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A novel role for FGF and extracellular signal–regulated kinase in gap junction–mediated intercellular communication in the lens

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Gap junction–mediated intercellular coupling is higher in the equatorial region of the lens than at either pole, a property believed to be essential for lens transparency. We show that fibroblast growth factor (FGF) upregulates gap junctional intercellular dye transfer in primary cultures of embryonic chick lens cells without detectably increasing either gap junction protein (connexin) synthesis or assembly. Insulin and insulin-like growth factor 1, as potent as FGF in inducing lens cell differentiation, had no effect on gap junctions. FGF induced sustained activation of extracellular signal–regulated kinase (ERK) in lens cells, an event necessary and sufficient to increase gap junctional coupling. We also identify vitreous humor as an in vivo source of an FGF-like intercellular communication-promoting activity and show that FGF-induced ERK activation in the intact lens is higher in the equatorial region than in polar and core fibers. These findings support a model in which regional differences in FGF signaling through the ERK pathway lead to the asymmetry in gap junctional coupling required for proper lens function. Our results also identify upregulation of intercellular communication as a new function for sustained ERK activation and change the current paradigm that ERKs only negatively regulate gap junction channel activity.

Introduction

Visual acuity is dependent on the proper development and function of the ocular lens. The vertebrate lens is composed of only two cell types: a monolayer of epithelial cells that overlies its anterior face and the elongated, crystallin-rich fiber cells that differentiate from the epithelial cells at the lens equator (for review see Piatigorsky, 1981; Wride, 1996; McAvoy et al., 1999). Although it slows postnatally, the process of epithelial-to-fiber differentiation continues throughout the lifetime of the organism. Because lens cells neither die nor are shed, the size of the lens increases with age (Harding et al., 1977).

The unique optical properties of the lens are due in part to the absence of blood vessels or nerves and to the extraordinarily tight cell-to-cell packing of the fiber cells. How, then, does this solid, ever-expanding mass of cells remain in metabolic and ionic homeostasis (and thus transparent) through a lifespan that can exceed 100 yr? A major mechanism by which this is accomplished is an extensive network of gap junctional intercellular channels that physically and functionally link the cells of the lens (for review see Goodenough, 1992). Gap junctions are clusters of transmembrane channels that connect the plasma membranes of two adjoining cells. Substances under ~1 kD in molecular mass, including current-carrying ions, nutritional metabolites, and second messengers, are transferred from the cytoplasm of one cell to the cytoplasm of the other by diffusion via these reversibly gated channels. Present in virtually all cell types, gap junctions serve to maintain metabolic continuity within, and relay signals between, connected cells. The only known structural components of gap junctions in vertebrates are connexins, members of a family of four-transmembrane integral plasma membrane proteins that differ from each other with respect to their channel permeabilities, modes of regulation, and ability to interact with other connexin species (Bruzzone et al., 1996a; for review see Goodenough et al., 1996). Three members of the connexin family have been identified in the lens: connexin 43 (Cx43), expressed in the epithelium, and connexins 50 and 46 (or, in the chick, their avian orthologues Cx45.6 and Cx56), expressed at very high levels in fiber cells (Musil et al., 1990a; Paul et al., 1991; White et al., 1992; Rup et al., 1993; Jiang et al., 1994). Between one third and one half of the cell surface of mature fiber cells has been reported to be occupied by gap junctions,

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the largest fraction in any tissue of the body (Kuszak et al., 1985). Elimination of either Cx50 (White et al., 1998) or Cx46 (Gong et al., 1997) expression in mice by targeted gene disruption results in the formation of cataracts without grossly affecting epithelial-to-fiber differentiation. The ocular phenotypes of the $Cx50^{-/-}$ and $Cx46^{-/-}$ mice are distinct, indicating that the two connexins have nonidentical roles in lens homeostasis.

In the species examined (Baldo and Mathias, 1992; Mathias et al., 1997), gap junction–mediated intercellular communication between lens fiber cells is not uniform, but is instead higher in the equatorial region than at either the anterior or posterior poles of the organ (see Fig. 10 for a diagram of lens anatomy). Based largely on vibrating probe current measurements (Robinson and Patterson, 1983; Parmelee et al., 1985) and impedance studies (Mathias et al., 1985; Baldo and Mathias, 1992), a model has been developed in which ionic current (carried mainly by sodium) enters the lens predominantly through the extracellular spaces between fiber cells at the poles, crosses the fiber cell membranes, and is then transferred from cell to cell via gap junctions back to the lens surface at the equator. Water and dissolved solutes are thought to follow, establishing a nonvascular microcirculatory system that brings nutrients deep into the lens and flushes out waste products. The asymmetric distribution of open fiber-to-fiber gap junctional channels is believed to dictate the direction of this flow and to therefore play an essential role in the maintenance of lens transparency (for review see Goodenough, 1992; Mathias et al., 1997).

It is not known how the regional differences in gap junctional coupling in the lens are generated. As assessed by immunofluorescence microscopy, none of the known fiber connexins are markedly more concentrated throughout the equatorial axis of the lens than at the poles (Gruijters et al., 1987; Berthoud et al., 1994; Dahm et al., 1999). A study in human lens using freeze-fracture electron microscopy, which detects gap junctions by their unique structural features instead of by immunoreactivity, failed to reveal quantitative differences in gap junction channel content between equatorial region and polar fiber cells (Vrensen et al., 1992). Therefore, there is no compelling evidence that the estimated 14–335-fold increase in intercellular electrical conductance in the lens equatorial region (Baldo and Mathias, 1992; Rae et al., 1996) is accompanied by a proportional increase in the number of channels assembled from either previously characterized or novel connexin species. Instead, the enhanced coupling at the equator appears to be due at least in part to greater flux through gap junctional channels in this region. As in other organs, gap junction–mediated intercellular communication in the lens is reversibly regulated by a wide variety of effectors, including intracellular acidification, transjunctional voltage, cyclic nucleotides, transforming viral oncogene products, and some classes of lipophilic compounds (for review see Bruzzone et al., 1996b). Although in most cases the mechanisms underlying these phenomena remain obscure, some effectors may interact directly with the connexin molecule and induce conformational changes that gate the gap junctional channel. For example, phosphorylation of mammalian Cx43 by extracellular signal–regulated kinase (ERK)*-type mitogen-activated protein (MAP) kinases has been reported to rapidly reduce channel permeability in both intact cells (Warn-Cramer et al., 1998; Zhou et al., 1999) and in lipid vesicles reconstituted with immunopurified connexin (Kim et al., 1999). In contrast, the function of connexins that lack ERK consensus phosphorylation sites (either naturally or as a result of mutation) was not affected (Warn-Cramer et al., 1998; Zhou et al., 1999).

In addition to having the highest level of gap junctional coupling in the lens, the equatorial region is also the site of epithelial-to-fiber differentiation. Over 30 yr of investigation have led to the widely accepted concept that fiber differentiation is initiated by a factor (or factors) in the posterior of the eye that diffuses out of the vitreous body and interacts with cells at the lens equator (McAvoy and Chamberlain, 1989; Hyatt and Beebe, 1993; Schulz et al., 1993). In mammals, both ex vivo and in vivo studies (including lens-specific expression of dominant negative mutant forms of FGF receptor 1 in transgenic mice) strongly implicate one or more members of the FGF family in the differentiation process (Schulz et al., 1993; Chow et al., 1995; Robinson et al., 1995a; McAvoy et al., 1999). We have recently demonstrated that primary lens cells from embryonic chick, previously thought to be unresponsive to FGF (Beebe et al., 1987; Hyatt and Beebe, 1993), in fact undergo differentiation when cultured in the presence of exogenously added FGF-1 or FGF-2 for periods (>5 h) longer than those used in prior investigations. Moreover, an activity with properties indistinguishable from an FGF was shown to be capable of diffusing out of whole chick vitreous bodies to effect fiber formation (Le and Musil, 2001). Taken together, these studies are indicative of a physiologically important role for FGF in both mammalian and avian lens development, although which of the more than 23 FGF family members participate in this process is not yet known.

