50* is both necessary and sufficient for proper ocular growth regulation.

During normal differentiation, lens cells eliminate intracellular organelles to achieve optical clarity. Cx50KI46 lenses were able to achieve and maintain the proper differentiated state of transparency. Dissected wild-type lenses were clear when viewed through the anterior surface (Fig. 3H). *Cx50* knockout lenses were smaller than wild-type lenses and had prominent

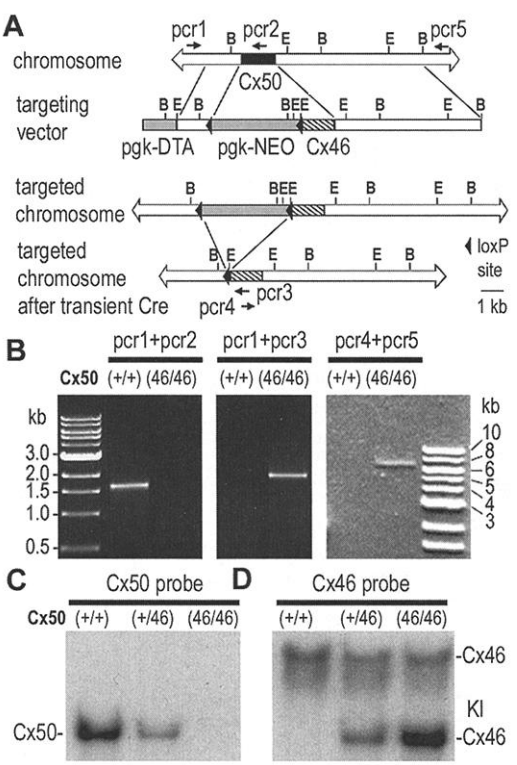

Fig. 1. Generation of Cx50KI46 mice. (**A**) Genomic structure near *Cx50*. A targeting vector replaced *Cx50* with *Cx46* and contained a pgk-NEO cassette flanked by loxP sites within an intron, and a pgk-DTA cassette at the 5′ end. After recombination, pgk-NEO was excised by transient Cre transfection. Arrows indicate PCR genotyping primers. (**B**) Genomic PCR confirmed recombination. At the 5′ end, a sense flanking primer (pcr1) was paired with either an antisense *Cx50* coding primer (pcr2) or a *Cx46* coding primer (pcr3). Primers 1+2 amplified a 1.6-kb band from wild-type *Cx50* (+/+) chromosomes, whereas primers 1+3 produced a 1.9-kb band from knock-in *Cx50* (46/46) chromosomes. At the 3′ end, an antisense flanking primer (pcr5) was paired with a sense *Cx46* coding primer (pcr4) that amplified a 7-kb band from knock-in chromosomes. (**C** and **D**) Southern blots of Bam H1–digested tail DNA. The *Cx50* gene was reduced in heterozygous (+/46) and absent in (46/46) animals. Native *Cx46* was present in all genotypes, and an appropriate knock-in band was detected in (+/46) and (46/46) mice. B, Bam HI; E, Eco RI.

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pulverulent opacities (Fig. 3I). Lenses from Cx50KI46 eyes lacked cataracts, although they were notably smaller than wild-type lenses (Fig. 3J). Therefore, unlike the targeted deletions of the *Cx46* or *Cx50* genes that resulted in cataract, replacement of *Cx50* by *Cx46* resulted in the normal attainment and maintenance of lens transparency.

Lens fiber development is further characterized by the accumulation of high concentrations of crystallins and major intrinsic protein (MIP, also known as aquaporin-0). Maintenance of crystallin solubility is the primary differentiated function of fibers and produces the high refractive index required for accommodation (*14*). Cx50KI46 lenses showed normal fiber differentiation with regard to both the synthesis and solubility of lens differentiation markers, as assayed by Western blotting after homogenization and separation into soluble and insoluble fractions (Fig. 3K). These data establish that knock-in lenses synthesized markers of lens differentiation at appropriate levels and maintained normal crystallin solubility. Thus, the targeted replacement of *Cx50* by *Cx46* prevented the loss of crystallin solubility that occurred after knockouts of either *Cx46* or *Cx50*, and it maintained the differentiated state of high refractive index.

It is not obvious why multigene families are needed to encode common structural components of cells. Mouse genetics allow this question to be probed, especially in families with simple gene structure such as connexins. In one recent study, *Cx43*, which is expressed in many tissues, was replaced by genetic knock-in. This study demonstrated that connexin specificity was required, but interpretation was greatly complicated by the animals' generally poor health resulting from *Cx43* substitution in multiple organs (*15*). In contrast, the lens is a simpler model system that requires the function of two distinct connexin genes for proper growth and differentiation (*5*). The targeted deletion of either *Cx46* or *Cx50* resulted in different phenotypes, which suggests that these outcomes were influenced by more than simple reductions in communication levels (which would occur in either knockout). It is known that the sequence diversity within the connexin family leads to functional diversity in vitro (*16*), but it has been difficult to assign in vivo consequences to the observed differences. In 50KI46 mice, reduced lens growth led to microphthalmia, similar to the growth defect resulting from *Cx50* deletion, whereas fibers were able to maintain their differentiated homeostasis and to avoid the crystalline precipitation that resulted after knockout of either *Cx46* or *Cx50*.

