Interaction between Connexin50 and Mitogen-activated Protein Kinase Signaling in Lens Homeostasis

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Both connexins and signal transduction pathways have been independently shown to play critical roles in lens homeostasis, but little is known about potential cooperation between these two intercellular communication systems. To investigate whether growth factor signaling and gap junctional communication interact during the development of lens homeostasis, we examined the effect of mitogen-activated protein kinase (MAPK) signaling on coupling mediated by specific lens connexins by using a combination of in vitro and in vivo assays. Activation of MAPK signaling pathways significantly increased coupling provided by Cx50, but not Cx46, in paired Xenopus laevis oocytes in vitro, as well as between freshly isolated lens cells in vivo. Constitutively active MAPK signaling caused macrophthalmia, cataract, glucose accumulation, vacuole formation in differentiating fibers, and lens rupture in vivo. The specific removal or replacement of Cx50, but not Cx46, ameliorated all five pathological conditions in transgenic mice. These results indicate that MAPK signaling specifically modulates coupling mediated by Cx50 and that gap junctional communication and signal transduction pathways may interact in osmotic regulation during postnatal fiber development.

INTRODUCTION

Multicellular organisms require regulated communication between cells for the normal growth and differentiation of complex organs. Such intercellular communication can be directly mediated between cells by the intercellular channels present in gap junctions or indirectly propagated by extracellular growth factors that bind receptors to activate signal transduction cascades. Both gap junctions and signal transduction pathways have been shown to play critical roles in the development of the ocular lens (Gerido and White, 2004; Lovicu and McAvoy, 2005; Robinson, 2006; Gong et al., 2007), but to date only a few studies have addressed the critical question of whether there is any interaction between these two different modes of intercellular communication (Le and Musil, 2001; Boswell et al., 2008a,b).

The lens is composed of three cell types: a monolayer of epithelial cells covering the anterior surface, differentiating fiber cells in the lens cortex, and mature fibers that fill the lens core (Piatigorsky, 1981). All three cell types are directly coupled to their neighbors by gap junctions containing three distinct connexins (Goodenough, 1992; Mathias et al., 1997). Cx43 is only present in the lens epithelium, Cx46 is expressed only in the differentiating and mature fiber cells, and Cx50 is found throughout both the epithelium and lens fibers (Beyer et al., 1989; Musil et al., 1990; Paul et al., 1991; White et al., 1992, 2007). Cx50 knockout animals exhibit significantly reduced postnatal mitosis, decreased lens and eye growth, delayed fiber denucleation, and mild cataracts (White et al., 1998; Rong et al., 2002; Sellitto et al., 2004). It has also been observed that mice bearing point mutations in Cx50 show profound defects in the differentiation of both primary and secondary fiber cells (Xia et al., 2006). These data have clearly established that intercellular communication mediated by Cx50 can contribute to the regulation of epithelial cell proliferation and fiber cell differentiation during lens development (Gong et al., 2007).

In addition to the regulation imposed by gap junctional coupling, it has long been known that lens development is also strongly influenced by growth factor signaling. For example, fibroblast growth factors (FGFs) and fibroblast growth factor receptors (FGFRs) regulate lens induction, epithelial cell proliferation and fiber differentiation (Robinson et al., 1995; Lovicu and McAvoy, 2001; Zhao et al., 2008). Consistent with these observations, lens-specific expression of a dominant-negative form of Ras reduces epithelial cell proliferation and delays fiber elongation (Xie et al., 2006). Although the defects in cell proliferation and differentiation that result from experimental manipulation of mitogen-activated protein kinase (MAPK) signaling are often more profound than those that arise after genetic mutation of the lens connexins, there is some overlap in the range of observed developmental deficiencies.

Epithelial cells differentiate into secondary fibers at the lens equator, in which high levels of MAPK signaling are stimulated by growth factors, including FGF (Le and Musil, 2001; Lovicu and McAvoy, 2005). Differentiating fiber cells at the lens equator also have the highest measured levels of gap junctional coupling compared with either the anterior and posterior poles, or the lens core (Baldó and Mathias, 1992; Mathias et al., 2007). This high coupling at the equator...
is critical in the establishment of an internal circulating current that drives the nonvascular microcirculatory system used by the lens to maintain transparency (Mathias et al., 1997; Donaldson et al., 2001). Previous in vitro work has shown that this developmentally regulated increase in junctional coupling is dependent on cross-talk between the FGF and bone morphogenetic protein signaling pathways (Le and Musil, 2001; Boswell et al., 2008a,b). Although these results support the notion that equatorial FGF signaling interacts with lens connexins to produce the observed asymmetry in gap junctional coupling, they have failed to identify the specific connexins that can interact with MAPK signaling in vivo.