The evolutionarily conserved response of lens cells to FGF and the high concentrations of this growth factor in vitreous humor (Mascarelli et al., 1987; Caruelle et al., 1989; Schulz et al., 1993) led us to consider whether FGF might be involved in the upregulation of gap junction function in the lens equatorial region. In this study, we show that FGF (either FGF-2 or FGF-1 plus its cofactor heparin) increases gap junction–mediated intercellular communication in primary cultures of chick lens epithelial cells in a reversible manner that does not involve an increase in either connexin expression or in gap junction assembly. Both purified recombinant FGF and the FGF-like activity of vitreous humor induced sustained activation of ERK in lens cells, an event necessary as well as sufficient to upregulate gap junctional coupling. Moreover, we show that FGF-induced activation of ERK in the intact lens is higher in the equatorial region than in polar and core fibers. These studies support a model in which the distribution of FGF-induced ERK signaling in the lens plays a central role in establishing the gradient of gap junctional coupling believed to be essential for lens transparency. The

*Abbreviations used in this paper: 18β-GA, 18β-glycyrrhetinic acid; BFA, brefeldin A; CA, constitutively active; DCDML, dissociated cell-derived monolayer; ERK, extracellular signal–regulated kinase; MAP, mitogen-activated protein; NCAM, neural cell adhesion molecule; TER, transepithelial electrical resistance.

lens is the first system in which ERKs have been demonstrated to positively regulate gap junction permeability.

Results

FGF specifically upregulates gap junction–mediated intercellular dye transfer in cultured embryonic chick lens cells

Although cells isolated from the lenses of several vertebrate species will take on fiber-like characteristics when maintained ex vivo, epithelial-to-fiber differentiation is most completely recapitulated in primary cultures of embryonic chick lens epithelial cells (Menko et al., 1984, 1987; TenBroek et al., 1994). This system also most faithfully reiterates fiber-type gap junction formation (FitzGerald and Goodenough, 1986; Menko et al., 1987; Jiang et al., 1993). DCDML cultures prepared from E10 chick lenses as described by Menko et al. (1984) (termed DCDMLs) are enriched in cells originating from the equatorial region of the lens, the area that undergoes both fiber differentiation and upregulation of gap junctional intercellular coupling in vivo. We have shown previously that DCDML cultures continue to divide and differentiate in a defined, growth factor–free medium (M199/BOTS), albeit to a more limited extent than in the presence of serum. They also remain coupled by gap junctions and express all three known chick lens connexins (Cx43, Cx45.6, and Cx56; Le and Musil, 1998). Addition of either purified recombinant FGF, insulin, or IGF-1 to serum-free DCDML cultures stimulates both cell proliferation and the expression of fiber differentiation markers (Le and Musil, 2001). We tested whether any of these growth factors affected gap junctional intercellular communication using the scrape-loading/dye transfer assay (el-Fouly et al., 1987). In the experiments depicted in Fig. 1 A, the membrane impermeant, low-molecular weight fluorescent dye Lucifer yellow was introduced into DCDML cultures by scraping the monolayer with a 27-gauge needle. As evaluated by the spread of Lucifer yellow from cells at the scrape border to adjoining unwounded cells, cultures maintained in the absence of added growth factor were moderately well coupled by gap junctions. Intercellular transfer of Lucifer yellow was increased an average of 2.72-fold (± 0.48; Fig. 2 A) when cells were cultured for 2 d in 15 ng/ml FGF-2. In contrast, neither 1 μg/ml insulin nor (not shown) 15 ng/ml of the IGF-1 analogue R^3IGF-1, although comparable to 15 ng/ml FGF-2 in their ability to upregulate expression of fiber differentiation markers (Le and Musil, 2001), had any detectable effect on Lucifer yellow transfer. Qualitatively similar results were obtained with biocytin, a gap junction–permeable compound with physical properties distinct from those of Lucifer yellow that is a more sensitive tracer of gap junctional communication in lens cells (Le and Musil, 1998; Fig. 1 B). Cultures treated with FGF were indistinguishable from those exposed to insulin/IGF with regard to cell number, protein content, and incorporation of [^{35}S]methionine into proteins synthesized during a 4 h pulse (data not shown). The increase in intercellular dye transfer in FGF-treated cells was prevented by the gap junction blocker 18β-GA (Davidson et al., 1986; Le and Musil, 1998), indicating a bona fide increase in gap junction–mediated intercellular communication (Fig. 2 A).

As shown in Fig. 2 A, upregulation of gap junctional intercellular dye transfer in DCDML cultures required concentrations of FGF-2 >1 ng/ml. The effect of 15 ng/ml FGF-2 was very similar to that of 50 ng/ml FGF-2; higher doses were deleterious to lens cells. FGF-1 also increased intercellular coupling, but only in the presence of its cofactor heparin which by itself was ineffective. Insulin (at up to 5 μg/ml) and R^3IGF-1 (5–50 ng/ml) had no effect on gap junction activity either in the absence or (not shown) presence of FGF, nor did the other purified growth factors tested (TGFα and EGF). Dye transfer was also not significantly increased by either a phosphodiesterase inhibitor/forskolin mix or a nonhydrolyzable cAMP analogue (8-CPT-cAMP), two previously characterized activators of protein kinase A that are known to enhance junctional communication in several nonlenticular cell types (Saez et al., 1986; Atkinson et al., 1995; van Rijen et al., 2000).

Time course experiments revealed that significant upregulation of cell-cell coupling required greater than 12 h of continuous exposure to FGF and reached a maximum by 24 h of treatment (Fig. 2 B). The level of cell–cell coupling achieved after 24 h of FGF exposure persisted for at least 6 d (the longest period tested) provided the cells were fed every 2 d with fresh FGF-containing medium. If the cells were instead refed with unsupplemented M199/BOTS (after washing out extracellular FGF in either the absence or presence of heparin), junctional coupling remained elevated for the next 24 h, but returned to basal levels by 48 h after FGF washout (Fig. 2 C). Therefore, the growth factor did not permanently change the junctional phenotype of lens cells. Insulin did not appreciably affect intercellular coupling at any of the time points tested (30 min, 1 h, 3, 4, or 6 d; not shown).

FGF-induced upregulation of gap junctional coupling is not mediated by an increase in connexin expression, gap junction assembly, or cell–cell adhesion

Although a direct demonstration was precluded by the long-term toxicity of inhibitors of protein and mRNA synthesis, the time course of the effect of FGF on intercellular coupling in DCDML cultures was suggestive of a change in gene expression. Pepper and Meda (1992) have reported that FGF increases the synthesis of Cx43 in cultured endothelial cells, leading to enhanced gap junction assembly and intercellular coupling. Similarly, FGF4 upregulates gap junction formation and function in undifferentiated posterior limb bud mesenchyme cells (Makarenkova et al., 1997). To investigate whether FGF increases connexin expression in lens cells, DCDMLs cultured for 2 d in either the absence or presence of added growth factor were analyzed for connexin content by immunoblotting. When normalized to total cellular protein, no reproducible effect of FGF on Cx43, Cx45.6, or Cx56 levels was observed (Fig. 3 A). Each connexin migrated on SDS-PAGE as multiple species, presumably as a result of posttranslational phosphorylation events. Phosphorylation of Cx43 to the lowest mobility form (Cx43-P_2) is closely correlated with its assembly into Triton X-100–insoluble, functional gap junctions in multiple cell