These data provide insight into gap junction functions that are replaceable and those that are not. The lens was able to segregate the contribution of *Cx50* to the control of normal growth from its contribution to maintenance of the differentiated state, and these functions were dependent on the repertoire, rather than the number, of connexin subunits available.

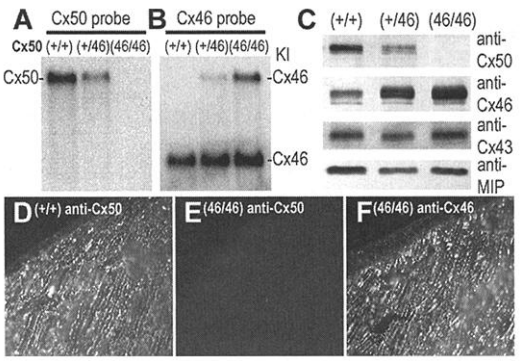

Fig. 2. Transcription, translation, and localization of Cx50KI46. (**A**) Hybridization of lens RNA with Cx50 demonstrated a reduction in (+/46) and absence in (46/46) samples. (**B**) Hybridization of identical blots with Cx46 showed that native transcript was abundant in all animals and the appropriate knock-in transcript appeared in (+/46) and (46/46) animals. (**C**) Western blotting of membrane fractions showed that Cx50 protein was reduced in (+/46) and depleted in (46/46) lenses. Cx46 synthesis increased in (+/46) and (46/46) animals. Cx43 and MIP levels were equivalent in all genotypes. (**D** and **E**) Cx50 was immunolocalized in fiber gap junctions from (+/+) but not (46/46) lenses. (**F**) Cx46 was detected in (46/46) lens in a typical punctate distribution identical to the absent Cx50.

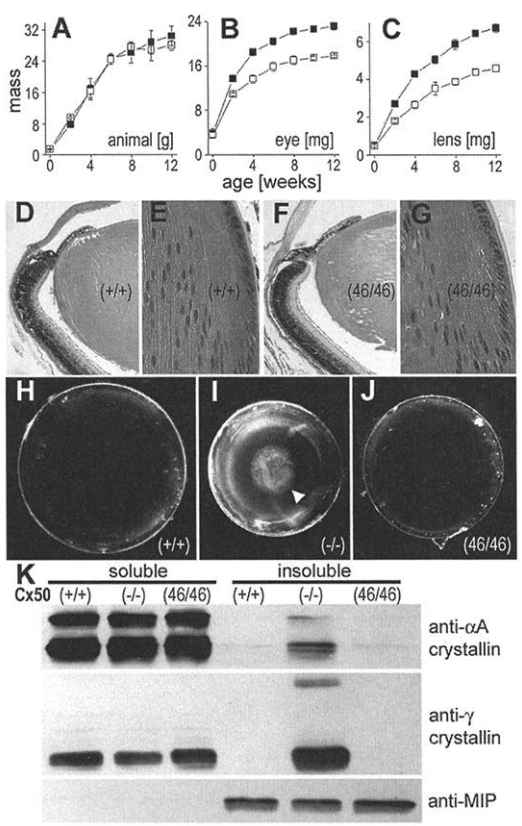

Fig. 3. Lens growth and development. (**A**) Total mass was identical for wild-type (■) and knock-in (□) mice at all ages. (**B**) By P14, knock-in eyes were 25% smaller than in the wild type. (**C**) P14 knock-in lens mass was reduced 34%. Eye and lens mass ratios remained constant thereafter (n = 4 to 9 animals per genotype at each time point, mean ± SD). (**D**) Hematoxylin- and eosin–stained section of a P7 (+/+) eye. (**E**) Cellular structure of the (+/+) lens bow region. (**F**) P7 (46/46) eyes were proportionately smaller but displayed normal anatomy. (**G**) In the bow region, (46/46) lens cells were not detectably different in size from controls. (**H**) Adult (+/+) lenses had no light-scattering opacities. (**I**) *Cx50* (–/–) lenses were smaller and contained nuclear cataracts (arrowhead). (**J**) In contrast, (46/46) lenses were smaller than (+/+) but remained clear. (**K**) Lenses were divided into soluble and insoluble fractions, subjected to Western blotting, and probed with antibodies to αA-crystallin, γ-crystallin, or MIP. αA-crystallin and γ-crystallin were present in the soluble fractions of all lenses and the insoluble fraction of (–/–), but not in the insoluble fraction of (+/+) or (46/46) lenses. MIP was equally abundant in the insoluble fractions from all genotypes.

References and Notes

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11. *Cx50* was replaced with *Cx46* by standard PCR techniques. A floxed pgk-neomycin resistance cassette was placed within an intron in the 5′ untranslated region of *Cx50*. A pgk-diphtheria toxin A chain cassette was placed outside the 5′ homology region. Homologous recombinants were identified by PCR screening at a frequency of 1 in 17 neo^r colonies. Two independent Cx50KI46 embryonic stem cell clones were transiently transfected with Cre recombinase to excise the pkg-NEO marker, leaving one 34–base pair loxP site within the intron. Both Cx50KI46 embryonic stem cell clones were injected into C57BL/6 blastocysts and produced founders exhibiting germ-line transmission. Cx50KI46 mice were maintained in the 129/SvEv genetic background. Analytical methods and antisera used were as described (*7*).
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