In the current study, we have investigated the mechanisms whereby gap junctional coupling and MAPK signaling may interact in the establishment of postnatal lens homeostasis. We have examined the functional interaction of lens connexins with MAPK signaling in vitro in paired Xenopus oocytes and in vivo in genetically engineered mice. We have found that activation of MAPK signaling significantly increased coupling provided by Cx50 but not Cx46 in vitro. Constitutively active MAPK signaling also specifically increased Cx50 functional activity between lens cells in vivo and produced macrophthalmia, cataracts, an increase in glucose accumulation, the formation of vacuoles in the bow region of the lens fibers, and lens rupture. The specific removal of Cx50, but not Cx46, as a potential target of constitutive MAPK signaling ameliorated all of these lens pathologies in mice. These results indicate that MAPK signaling specifically modulates Cx50, but not Cx46, and that Cx50-mediated gap junctional communication and signal transduction pathways may work together in the establishment of osmotic homeostasis during postnatal fiber development.

**MATERIALS AND METHODS**

**In Vitro Transcription, Oocyte Microinjection, and Pairing**

Cx50, Cx46, Xenopus FGF receptor, and constitutively active mitogen-activated protein kinase kinase (MEK) 1(E) coding sequences were subcloned into pcDNA3+, linearized, and used as the template to produce cRNAs by using the SP6 mMessage mMachine (Ambion, Austin, TX). Cells were injected with 40 nl of an antisense SP6 mMessage mMachine (Ambion, Austin, TX). Oocytes were isolated from Xenopus (Sigma-Aldrich, St. Louis, MO) for 2 h and then recorded. In some experiments, the MEK inhibitor U0126 (DuPont, Wilmington, DE) was added 30 min before pairing. For experiments testing MEK1(E), cells were injected with connexin cRNA. For experiments testing MEK1(E), cells were injected with Connexin 38 or a mixture of antisense plus cRNA (40 ng/cell) by using a Nanoject II injector (Drummond, Broomall, PA) and allowed to recover for 24 h. To pair cells, mRNPs were resuspended in SD2 sample buffer (2 μl/oocyte), separated on 10% SD2-PAGE gels, and transferred to nitrocellulose membranes. Membrane protein blots were probed with polyclonal Cx50 or Cx46 antibody bodies (Paul et al., 1991; White et al., 1992) at a 1:1,000 dilution followed by incubation with alkaline phosphatase-conjugated anti-rabbit secondary antibody (Jackson Immunoresearch Laboratories, West Grove, PA). Soluble protein blots were probed with rabbit polyclonal phospho-(p)-extracellular signal-regulated kinase (ERK) or total ERK antibodies (Cell Signaling Technology, Danvers, MA) at 1:1,000 dilution, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

**Mouse Breeding**

MEK1(E) transgenic mice have mice that express a constitutively active mutant of MEK1 under the αa-crystallin promoter (Gong et al., 2001) and were interbred with Cx50KO (White et al., 1998), Cx46KO (Gong et al., 1997), or Cx46KO mice (White, 2002) to produce MEK1(E)-Cx50KO, MEK1(E)-Cx46KO, or MEK1(E)-Cx50KO mice. Genomic DNAs isolated from tail biopsies were genotyped by polymerase chain reaction (PCR) (Gong et al., 1997, 2001; White et al., 1998; White, 2002).

**Lens Epithelial Cell Isolation**

Lenses were dissected from eyes and transferred to calcium- and magnesium-free phosphate-buffered saline (PBS). The lens capsule was then peeled away from the fiber cell mass by using fine forceps. For Western blotting, capsules were transferred to 2× sample buffer (15 μl/lens), separated on SD2-PAGE gels, and transferred to nitrocellulose membranes. Blots were probed with polyclonal Cx50, Cx46, p-ERK, or total ERK antibody bodies (Cell Signaling Technology, Danvers, MA) at 1:1,000 dilution, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

**Dual Whole-Cell Patch Clamp**

Junctional conductance was measured between cell pairs by using dual whole-cell patch-clamp with Axopatch 1D patch-clamp amplifiers (Axon Instruments) at room temperature. The solution bathing the cells contained 135 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 5 mM HEPES, 5 mM dextrose, 2 mM pyruvate, and 1 mM BaCl2. pH 7.4. Patch electrodes had resistances of 3–5 MΩ when filled with internal solution containing 125 mM CsCl, 10 mM EGTA, 0.5 mM CaCl2, and 10 mM HEPES, pH 7.2. Macroscopic and single-channel recordings were acquired by using PCLAMP8 software (Axon Instruments), sampled at 1–2 kHz and filtered at 0.2–0.5 kHz, and analysis was performed with PCLAMP8 and ORIGIN 6.0 software (MicroCal Software, Northampton, MA). Each cell of a pair was initially held at a common holding potential of 0 mV. To evaluate junctional coupling, 200-ms hyperpolarizing pulses from the holding potential of 0 mV to −260 mV were applied to one cell to establish a transjunctional voltage gradient (Vj), and junctional current was measured in the second cell (held at 0 mV). Quinine (Sigma-Aldrich) was used to selectively block Cx50 conductance because its effects are fully and rapidly reversible, and it has no effect on Cx46 or Cx43 junctional currents (Srivastava et al., 2001; Cruikshank et al., 2004; Bai et al., 2006). Quinine solutions were applied with a gravity-fed perfusion system, and solution exchange was complete within 10 s. The magnitude of inhibition caused by quinine is expressed as the percentage of the conductance inhibited by the drug.