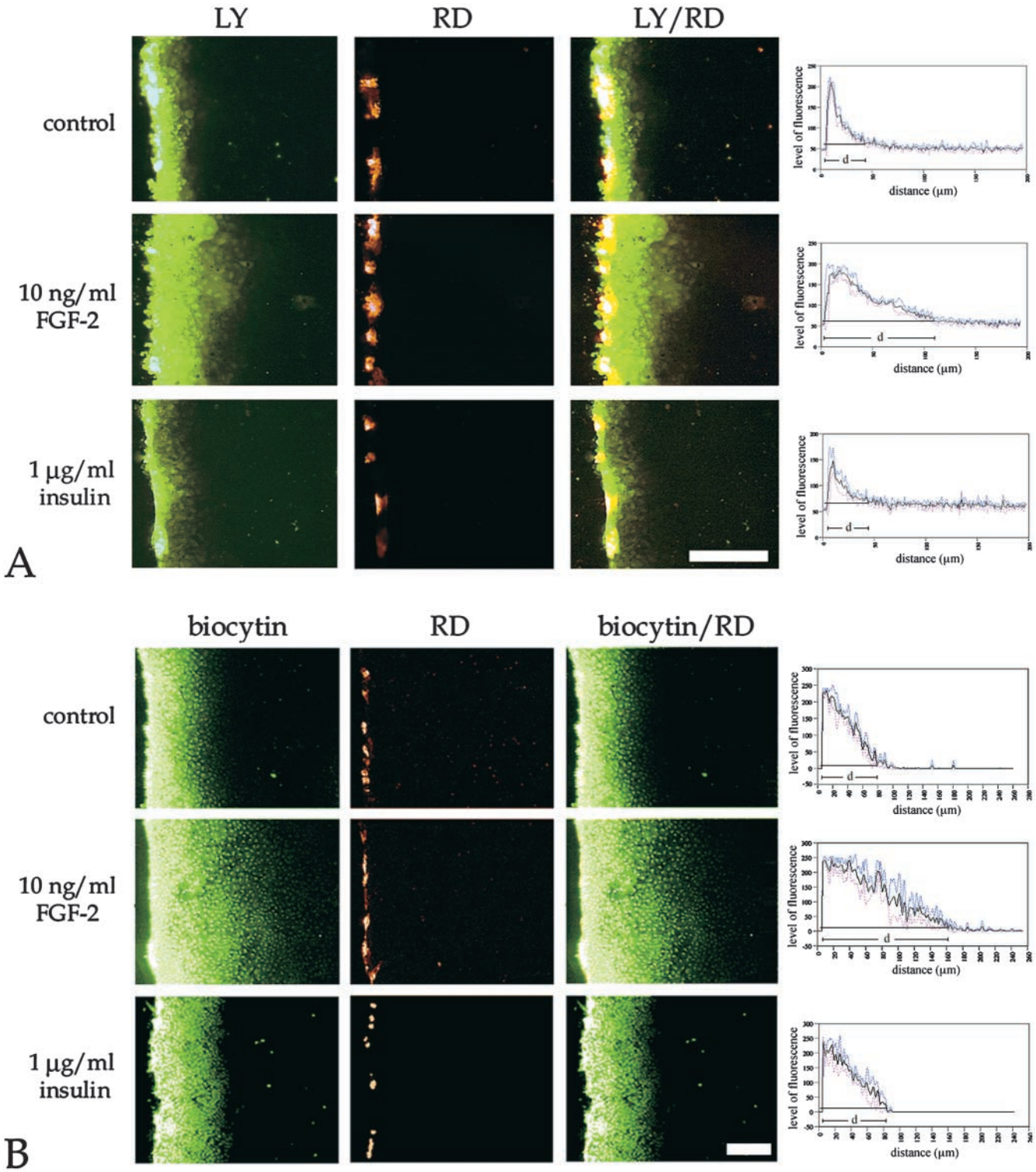
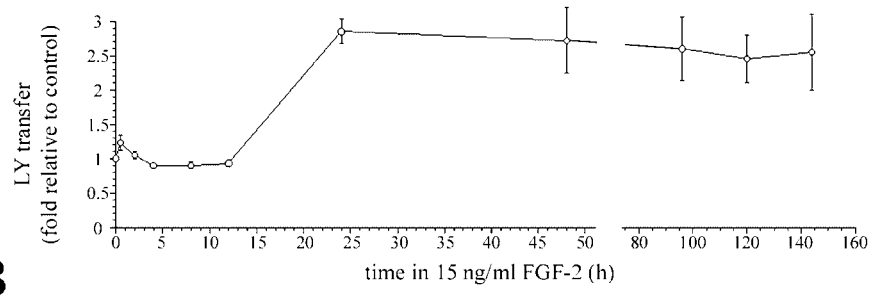


Figure 1. **FGF, but not insulin, increases gap junctional intercellular communication in chick lens DCDMLs.** 1-d-old DCDML cultures prepared from E10 chick lenses were incubated for an additional 48 h in either the absence (control) or presence of the indicated growth factor. Gap junction–mediated intercellular communication was then assessed using the scrape-loading/dye transfer assay as described in Materials and methods. Cells were scrape-loaded with rhodamine-dextran mixed with either Lucifer yellow (A) or biocytin (B). After either 8 (A) or 2 min (B), the cells were fixed and the dyes visualized by fluorescence microscopy. The M_r = 10 kD rhodamine-dextran (A and B; RD) remained confined to the cells at the wound edge into which dye had been directly introduced during the scrape-loading process. In contrast, Lucifer yellow (A; LY) and biocytin (B; biocytin) were transferred to adjacent cells via open gap junctional channels. Each panel depicts a portion of the right half of the scrape/load wound. Superposition of the staining pattern of the two dyes is shown to the immediate right of the rhodamine-dextran images (LY/RD and biocytin/RD) Graphs at far right: averaged fluorescence plot profiles of the corresponding Lucifer yellow (A) or biocytin (B) images. In each case, the solid line represents the average of three fluorescence profiles generated as described in Material and methods, and the dotted lines indicate standard deviation (blue and magenta, respectively). The distance of gap junction–mediated dye transfer into the monolayer (d) is indicated; d for FGF-treated monolayers was essentially the same if the concentration of Lucifer yellow was reduced from 1 to 0.04% (data not shown). Bars, 100 μm.

A

treatment	fold LY transfer relative to control
control	1 (n=30)
15 ng/ml FGF-2	2.72 +/- 0.48 (n=30)*
15 ng/ml FGF-2 + 10 μM 18β-GA (1h)	0.7 +/- 0.1 (n=4)*
1 ng/ml FGF-2	0.96 +/- 0.15 (n=5)
15 ng/ml FGF-1 + 2 μg/ml heparin	3.83 +/- 0.71 (n=3)*
15 ng/ml FGF-1	1.03 +/- 0.04 (n=3)
2 μg/ml heparin	1 +/- 0.13 (n=3)
50 ng/ml FGF-2	2.73 +/- 0.30 (n=3)*
100 ng/ml insulin	0.99 +/- 0.17 (n=5)
1 μg/ml insulin	0.98 +/- 0.4 (n=4)
5-50 ng/ml IGF-1	0.93 +/- 0.09 (n=3)
40 μM forskolin/50 μM RO-20-1724	1.37 +/- 0.22 (n=3)
170 μM 8-CPT	1.15 +/- 0.14 (n=9)
10 ng/ml TGFα	1.01 +/- 0.12 (n=3)
100 ng/ml EGF	0.95 (n=2)

B



C

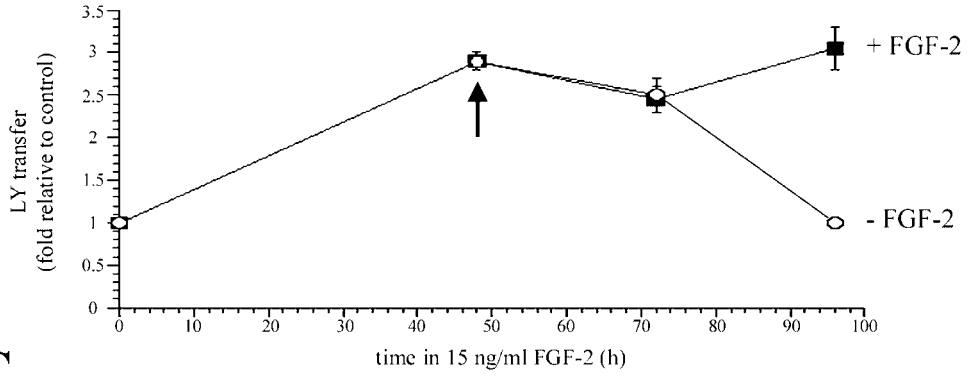
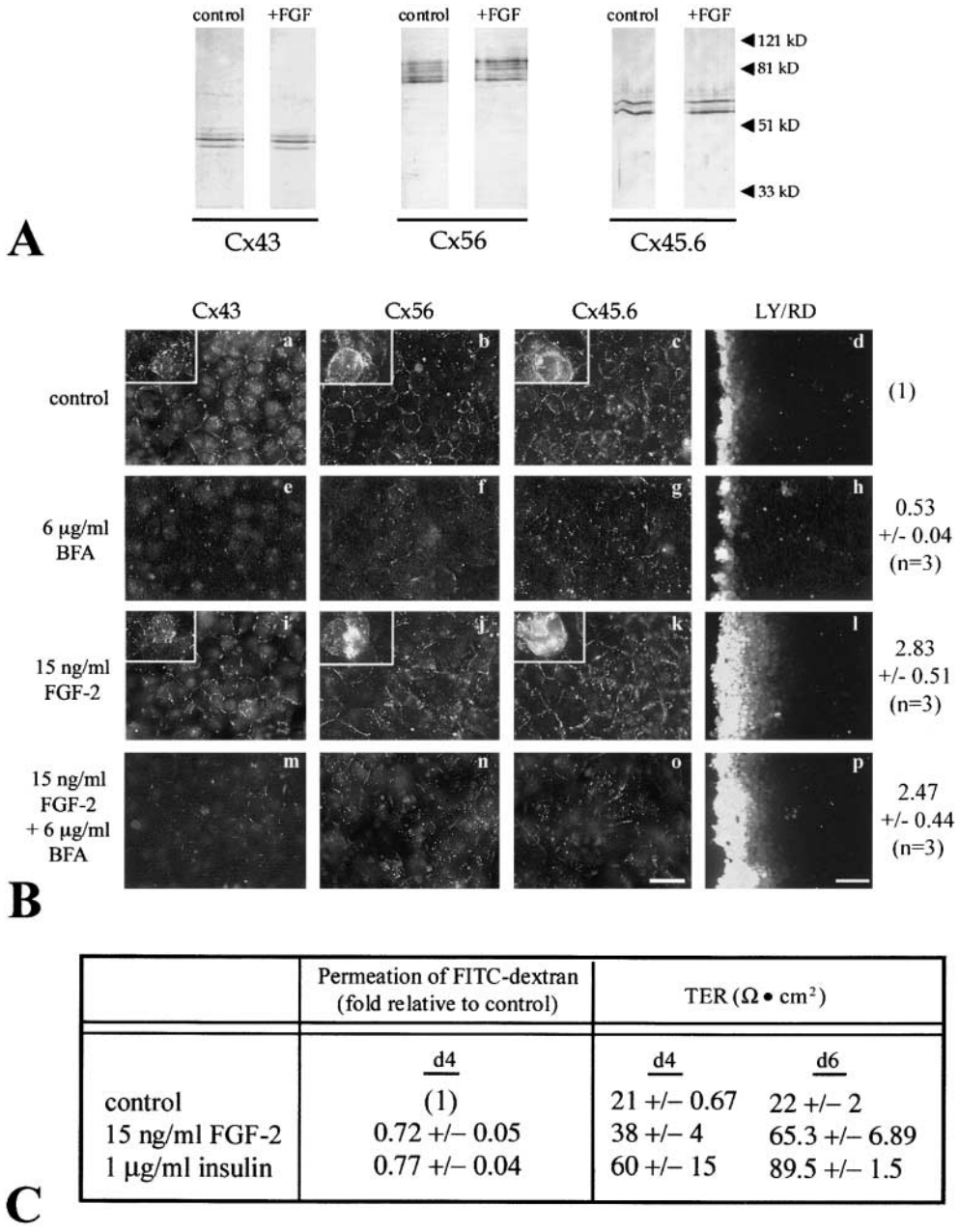


Figure 2. **Summary of effects of growth factors on gap junctional intercellular communication in chick lens DCDML cultures.** (A) 1-d-old DCDML cultures were incubated for an additional 48 h in either the absence (control) or presence of the indicated additive(s) and then assayed for gap junction–mediated intercellular communication as described in the legend to Fig. 1 A. The averaged results were expressed relative to Lucifer yellow transfer in control cells within the same experiment. The effect of the gap junction blocker 18β-GA was assessed by culturing cells for 3 d with 15 ng/ml FGF-2 in M199/BOTS lacking bovine serum albumin (which binds 18β-GA) and then measuring dye transfer 1 h after addition of 10 uM 18β-GA. Each value presented is the mean of three or more independent experiments (n, number of experiments) ± standard deviation, except that for 100 ng/ml EGF, which is the average of two experiments (LY transfer, 0.9-fold and 1.0-fold relative to control). The asterisks denote values significantly different from control ($P < 0.05$) as assessed by the two-tailed paired Student's *t* test. (B) DCDMLs were incubated with 15 ng/ml FGF-2 for 0.5, 2, 4, 8, 12, 24, 48, 96, 120, or 144 h, after which intercellular transfer of Lucifer yellow was quantitated as in A. (C) 1-d-old DCDMLs were cultured for an additional 48 h in 15 ng/ml FGF-2, at which time FGF was stripped from the cell surface with heparin as described in Materials and methods. The cells were then incubated for either 0, 24, or 48 additional hours in fresh M199/BOTS medium with (solid squares) or without (open ovals) 15 ng/ml FGF and assessed for intercellular transfer of Lucifer yellow as in A. The results plotted in B and C are the means from three or more experiments ± standard deviation.



C

	Permeation of FITC-dextran (fold relative to control)	TER ($\Omega \cdot cm^2$)	
	d4	d4	d6
control	(1)	21 +/– 0.67	22 +/– 2
15 ng/ml FGF-2	0.72 +/– 0.05	38 +/– 4	65.3 +/– 6.89
1 μg/ml insulin	0.77 +/– 0.04	60 +/– 15	89.5 +/– 1.5

Figure 3. **FGF's effect on intercellular coupling in chick lens cells is not mediated by an increase in connexin protein expression, gap junction assembly, or cell–cell adhesion.** (A) 1-d-old DCDMLs were cultured for two additional days in M199/BOTS in the absence (control) or presence of FGF-2 or insulin. Cells were solubilized in SDS and whole cell lysates (1 μg protein/lane) probed for Cx43, Cx56, or Cx45.6 by immunoblotting. In some experiments, FGF appeared to increase the amount of the slowest migrating form of Cx56. This effect was shared with insulin, which does not upregulate junctional communication (not shown). Molecular mass markers are indicated on the right. (B) DCDMLs cultured for 2 d in M199/BOTS in the absence (control) or presence of 15 ng/ml FGF-2 were incubated for an additional 3 h either with or without 6 μg/ml BFA. The cultures were then immunostained for Cx43, Cx56, or Cx45.6, or assayed for intercellular transfer of Lucifer yellow as in Fig. 1 A. Insets, connexin immunoreactivity in differentiating lentoids. The values given to the right of the micrographs represent the fold Lucifer yellow transfer (± standard deviation) relative to (−BFA, −FGF) controls within the same experiment; n, number of independent experiments. *P* for +BFA compared with (−BFA, −FGF) control was 0.02; +FGF +BFA was not significantly different from +FGF ($P = 0.25$) as assessed by the two-tailed paired Student's *t* test. (C) DCDML cells were cultured on Transwell filter inserts in unsupplemented M199/BOTS (control) or in the presence of FGF-2 or insulin. On the indicated days, the paracellular permeability of the cell monolayer to either FITC-dextran or current-carrying ions (TER) was measured as described in Materials and methods. FITC-dextran permeation was expressed relative to growth factor–free controls within the same experiment to compensate for minor interexperimental differences in cell confluence. Each value presented is the mean (± standard deviation) of three experiments. Bars, 20 μm.

types (Musil and Goodenough, 1991; Laird et al., 1995; Nagy et al., 1997), and modification of Cx56 and Cx45.6 to phosphospecies resolvable on SDS-PAGE has been suggested to play a role in their function (Berthoud et al., 1997; Yin et al., 2000). However, FGF treatment did not reproducibly affect the banding pattern of any of these proteins or (data not shown) the sensitivity of Cx43 to Triton X-100. Pulse–chase analysis of Cx43 and Cx56 also failed to reveal

FGF-dependent effects on turnover rate or electrophoretic mobility; Cx45.6 was not examined due to limited antibody availability (not shown).

Assembled gap junctions are visualized by immunofluorescence microscopy as punctate or linear concentrations of anticonnexin staining at cell–cell interfaces. FGF did not appear to qualitatively or quantitatively affect the pattern of Cx43, Cx45.6, or Cx56 immunoreactivity in DCDMLs (Fig. 3 B; compare a–c with i–k). In both treated and untreated cultures, Cx45.6 and Cx56 staining was especially strong in lentoids, clusters of enlarged cells previously shown to be enriched in fiber differentiation markers (Menko et al., 1984; Le and Musil, 1998). More definitive proof that FGF did not enhance intercellular communication by increasing the number of gap junctions was provided by experiments in which cultures were incubated for 3 h with brefeldin A (BFA). BFA blocks the transport of newly synthesized connexins to the cell surface without affecting the degradation of preexisting gap junctions (Musil and Goodenough, 1993; Laird et al., 1995). In both FGF-treated and control (no FGF) cultures, BFA dramatically decreased the amount of Cx43, Cx56, and Cx45.6 detectable at cell–cell interfaces, as expected given the rapid rate at which connexins turn over (compare a–c with e–g, and i–k with m–o). If FGF enhanced intercellular dye transfer by increasing gap junction assembly, then using BFA to reduce the number of immunofluorescently detectable gap junctions in FGF-treated cells to below the number observed in untreated (−BFA, −FGF) control cells should correspondingly reduce the level of intercellular dye coupling to less than that in the untreated controls. If FGF acted instead by increasing the permeability (but not the quantity) of gap junctional channels, then junctional coupling may remain elevated in FGF-treated cells after BFA exposure despite the reduction in the number of gap junctional plaques. As shown in Fig. 3 B, our results were consistent with the latter, but not the former, possibility: although the large decrease in gap junctional plaques elicited by BFA reduced intercellular transfer of Lucifer yellow to near background levels in control (no FGF) cells (compare d with h), it had no statistically significant impact ($P = 0.25$; $n = 3$) on the extent of Lucifer yellow transfer in FGF-treated cells (compare l with p). This finding suggests that the average amount of dye transferred per immunocytochemically detectable gap junction channel is higher in FGF-treated cells than in untreated controls, but does not identify which connexin species are involved in this effect.