**5-Bromo-2'-deoxyuridine (BrDU) Injection**

Postnatal mouse pups were injected intraperitoneally with 100 μg/g body weight of BrDU (Sigma-Aldrich). BrDU at 10 mg/ml was dissolved in PBS at 37°C just before use. Injected pups were returned to their mothers for 1 h.
incubation, euthanized, and lenses were dissected. The lens capsule was then peeled away from the fiber cell mass by using fine forceps and pinned down on an encapsulant (Sygard; Dow Corning, Midland, MI) coated 35-mm Petri dish and fixed for 30 min in 2% formaldehyde in PBS. Fixed capsules were rinsed with PBS, incubated with 100% MeOH at −20°C for 5 min, mounted on microscope slides, and allowed to air dry. BrdU was immunolabeled with an in situ detection kit (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer’s instructions, with the exception that endogenous peroxidase was quenched with 0.3% hydrogen peroxide diluted in absolute methanol, and all antibody incubations were carried at 37°C. BrdU-negative nuclei were counterstained with aqueous hematoxylin (Sellitto et al., 2004). Stained sections were viewed and photographed with a digital camera (MagnaFire; Optronics, Goleta, CA).

Quantitation of BrdU Labeling
The diameters of BrdU-labeled capsules were individually measured for each genotype at each developmental age (P2 or P5). A rectangular area with a length of three-fifths diameter and a height of one-fifth diameter was drawn for each capsule. The rectangle was positioned to cover the area from the lens equator to the center of the lens. BrdU-positive nuclei were counted within the rectangular region and divided by the area to calculate the density of BrdU-positive nuclei. Statistical analysis between wild-type, MEK1(E), Cx50KO, and MEK1(E)-Cx50KO lenses was performed using the three-way analysis of variance (ANOVA).

Growth Analysis and Lens Photography
Eyes of age-matched male animals were dissected, blotted dry on tissue paper, and individually weighed. Eyes were transferred to a Petri dish containing 37°C M199 medium (Sigma-Aldrich) with 10 mM HEPES, pH 7.4, on a warm stage. Lenses were dissected and transferred to a prewarmed Petri dish with a glass bottom (WPI, Sarasota, FL) filled with M199 medium. Lenses were visualized and photographed through a SZX9 dissecting microscope equipped with a C3030 zoom digital camera (Olympus of America, Lake Success, NY), blotted dry on tissue paper, and individually weighed (Martinez-Wittinghan et al., 2003).

Histology
Mouse eyes were dissected and fixed in a 4% formaldehyde solution in PBS for 16–24 h at room temperature. Fixed eyes were rinsed with PBS, dehydrated through an ethanol series, and embedded in paraffin. Sections of 2–3 μm were cut on a diamond knife, deparaffinized, and stained with hematoxylin-eosin. Histological sections were viewed on a BX51 microscope (Olympus of America) and photographed with a digital camera (MagnaFire; Optronics).

Glucose Concentration Measurement
Six lenses from 3-wk-old wild-type, Cx50KO, MEK1(E), and MEK1(E)-Cx50KO lenses were dissected and pooled for each measurement. Lenses were deproteinized by homogenization with a 5-mm Polytron (Brinkmann Instruments, Westbury, NY) in 300 μl of 6% HClO 4 solution, followed by centrifugation at 18,000 × g for 10 min at 4°C. Supernatants were neutralized with 70 μl of 2 M K 2 CO 3 and centrifuged at 18,000 × g for 10 min at 4°C. Glucose was quantified with the Glucose (HK) Assay Kit (Sigma-Aldrich) according to the manufacturer’s instructions. Lens glucose concentration was calculated from the total glucose amount per lens and lens volume. Lens volume was calculated assuming the lens was a sphere based on 1/6 πD 3, using the equatorial diameter (D).