In some cell types, the efficiency with which cell surface connexins are assembled into functional gap junctional channels is positively correlated with the strength of intercellular adhesion (Keane et al., 1988; Musil et al., 1990b). Because growth factors have been reported to increase cell–cell adhesion in some systems (Bracke et al., 1993; Guvakova and Surmacz, 1997), we used a previously established assay (Martin-Padura et al., 1998) to investigate whether FGF's effect on gap junctional communication might be a downstream consequence of increased cell–cell apposition (Fig. 3 C). DCDML cultures were established on Transwell filter inserts in either the absence or presence of added growth factor. Confluent cultures were then tested for their ability to impede the paracellular movement of apically added FITC-dextran (Mr = 40 kD) through the monolayer. FGF treatment reproducibly reduced the amount of FITC-dextran that diffused through the intercellular space to the basolateral medium, indicative of an increase in cell–cell adhesion. However, this response did not correlate with upregulation of junctional communication, insomuch as insulin had a similar effect despite its inability to stimulate intercellular coupling. Both insulin and FGF also modestly increased the resistance of the cell monolayer to the paracellular passage of current-carrying ions (transepithelial electrical resistance [TER]). Because development of TER is indicative of the formation of a tight junction barrier, the latter finding implies that cultured embryonic chick lens cells retain their in vivo ability (Goodenough et al., 1980) to form tight junctions. FGF treatment had no detectable effect on the immunostaining pattern of two known lens intercellular adhesion molecules, N-cadherin and NCAM (data not shown). We conclude that enhanced intercellular apposition could contribute to, but cannot solely account for, FGF's effect on gap junction function.

Taken together, the studies presented in Fig. 3 do not support increased connexin expression or assembly, as the mechanism by which FGF upregulates intercellular communication in cultured lens cells. Therefore, FGF most likely acts at the level of gating of the gap junctional channel (see Discussion). Elevated gap junction–mediated intercellular coupling without a proportional increase in gap junction number is also a feature of the equatorial region of the lens in vivo.

Role of ERK in FGF-mediated upregulation of lens cell coupling

In the lens, as in many other tissues, FGF activates the ERK subclass of MAP kinases (Chow et al., 1995; Le and Musil, 2001). Activation of ERKs by growth factors or other stimuli has been causally linked to an inhibition of Cx43-mediated intercellular communication in several nonlenticular cell types (Hossain et al., 1998; Warn-Cramer et al., 1998; Zhou et al., 1999) but not, to our knowledge, to a posttranslational increase in the function of any connexin species. The ability of FGF to upregulate gap junctional coupling in cultured lens cells could therefore either be due to an ERK-independent activity of the growth factor or be the result of a previously unknown effect of the MAP kinase cascade on gap junction function. To distinguish between these two possibilities, we used UO126, a potent, cell permeable, nontoxic, and highly specific inhibitor of the kinases (MEK 1/2) immediately upstream of ERK in the MAP kinase cascade (Favata et al., 1998; Le and Musil, 2001). As assayed by Western blotting with an antibody specific for the dually phosphorylated, active form of ERK (Khokhlatchev et al., 1997), UO126 completely blocked the ability of FGF to activate the ERK pathway in lens cells (Fig. 4 A). This effect persisted for at least a week provided the cells were refed with fresh UO126-containing medium every 2 d. Scrape-load dye transfer analysis demonstrated that UO126 pretreatment completely prevented FGF from increasing intercellular coupling in lens cultures. In contrast, upregulation of junctional communication in response to 15% fetal calf serum was in-

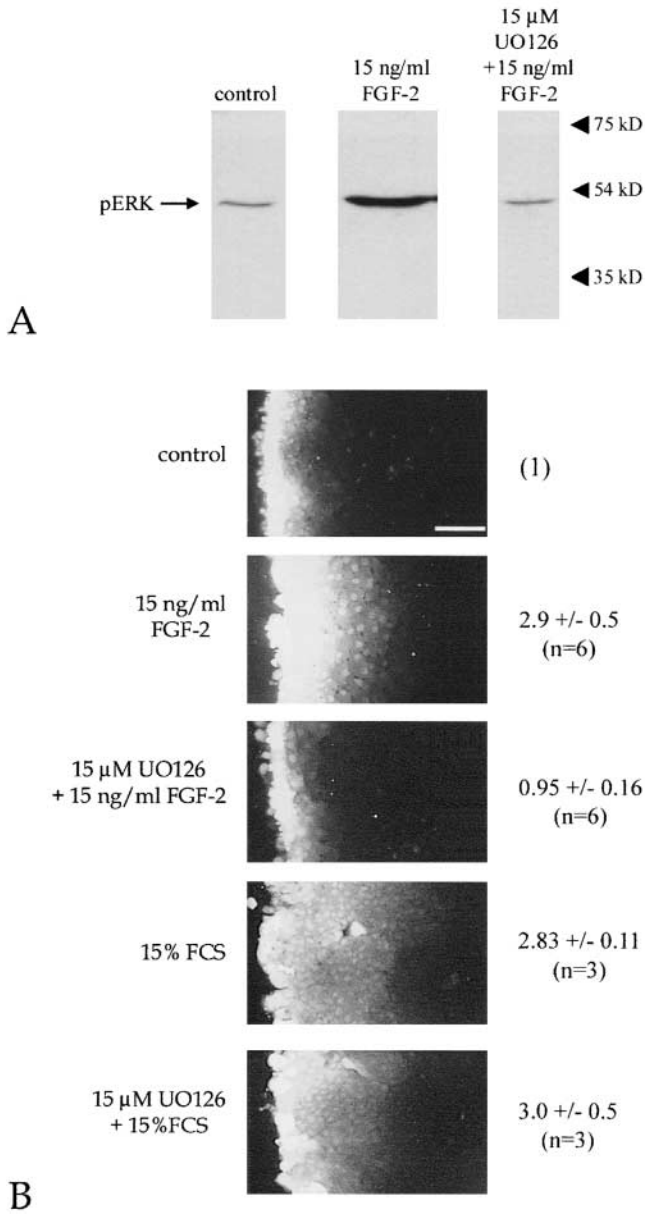


Figure 4. **ERK activation is required for FGF to increase gap junctional intercellular communication in chick lens cells.** (A) 3-d-old DCDMLs, cultured in M199/BOTS, were incubated for 15 min at 37°C with no additions (control), with 15 ng/ml FGF-2, or with 15 μM UO126 plus 15 ng/ml FGF-2 after a 30 min pretreatment with 15 μM UO126 alone. The samples were then immediately solubilized in SDS and whole cell lysates assessed for activation of ERK by immunoblotting with the phospho-specific anti-p44/42 MAP kinase E10 monoclonal antibody. Molecular mass markers are indicated on the right. Note that chick lens cells express only the 42 kD (ERK2) MAP kinase species. (B) 1-d-old DCDMLs were incubated for 30 min with or without 15 μM UO126, after which they were incubated for an additional 48 h with no additions (control), 15 ng/ml FGF-2, or 15% fetal calf serum, in the absence or presence of 15 μM UO126 as indicated. The cells were then assayed for gap junction–mediated intercellular communication as described in the legend to Fig. 1 A. Only Lucifer yellow immunofluorescence is presented; rhodamine-dextran was confined to a single row of cells immediately bordering the wound (see Fig. 1). The values given to the right of the micrographs represent the fold Lucifer yellow transfer (± standard deviation) relative to untreated controls within the same experiment; n, number of independent experiments. *P* for UO126 +FGF-2 compared with +FGF-2 was 0.0004; UO126 +FCS was not significantly different from +FCS ($P = 0.55$). UO126 did not affect the amount of total (activated plus inactive) ERK protein, nor did it influence the basal level of intercellular dye transfer (not shown). Bar, 50 μm.

sensitive to inhibition of the ERK pathway (Fig. 4 B). The latter finding demonstrated that UO126 is not a general inhibitor of gap junction function and reveals the existence of ERK-independent (fetal calf serum–mediated) in addition to ERK-dependent (FGF-mediated) pathways of gap junction upregulation in lens epithelial cells. Because ocular fluids contain only very low levels of serum proteins (Beebe et al., 1986), the effect of fetal calf serum on lens cell junctional coupling is unlikely to be of physiological relevance.

Constitutive activation of ERK stimulates intercellular dye transfer in DCDMLs

To determine whether ERK activation was sufficient to increase gap junctional coupling in lens cells, DCDML cultures were transiently transfected with a plasmid encoding a constitutively active form of MEK1 (CA-MEK) (Cowley et al., 1994; Le and Musil, 2001). Immunocytochemistry with antiphosphoERK and anticonnexin antibodies revealed that the CA-MEK construct activated endogenous ERK in 60–70% of the cells in the culture without detectably altering the immunostaining pattern of Cx43, Cx45.6, or Cx56 (data not shown). Expression of CA-MEK1 induced a large increase in the amount of phosphorylated ERK detectable by immunoblotting (Fig. 5, top) and increased the intercellular transfer of Lucifer yellow (Fig. 5, bottom) in cells cultured in the absence of FGF or other added growth factors by an average of 2.3-fold. Both effects were completely blocked by the MEK inhibitor UO126. Neither wild-type MEK1 (Fig. 5; WT-MEK) nor the irrelevant transfection control β-galactosidase (not shown) stimulated ERK activation (Fig. 5, top) or gap junctional coupling (Fig. 5, bottom). Culture medium conditioned by CA-MEK–expressing cells failed to increase Lucifer yellow transfer in untransfected cells, suggesting that the effect of the activated kinase was cell autonomous (data not shown). We conclude that ERK family kinases are positive effectors of gap junctional intercellular communication in lens cells.

FGF-mediated upregulation of gap junction function requires sustained activation of ERK

The demonstration that ERK activation is both necessary and sufficient to upregulate intercellular coupling in DCDMLs created an apparent paradox in that insulin and IGF-1 also stimulate ERKs in chick and rodent lens cells (Le and Musil, 2001), yet have no effect on gap junctions. One of the most important determinants of the biological outcome of MAP kinase signaling is the length of time that a stimulus activates ERKs (Marshall, 1995). As assessed by quantitative antiphosphoERK immunoblotting, 1–15 ng/ml FGF-2, FGF-1 with or without its cofactor heparin, 1 μg/ml insulin, or (not shown) 15 ng/ml IGF-1 all comparably activated ERK within 15 min of addition to DCDML cultures (Fig. 6). Only the treatments capable of upregulating junctional communica-

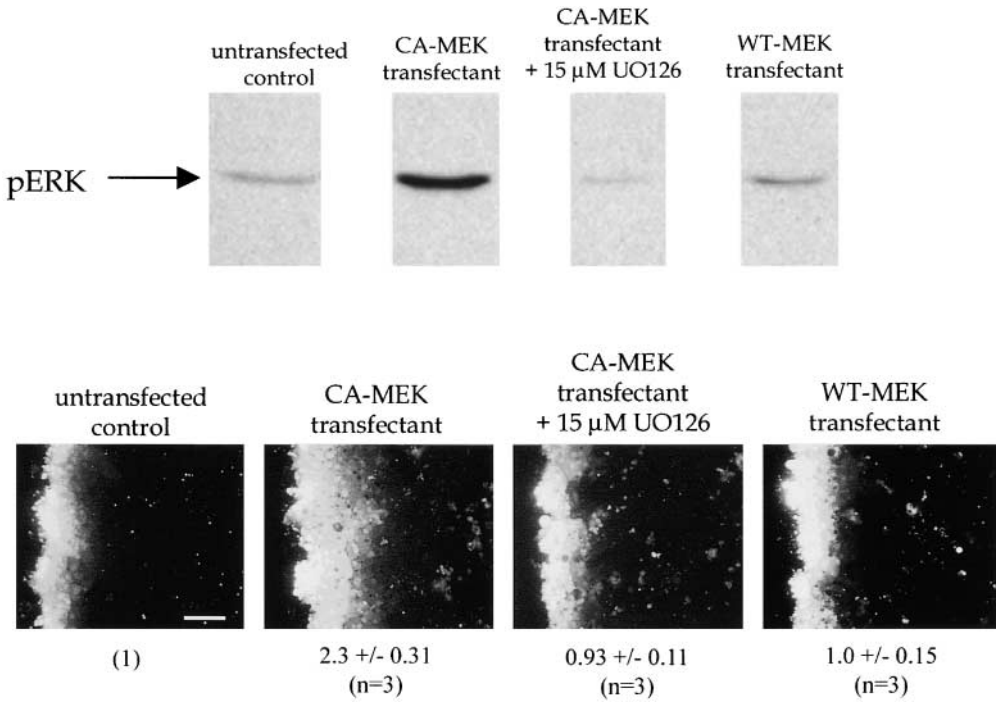


Figure 5. **ERK activation is sufficient to upregulate gap junctional intercellular communication in chick lens cells.** DCDMLs were transfected the day after plating with plasmids encoding either a mutant form of MEK1 that constitutively activate ERKs (CA-MEK), or wild-type MEK1 (WT-MEK). Where indicated, the MEK inhibitor UO126 (15 μM) was added 3 h after transfection. Top, whole cell lysates were prepared 48 h after transfection and assessed for activation of ERK by immunoblotting with the phospho-specific anti-p44/42 MAP kinase E10 monoclonal antibody. Phospho-ERK immunoreactivity is lower in UO126-treated transfectants than in untransfected controls because UO126 reduces the level of basal ERK activity in DCDML cultures after long-term (>4 h) treatment (Le and Musil, 2001). Bottom, gap junctional intercellular communication was assessed 48 h after transfection as described in the legend to Fig. 1 A. Only Lucifer yellow immunofluorescence is presented; rhodamine-dextran was confined to a single row of cells immediately bordering the wound (see Fig. 1). The values given under the micrographs represent the fold Lucifer yellow transfer (± standard deviation) relative to untransfected controls within the same experiment; n, number of independent experiments. Only the value obtained for CA-MEK was significantly higher than control ($P > 0.05$). Bar, 50 μm.

tion (15 ng/ml FGF-2 or FGF-1 plus heparin; Fig. 2 A), however, sustained ERK activation for more than 8 h.

To determine whether the observed correlation between FGF-mediated sustained ERK activation and upregulation of intercellular coupling reflected a cause-and-effect relationship, the duration of ERK activity was varied using the MEK inhibitor UO126 (Fig. 7). 15 ng/ml FGF-2 was added to lens cells and, after 4, 8, or 12 h, UO126 was included and the incubation continued for a total of 24 h in FGF. PhosphoERK blots verified that addition of UO126 reduced the level of activated ERK to near that of untreated controls within 90 min (Fig. 7 A, lane 3), and that this inhibition persisted for over 8 h (lane 5). Scrape-load dye transfer analysis revealed that ERK had to be active for at least 12 h in order for FGF to increase gap junctional coupling (Fig. 7 B). Given that expression of CA-MEK also induces long-term activation of ERKs and increased junctional coupling (Fig. 5), these findings establish a new role for sustained ERK activation in the regulation of intercellular communication.

Vitreous humor is an in vivo source of an FGF-like communication-promoting activity

For FGF to play a physiologically important role in the regulation of gap junction–mediated intercellular communication in the lens, it must be present in an appropriate location in the ocular environment. In both the mammalian and avian eye, FGFs (derived largely from the retina) are concentrated in vitreous humor, the liquid component of the gel-like vitreous body that occupies most of the posterior chamber (Mascarelli et al., 1987; Caruelle et al., 1989; Schulz et al., 1993). We have recently shown that crude vitreous humor diluted 1:2.3 in M199/BOTS medium (termed 30% vitreous humor [VH]) increases the expression of fiber differentiation markers including δ-crystallin in embryonic chick lens DCDML cultures. Moreover, M199/BOTS conditioned overnight with whole vitreous bodies has a similar effect, demonstrating that the active factor in vitreous humor is capable of diffusing out of the vitreous body and affecting lens cell fate (Le and Musil, 2001). Both 30% VH (from either embryonic chick or adult mouse) and vitreous body conditioned medium (VBCM) increased intercellular transfer of Lucifer yellow to an extent comparable to 15 ng/ml purified recombinant FGF (Fig. 8 B). Moreover, they also induced sustained activation of ERKs as assessed by antiphosphoERK immunoblotting (Fig. 8 A). The effect of vitreous on junctional communication, like that of recombinant FGF, was sensitive to the gap junction blocker 18β-GA, required a minimum of 12–24 h of treatment, and did not detectably alter the immunostaining pattern of either Cx43, Cx45.6, Cx56, NCAM, or N-cadherin (data not shown).