RESULTS
MEK1 Activity Increases Cx50 Functional Conductance in Paired Oocytes
If MAPK signaling interacts with gap junctional coupling in the development of lens homeostasis, then activation of the MAPK pathway may have specific functional effects on intercellular channels composed of lens connexins. To test this idea, we coexpressed a constitutively active form of MEK1 kinase, MEK1(E), with either Cx46, or Cx50, in an in vitro paired Xenopus oocyte assay and measured junctional conductance (G j). As shown in Figure 1, injection of oocyte pairs with either water or MEK1(E) alone failed to induce appreciable cell-to-cell coupling. Pairs expressing Cx50 were well coupled with a mean G j of 6.1 μS. Oocytes coexpressing Cx50 with MEK1(E) displayed a 3.4-fold increase in gap junctional conductance (p < 0.05, Student’s t test) over pairs containing Cx50 alone (Figure 1A). This significant enhancement of Cx50-mediated junctional conductance could have resulted from either an increase in the level of connexin protein, or from the activation of quiescent Cx50 channels by MAPK signaling. To distinguish between these possibilities,
homogenates were prepared from oocytes injected with water, MEK1(E), Cx50, or Cx50 and MEK1(E) and analyzed for protein expression by Western blotting. When blots were probed with an anti-Cx50 antibody, qualitatively similar levels of an ~60-kDa band corresponding to Cx50 were visualized in cells injected with Cx50 cRNA alone or in combination with MEK1(E). No signal was detected from cells receiving water or MEK1(E) alone (Figure 1B). To examine MAPK signaling, Western blots were probed with anti-total-ERK and anti-p-ERK antibodies. Injection of the constitutively active MEK1(E) cRNA did not alter the endogenous levels of total ERK (Figure 1C). However, MEK1(E) expression activated the MAPK pathway in oocytes as shown by the large increase in phosphorylated (p)-ERK detection in cells that were injected with MEK1(E) alone or in combination with Cx50 (Figure 1D). Together, these data suggest that Cx50 and MEK1(E) functionally interact in vitro and that the augmentation of Cx50-mediated conductance was not due to an increase in connexin protein expression but was directly correlated with ERK phosphorylation.

Cx46 Does Not Functionally Interact with MEK1(E)

We have previously shown that the in vivo replacement of Cx50 with Cx46 by genetic knockin was not sufficient to restore defects in lens development caused by Cx50 knockout (White et al., 1998; White, 2002; Rong et al., 2002). If interactions between Cx50 and MAPK signaling are required for normal development, then part of the failure of Cx46 to substitute for Cx50 could be caused by an inability to functionally interact with MAPK signaling. To examine this possibility, we repeated our oocyte experiments using Cx46 cRNA (Figure 1E). As expected, oocytes injected with water or MEK1(E) alone were uncoupled and cell pairs expressing Cx46 alone were robustly coupled, with a mean Gj of 10.8 μS. In contrast to the Cx50 data, oocyte pairs that coexpressed Cx46 with MEK1(E) had conductance values that were not significantly different from the pairs expressing Cx46 alone (Gj = 12.5 μS; p > 0.05). Analysis of Cx46 expression (Figure 1F) confirmed qualitatively equivalent protein levels in oocytes injected either with Cx46 alone or in combination with MEK1(E). Examination of total and phosphorylated ERK showed that the expression of MEK1(E) greatly increased p-ERK levels in oocytes, whether or not they coexpressed Cx46 (Figure 1H). These data imply that coupling mediated by Cx46 is not affected by MEK1(E) and that there is a connexin specific interaction between MAPK signaling and Cx50.

FGF Receptor Activation Also Increases Cx50-mediated Coupling

MEK1 is the penultimate kinase in the canonical MAPK signaling pathway, and our data show that a constitutively active form, MEK1(E), specifically increases coupling mediated by Cx50 but not Cx46. During lens development, the MAPK signaling pathway is normally activated by the binding of growth factors to their specific receptors. FGF receptor signaling is known to play a key role in lens development (Robinson, 2006) and mediates many of its effects through the MAPK pathway (Lovicu and McAvoy, 2005), leading us to predict that activation of the FGFR may also have an effect on junctional coupling mediated by Cx50. To evaluate this possibility, we coexpressed the *Xenopus* fibroblast growth factor receptor with Cx50 to activate the MAPK signaling pathway via a receptor–ligand interaction. Oocytes were injected with Cx50 and FGFR individually or together and then paired and incubated with 15 ng/ml FGF before analyzing intercellular coupling (Figure 2A). Cells expressing the FGFR alone failed to induce cell-to-cell coupling above the levels seen in the water-injected negative control pairs. Oocytes expressing Cx50 alone were coupled at a level >50-fold higher than background, with a mean Gj of 4.6 μS. Cells coexpressing Cx50 with FGFR displayed a 4.6-fold increase in coupling (p < 0.05, Student’s t test) over pairs containing Cx50 alone in the presence of FGF ligand. To test whether MAPK signaling was the mediator of FGFR action, we repeated the experiments in the presence of the MEK inhibitor U0126 (Figure 2B). Addition of 15 μM U0126 30 min before FGF application blocked the FGFR induced increase in Cx50 coupling. These data show that activation of MAPK signaling at the level of receptor–ligand interactions has the same effect on Cx50 mediated communication as coexpression of MEK1(E).

Cx50-mediated Gap Junctional Coupling Is Increased in MEK1(E) Transgenic Lenses

Our in vitro results showed that activation of MAPK signaling by two different approaches significantly increased Cx50-mediated coupling. To examine whether MAPK signaling and Cx50 could also interact in vivo, we analyzed a transgenic mouse model that expressed MEK1(E) in the lens under the control of the α-crystallin promoter (Gong et al., 2001). Lens fiber cells from MEK1(E) transgenic animals were shown previously to be severely compromised, but their epithelial cells remained intact (Gong et al., 2001). We have previously documented that Cx50 contributes to epithelial cell coupling in a developmentally regulated manner and that its activity can be distinguished from that of Cx43 or Cx46 by the drug quinine, which specifically inhibits Cx50 (Srinivas et al., 2001; White et al., 2007). To test for an interaction between MAPK signaling and Cx50 in vivo, we isolated primary epithelial cell pairs from wild-type and MEK1(E) lenses and measured the quinine sensitive component of their gap junctional conductance. First, postnatal day
show that epithelial cells in MEK1(E) transgenic lenses have an elevated level of MAPK signaling.

To test for a MEK1(E) effect on Cx50-mediated coupling, P28 epithelial cells were dispersed into pairs, and junctional currents (Ij) were directly measured by dual whole-cell patch clamp before and after perfusion with 300 μM quinine, a concentration that reversibly blocks Cx50, but not Cx43, channels (Srinivas et al., 2001). P28 cells were chosen because Cx50 activity is normally minimal at this age (White et al., 2007), and a MEK1(E)-induced increase in Cx50 activity would be easy to distinguish using quinine. Wild-type cell pairs displayed a 25% reduction in Ij upon perfusion with quinine (Figure 3C), consistent with our previous report that Cx50 is normally a minor contributor to junctional conductance at this developmental age (White et al., 2007). In contrast, P28 MEK1(E) cell pairs had a macroscopic conductance that was twofold higher than wild type, on average, and 66% of the total Ij was quinine sensitive (Figure 3D). If the doubling of magnitude in coupling was due entirely to Cx50 activation by MEK1(E), and there was no Cx43 inactivation, then the Cx43 quinine-insensitive contribution would be expected to drop to 37.5% from 75%, a number in very good agreement with the observed value of 34%. A summary of all perfusion experiments is shown in Figure 3E. Expression of the MEK1(E) transgene increased the quinine-sensitive component of junctional conductance (i.e., Cx50) 2.6-fold (p < 0.05), consistent with our in vitro studies conducted in Xenopus oocytes. Together, these results show that the contribution of Cx50 to lens cell coupling can also be increased by the activation of MAPK signaling in vivo.

**Postnatal Mitosis Stimulated by the MEK1(E) Transgene Is Diminished in the Absence of Cx50**

During normal lens development, Cx50 activity in the epithelium is maximal during peak postnatal mitosis (P2–P3) and declines significantly as the lens matures into an adult organ (P12–P28). In addition, deletion of Cx50 results in a decrease in postnatal mitosis (Sellitto et al., 2004; White et al., 2007). The MEK1(E) transgene significantly increased Cx50 channel activity in the postnatal lens epithelium, maintaining it at a high level as late as P28. To investigate whether this increase in Cx50 coupling correlated with altered mitotic activity in lens capsules, we assayed BrdU incorporation in wild-type, MEK1(E), and MEK1(E)-Cx50KO lenses during the first postnatal week. As shown in Figure 4A, there was no difference in the density of BrdU-labeled cells between wild-type and MEK1(E) lenses on P2, a day when postnatal mitosis and Cx50 activity are normally at their maximum levels (Sellitto et al., 2004; White et al., 2007). Deletion of Cx50 from MEK1(E) lenses reduced BrdU labeling by ~30% (p < 0.05), consistent with our previous studies (Sellitto et al., 2004; White et al., 2007). On P5, BrdU incorporation was reduced in all lenses compared with P2, but the P5 MEK1(E) transgenic epithelial cells had a density of labeling that was 60% increased over P5 wild type (p < 0.05). Deletion of Cx50 from the MEK1(E) animals restored BrdU labeling to wild-type levels on P5 (Figure 4B). In addition to reducing the magnitude of BrdU incorporation on P5, deletion of Cx50 from the MEK1(E) lenses also altered the spatial distribution of mitotic cells. In P5 MEK1(E) lenses, BrdU positive cells were detected across the entire lens capsule (Figure 4C), including the central epithelium (Figure 4D). In contrast, P5 MEK1(E)-Cx50KO capsules had strong labeling in the equatorial “germinative zone” (Figure 4E) but greatly reduced BrdU incorporation in the central epithelium (Figure 4F). These data suggest that MEK1(E) transgene caused a modest
progression of the cataract phenotype. Wild-type lenses Cx46KO, or Cx50KI46 animals and monitored the postnatal MEK1(E) transgenic mice, we crossed them with Cx50KO, contributed to the development of lens opacity in the whether interactions between Cx50 and MAPK signaling atered coupling in MEK1(E) transgenic lens cells. To examine 2001), and our present data showed increased Cx50-medi-

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Figure 4. MEK1(E) stimulated mitosis is reduced by Cx50 knock-

out. (A) On P2, there were no differences in the density of BrdU-

labeled cells between wild-type and MEK1(E) lenses, although deletion of Cx50 from MEK1(E) lenses reduced BrdU labeling by 30%. (B) On P5, the MEK1(E) animals had a density of labeling that was 60% increased over wild-type (p < 0.05), and this increase was eliminated by knockout of Cx50 from the MEK1(E) animals. Deletion of Cx50 from the MEK1(E) lenses also altered the spatial dis-

tribution of mitotic cells on P5. (C) In P5 MEK1(E) lenses, BrdU-
labeled cells were found across the entire capsule, including the central epithelium (D). (E) In P5 MEK1(E)-Cx50KO capsules, BrdU staining was strong in the equatorial zone but greatly reduced in the central epithelium (F).

increase in mitosis on P5 that was dependent on the presence of Cx50 gap junction channels.