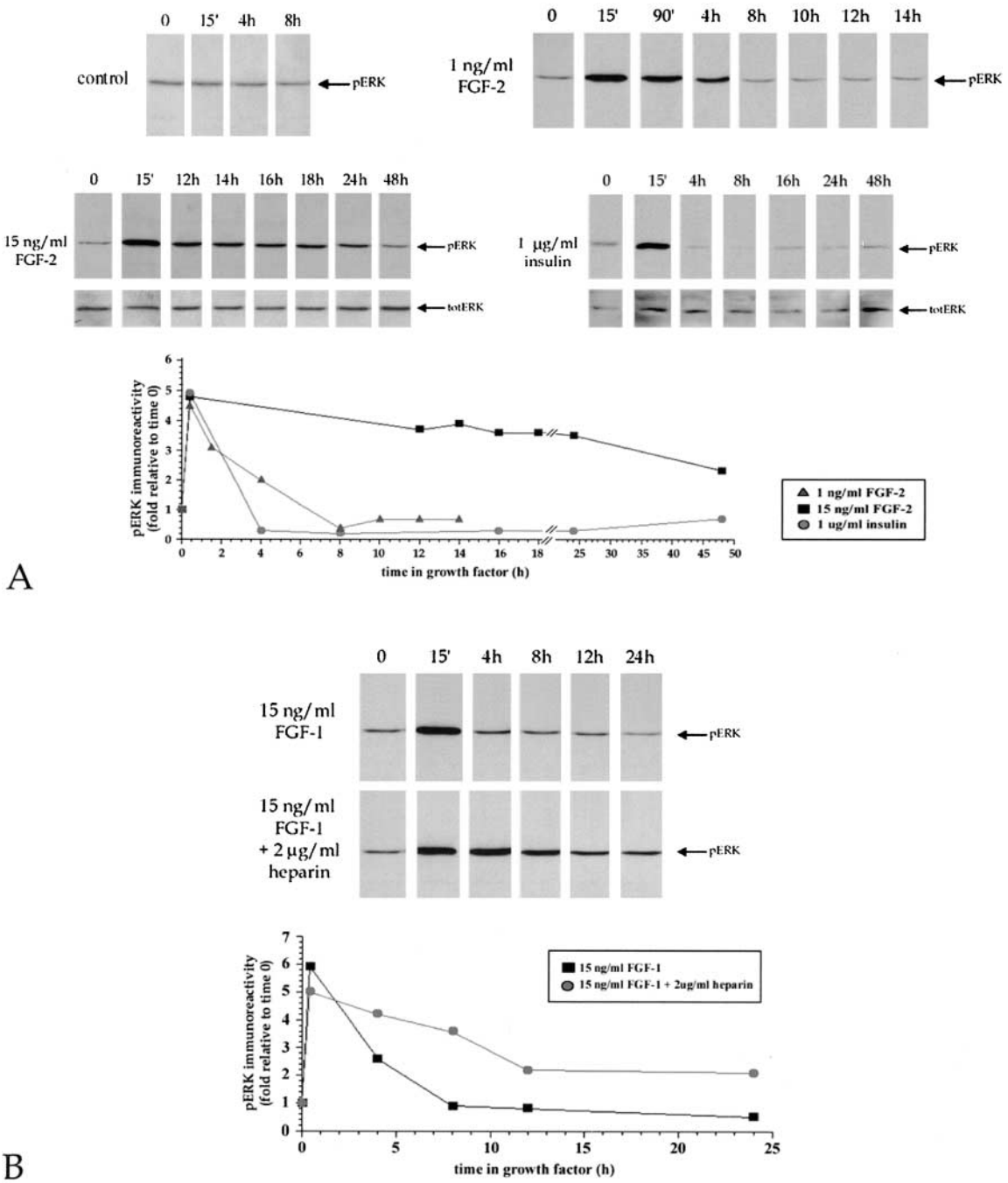


Figure 6. **FGF-mediated upregulation of gap junctional intercellular communication in chick lens cells correlates with sustained activation of ERK.** (A and B) The indicated growth factor was added directly to the culture medium of 3-d-old DCDMLs plated and maintained in M199/BOTS. After the indicated period (15 min to 48 h) at 37°C, the cultures were solubilized in SDS and whole cell lysates were assessed for activation of ERK by immunoblotting with the phospho-specific anti-p44/42 MAP kinase E10 monoclonal antibody. Control cells received no added growth factor. After quantitation of the pERK bands by densitometry, the blots were stripped and reprobed for total ERK (both activated and inactive; totERK) to verify that the level of ERK protein remained constant throughout the experiment; results are shown for 15 ng/ml FGF-2 and 1 µg/ml insulin. The data are graphed as the fold increase in pERK immunoreactivity in treated cells relative to the amount of pERK in untreated time 0 controls (first panel in each time course) within the same experiment. The data shown are representative of three independent experiments. Note that the time points analyzed in Fig. 6 A differ between the various treatments.

A defining characteristic of all members of the FGF family is their high affinity for heparin, which distinguishes them from other growth factors known or suspected to be present in the eye (Ornitz, 2000). Control experiments verified that the gap junctional communication-promoting activity of 50 ng/ml recombinant FGF was quantitatively bound to heparin-Affigel beads in 0.1 M NaCl and was eluted from the beads only when the salt concentration was raised to 2.5 M (Fig. 9, top row). The ability of 30% VH (or, not shown, vitreous body conditioned medium) to enhance intercellular transfer of Lucifer yellow was abolished if the sample was incubated with immobilized heparin in 0.1 M NaCl, and only