Removal of Cx50 Delays Cataract Progression in MEK1(E) Transgenic Lenses

Transgenic overexpression of MEK1(E) in the lens has been reported previously to produce severe cataract (Gong et al., 2001), and our present data showed increased Cx50-mediated coupling in MEK1(E) transgenic lens cells. To examine whether interactions between Cx50 and MAPK signaling contributed to the development of lens opacity in the MEK1(E) transgenic mice, we crossed them with Cx50KO, Cx46KO, or Cx50KI46 animals and monitored the postnatal progression of the cataract phenotype. Wild-type lenses were transparent between 1 and 7 wk of age (Figure 5, A–D). In contrast, MEK1(E) transgenic lenses contained both nuclear and cortical cataracts at 1 wk (Figure 5E) that progressively worsened to a total lens opacity by 5 wk of age (Figure 5G) and frequently displayed lens rupture by 7 wk (Figure 5H). Deletion of Cx46 from the MEK1(E) transgenic animals had no effect on cataract development (Figure 5, I–L). However, when Cx50 was deleted, the lens showed only a mild nuclear cataract at 1 wk of age (Figure 5M), a phenotype shared by the Cx50 KO lens in the absence of the MEK1(E) transgene (White et al., 1998). At 3 wk, both the central and cortical cataracts were much less severe in the MEK1-Cx50KO lens. By 5 wk, the central cataract was similar to that seen in the MEK1(E) transgenic lens (Figure 5O), but the lens cortex remained cataract free in the MEK1(E)-Cx50KO lens through 7 wk of age (Figure 5P). MEK1(E)-Cx50KI46 lenses, where the coding sequence of Cx50 has been replaced with that of Cx46 (White, 2002), were similar to the MEK1(E)-Cx50KO lenses, with a delay in both the progression and severity of the cataract (Figure 5, Q–T). These results show that the specific removal of Cx50, but not Cx46, can delay cataract onset caused by the constitutive activation of MAPK signaling in the MEK1(E) transgenic lens.

Deletion of Cx50, but Not Cx46, Prevents Rupture in MEK1(E) Lenses

While monitoring cataract progression, we observed that lens rupture frequently accompanied the severe cataract that developed in adult MEK1(E) transgenic mice. To determine whether this process also involved a specific interaction between MAPK signaling and Cx50, we monitored the frequency of lens rupture between 1 and 7 wk for wild-type, MEK1(E), MEK1(E)-Cx46KO, MEK1(E)-Cx50KO, and MEK1(E)-Cx50KI46 transgenic mice. Wild-type lenses never exhibited lens rupture in the time period examined, whereas MEK1(E) lenses began to rupture at 3 wk of age and increased to an incidence of 57% rupture by 7 wk (Figure 6A). Lenses from MEK1(E)-Cx46KO mice were also frequently ruptured, beginning at 3 wk and reaching a level of 45% by 7 wk of age. Interestingly, MEK1(E)-Cx50KO lenses failed to exhibit lens rupture during the 7 wk of observation, and a similar low level of rupture was seen with MEK1(E)-Cx50KI46 lenses (Figure 6B). Together, these data suggest that the deletion, or replacement, of Cx50 as one possible target of MAPK signaling rescued the lens rupture induced by MEK1(E).

Cx50 Is Required for the Development of Macrophthalmia in MEK1(E) Mice

MEK1(E) transgenic animals were shown previously to develop macrophtalmia, with a 32% increase in eye mass and a >40% increase in lens mass compared with wild-type mice (Gong et al., 2001). To determine whether changes in size of the lens and eye induced by constitutively active MAPK signaling specifically required Cx50, we plotted eye and lens mass as a function of age for wild-type, MEK1(E), MEK1(E)-Cx46KO, MEK1(E)-Cx50KO, and MEK1(E)-Cx50KI46 transgenic mice. Eye growth for Cx46KO mice was indistinguishable from wild-type, whereas Cx50KO and Cx50KI46 mice had reduced eye mass (Figure 7A), in good agreement with previous data (Gong et al., 1998). Eye growth for Cx46KO mice was indistinguishable from wild-type, whereas Cx50KO and Cx50KI46 mice had reduced eye mass (Figure 7B), as reported previously (Gong et al., 1997; White et al., 1998; White, 2002). Interbreeding MEK1(E) transgenic mice with the different knockin and knockout mice revealed that MEK1(E)-Cx46KO eye growth was identical to that of the MEK1(E) transgenic alone, whereas MEK1(E)-Cx50KO and MEK1(E)-Cx50KI46 were
both significantly reduced ($p < 0.05$, ANOVA) from that of the MEK1(E) transgenic (Figure 7C). Lens growth followed a similar pattern, with the MEK1(E) transgene promoting a significant increase in lens mass that was abolished by deletion or replacement of Cx50 but that was unaffected by deletion of Cx46 (Figure 7, D–F). The significant decreases in eye and lens mass for MEK1(E)-Cx50KO and MEK1(E)-Cx50K146 animals in comparison with MEK1(E) mice suggest that MAPK signaling specifically interacts with Cx50 in the generation of macrophthalmia.

**Histological Analysis of Wild-Type, MEK1(E), and MEK1(E)-Cx50KO Lenses**

Our data have shown that MEK1(E) increases coupling mediated by Cx50 but not Cx46. We have further documented that the specific removal of Cx50 from MEK1(E) transgenic
lenses delays cataractogenesis, eliminates lens rupture, and abolishes macrophthalmia. To gain mechanistic insight into how these observations were possibly linked, we examined histological sections from mouse eyes that were stained with hematoxlyin and eosin. On P7, wild-type eyes revealed normal ocular development with an absence of lens pathology in the central and cortical regions (Figure 8, A and D). In contrast, sections of P7 MEK1(E) eyes were characterized by a pronounced loss of eosin staining in the lens nucleus (Figure 8B) and the appearance of numerous vacuoles in the bow region of the lens cortex (Figure 8E), features consistent with the gross morphology of the intact lens at this age (Figure 5E). Sections from MEK1(E)-Cx50KO lenses exhibited a reduced loss of eosinophilic staining in the core of the lens (Figure 8C) compared with MEK1(E) mice, consistent with the milder cataract seen in intact lenses (Figure 5M). In addition, vacuole formation was never observed in the lens cortex of MEK1(E)-Cx50KO animals (Figure 8F). These data suggest that increased Cx50 coupling induced by MAPK signaling leads to the formation of vacuoles at the lens equator in MEK1(E) transgenic mice. Incorporating these data with previous studies showing that MAPK signaling plays an important role in the increase in gap junctional coupling at the lens equator during fiber differentiation (Mathias et al., 1997; Le and Musil, 2001) further implies that a specific interaction between MAPK signaling and Cx50 may be required for the normal development of lens transport pathways, and that the formation of vacuoles in the MEK1(E) lenses may result from defects in lens transport.

Deletion of Cx50 Reduces MEK1(E)-stimulated Lens Glucose Accumulation in the Lens

An elevation of glucose concentration has been reported previously in MEK1(E) transgenic lenses (Gong et al., 2001). Our lens histology results suggested that removal of Cx50 from the MEK1(E) mice prevented vacuole formation that in turn may have been driven by defects in lens transport. We would predict that if Cx50 deletion ameliorated lens transport defects in MEK1(E) lenses, the previously documented rise in glucose concentration could also be eliminated by Cx50 deletion. To test this possibility, we assayed the glucose concentration in lenses from wild-type, MEK1(E), Cx50KO, and MEK1(E)-Cx50KO mice at 3 wk of age. As shown in Figure 9, the MEK1(E) transgenic lenses exhibited a 60% increase in glucose concentration compared with wild type. Deletion of Cx50 by itself did not significantly alter the lens glucose concentration from wild-type levels. In contrast, MEK1(E)-Cx50KO lenses were significantly different from the MEK1(E) transgenic alone (p < 0.05, Student’s t test) and restored lens glucose concentration to the wild-type level. These results suggest that the removal of Cx50 in the presence of constitutive MAPK signaling restores lens transport at the equator, which in turn eliminates the formation of vacuoles that are potentially formed by the inappropriate accumulation of solutes like glucose.

**DISCUSSION**

We have demonstrated specific functional interactions between Cx50 and MAPK signaling in vivo and in vitro.
MEK1(E) or FGFR activation produced significant increases in junctional coupling mediated by Cx50, but not Cx46, in paired Xenopus oocytes and primary lens epithelial cells. Deletion or replacement of Cx50 in transgenic mice alleviated a variety of lens pathologies induced by transgenic MEK1(E) expression, whereas knockout of Cx46 had no effect. These improvements included a delay in lens opacification, elimination of lens rupture, a reduction of fiber vacuolization in the equatorial region, abolition of microphthalmia, restoration of glucose levels, and normalization of epithelial proliferation. These results suggest that MEK1 specifically modulates coupling mediated by Cx50, but not Cx46, and that gap junctional communication and MAPK signaling may act synergistically in the development of the internal lens circulation, and the maintenance of osmotic balance.

Although we have documented a significant increase in Cx50-mediated coupling induced by MAPK signaling, the mechanism responsible remains unclear. At present, we have no evidence that Cx50 is phosphorylated after MEK activation, although this remains an intriguing possibility. The coupling changes could have resulted from an increase in unitary conductance, or channel open probability. Alternatively, it may have been because of an augmented rate of channel assembly. All of these parameters can be influenced
by phosphorylation of connexin subunits (Moreno and Lau, 2007) and could potentially result from Cx50 being phosphorylated either directly by MAPKs or indirectly via other kinases activated by the MAPK pathway. Future investigation of these parameters may help explain why Cx50 junctional conductance was specifically increased compared with that mediated by Cx46.

We have identified reciprocal roles for MEK1 and Cx50 in osmotic regulation during postnatal fiber development and have summarized the rescue of osmotic defects caused by MEK1(E) by deletion or replacement of Cx50 in Table 1. Fiber gap junctional coupling contributes to an internal circulating current that underpins the nonvascular microcirculatory system used by the lens to maintain transparency (Mathias et al., 1997; Donaldson et al., 2001). Sodium is the primary ion carrying the circulating current, and it enters the lens at the anterior and posterior poles and flows inward along the extracellular spaces. Na+ is driven across fiber cell membranes by its electrochemical gradient, and in the fiber cytoplasm the direction of flow is reversed, with the current moving back toward the lens surface through gap junction channels (Mathias et al., 2007). Because the coupling of differentiating fibers is normally highest at the equator and lowest at the poles (Baldo and Mathias, 1992), the intercellular current is directed to the equatorial epithelium where Na+/K+ -ATPase activity is also concentrated (Gao et al., 2000; Candia and Zamudio, 2002; Tamiya et al., 2003), and sodium is pumped out of the lens to complete the circuit. Water and dissolved metabolites follow the Na+ current creating the microcirculatory system for the avascular lens.

Our data support a model in which the normal gradient in junctional communication is established by specific interaction between MAPK signaling and Cx50. The tremendous increase in lens mass resulting from the MEK1(E) transgene has been shown previously to be primarily a result of water accumulation within the lens (Gong et al., 2001), implying an osmotic imbalance that could be driven by ion and solute accumulation within the lens. This notion is supported by the vacuole formation and glucose accumulation in MEK1(E) lenses that accompany the increase in water content. The highest levels of MAPK signaling are normally found at the lens equator (Le and Musil, 2001; Lovicu and McAvoy, 2005), and in mice expressing constitutively active MEK1(E) throughout the lens, this gradient is likely to be disrupted. The specific interaction between MEK1(E) and Cx50 could result in increased fiber coupling in other regions of the lens than the equator, disrupting the normal angular variation in fiber cell junctional coupling. Because the extensive vacuolization of fiber cells in the MEK1(E) lenses, it impossible to directly test this hypothesis by measuring the angular variation in coupling by using whole-lens impedance techniques (Martinez-Wittingham et al., 2003). However, this idea is supported by the elimination of glucose accumulation, fiber vacuolization, macrophthalmia, and lens rupture by the specific deletion of Cx50 from MEK1(E) transgenic mice.

Constitutive activation of a potent signal transduction pathway would be expected to wreak havoc with normal processes of development, as was found to be the case with MEK1(E) transgenic animals (Gong et al., 2001). In recent years an unexpected role for connexins in lens development has emerged from numerous studies of genetically engineered mice (White et al., 1998; Kong et al., 2002; Sellitto et al., 2004; Xia et al., 2006). Although the deficiencies in cell proliferation and differentiation that result from genetic mutation of Cx50 are less severe than the developmental defects that arise from the experimental manipulation of MAPK signaling (Robinson et al., 1995; Lovicu and McAvoy, 2001; Xie et al., 2006; Zhao et al., 2008), there is potential overlap in regulation of some aspects of development. Here, we have shown that simply removing Cx50 as one potential target for MAPK signaling can significantly diminish the developmental chaos induced by MEK1(E) expression in the lens. These data suggest that signal transduction and gap junctional communication may specifically interact in the maintenance of osmotic balance, and broaden the paradigm of how intercellular communication contributes to the regulation of development of specialized organs like the lens.

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REFERENCES


Table 1. Summary of osmotic defects in the different mouse models

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Cx46 gene</th>
<th>Cx50 gene</th>
<th>MEK1(E)</th>
<th>Macrophthalmia</th>
<th>Lens rupture</th>
<th>Vacuole formation</th>
<th>Glucose accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>+/+</td>
<td>+/+</td>
<td>–</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>MEK1(E)</td>
<td>+/+</td>
<td>+/+</td>
<td>+</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Cx46KO</td>
<td>/–</td>
<td>+/+</td>
<td>–</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cx50KO</td>
<td>+/+</td>
<td>–/–</td>
<td>–</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Cx50K14h</td>
<td>+/+</td>
<td>46/46a</td>
<td>–</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cx46KO-MEK1(E)</td>
<td>/–</td>
<td>+/+</td>
<td>+</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cx50KO-MEK1(E)</td>
<td>+/+</td>
<td>–/–</td>
<td>+</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Cx50K14h-MEK1(E)</td>
<td>+</td>
<td>46/46a</td>
<td>+</td>
<td>46/46a</td>
<td>46/46a</td>
<td>46/46a</td>
<td>46/46a</td>
</tr>
</tbody>
</table>

N.D., not determined.

* 46/46, knockin mice which lack endogenous Cx50 and have extra copies of Cx46 in its place (White, 2002).