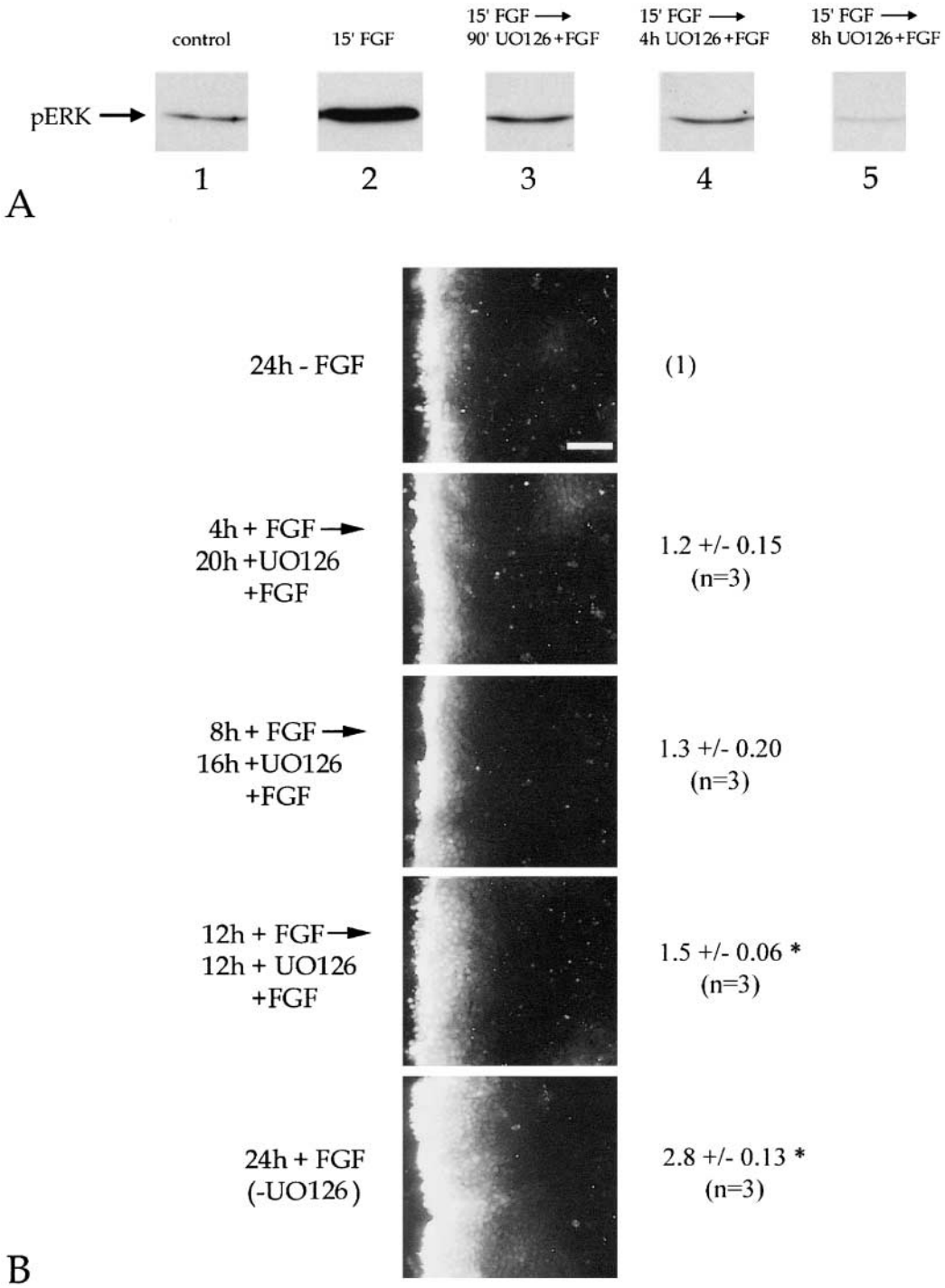


Figure 7. **FGF-mediated upregulation of gap junctional intercellular communication in chick lens cells requires sustained activation of ERK.** (A) 3-d-old DCDMLs cultured in M199/BOTS were incubated for 15 min at 37°C in the absence (lane 1; control) or presence of 15 ng/ml FGF-2. In lanes 3–5, 15 μM UO126 was then added to inhibit further activation of ERK. At the indicated times, the cells were solubilized in SDS and whole cell lysates were assessed for phosphoERK immunoreactivity. pERK immunoreactivity is lower in lane 5 than in the lane 1 control because UO126 reduces the level of basal ERK activity in DCDML cultures after long-term (>4 h) treatment (Le and Musil, 2001). (B) 3 d after plating, DCDML cells were incubated for an additional 24 h in either the absence of FGF (24 h −FGF), in the presence of 15 ng/ml FGF-2 (24 h + FGF), or for 4, 8, or 12 h in the presence of 15 ng/ml FGF-2 before addition of 15 uM UO126 and a further 20, 16, or 12 h incubation (respectively) in FGF plus UO126. The cells were then assayed for gap junction–mediated intercellular communication as described in the legend to Fig. 1 A. Only Lucifer yellow immunofluorescence is presented; rhodamine-dextran was confined to a single row of cells immediately bordering the wound. The values given to the right of the micrographs represent the fold Lucifer yellow transfer (± standard deviation) relative to untreated controls within the same experiment; n, number of independent experiments. The asterisks denote values significantly different from control ($P < 0.05$) as assessed by the two-tailed paired Student's *t* test; *P* for 12 h plus FGF compared with untreated control was 0.01. Note that at least 12 h of ERK activation was required for FGF to stimulate intercellular transfer of Lucifer yellow. Bar, 50 μm.

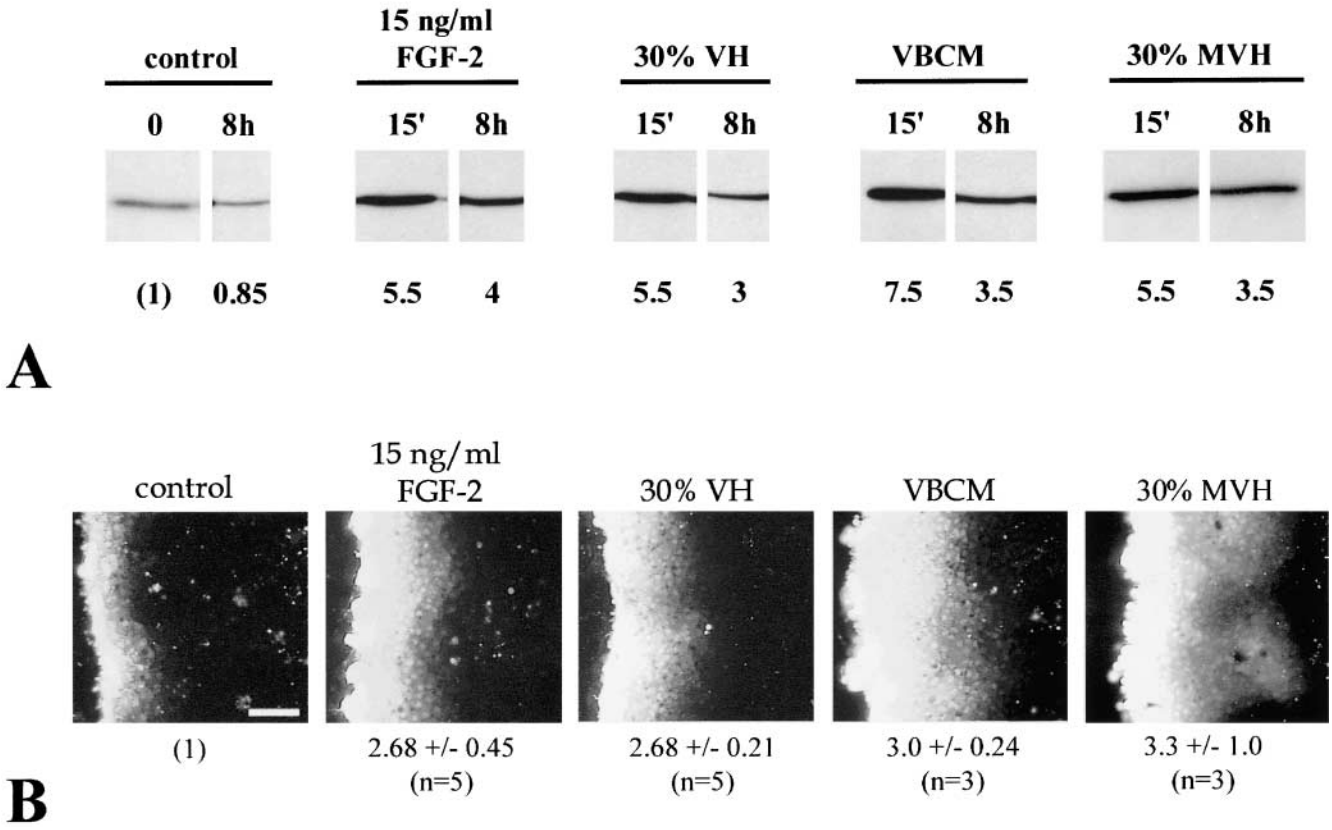


Figure 8. **Vitreous humor induces sustained ERK activation and increases gap junctional intercellular communication in chick lens cells.** (A) 3-d-old DCDMLs cultured in M199/BOTS were incubated at 37°C in fresh M199/BOTS medium without additions (control), in fresh M199/BOTS with 15 ng/ml FGF-2, in chick vitreous humor diluted with 2.3 vol of M199/BOTS (30% VH), in M199/BOTS conditioned with intact E10 chick vitreous bodies (VBCM), or in 30% vitreous humor prepared from adult mouse (30% MVH) as indicated. After either 15 min or 8 h, the cells were solubilized in SDS and whole cell lysates assessed for activation of ERK by immunoblotting with the phospho-specific anti-p44/42 MAP kinase E10 monoclonal antibody. The numbers under the blots are the fold increase in pERK immunoreactivity in treated cells at the indicated time relative to the amount of pERK in control cells at time 0. (B) Gap junctional intercellular communication in DCDMLs cultured for 2 d in the absence (control) or presence of the agents described in A was assessed as in Fig. 1 A. Only Lucifer yellow immunofluorescence is presented; rhodamine-dextran was confined to a single row of cells immediately bordering the wound. The values given underneath the micrographs represent the fold Lucifer yellow transfer (± standard deviation) relative to untreated controls within the same experiment; n, number of independent experiments. All treatments significantly elevated dye coupling ($P \leq 0.05$). Bar, 50 μm.

an insignificant amount of activity remained if the adsorption was carried out in 0.6 M salt (Fig. 9, bottom row). Incubation of the vitreous-treated heparin beads with 2.5 M NaCl released an activity that increased intercellular dye transfer an average of 2.1-fold and (not shown) induced sustained activation of ERK. This elution behavior is typical of an FGF (Seed et al., 1988) and has been used to purify FGFs from bovine (Schulz et al., 1993) and chick (Mascarelli et al., 1987) vitreous humor. The δ-crystallin–promoting activity of vitreous also binds to heparin with high affinity (Le and Musil, 2001), strongly supporting (although not unequivocally proving) a role for one or more of the ~23 members of the FGF family in both fiber differentiation and upregulation of intercellular communication.

Localization of FGF-induced ERK activation in the intact lens

If vitreous-derived FGF plays a role in increasing junctional communication at the lens equator in vivo, then the cells in this region must be able to respond to extralenticular FGF by sustained activation of ERK. To address this issue, intact lenses were dissected from embryonic chick eyes, incubated overnight in unsupplemented M199 medium to reduce the level of endogenous activation of ERK (Chow et al., 1995), and the still viable lenses treated with 25 ng/ml recombinant FGF-2 for either 2 or 8 h. The lenses were then microdissected, under conditions designed to maintain the in vivo phosphorylation state, into three fractions: the central epithelium, the equatorial region, and the polar and core fibers that constitute the remainder of the lens (see diagram, Fig. 10). Each fraction was assayed by immunoblotting for activated (pERK) as well as for total (totERK) ERK. As shown in Fig. 10, FGF efficiently induced ERK activation in the central epithelium and in the equatorial region that was sustained for more than 8 h. In contrast, pERK immunoreactivity was much less enhanced by FGF in the polar and core fiber fraction despite concentrations of total ERK protein in excess of those in the equatorial region. The distribution of FGF-activated ERK supports a model in which regional differences in FGF signaling underlie the asymmetry of gap junctional intercellular coupling in the lens (see Discussion).

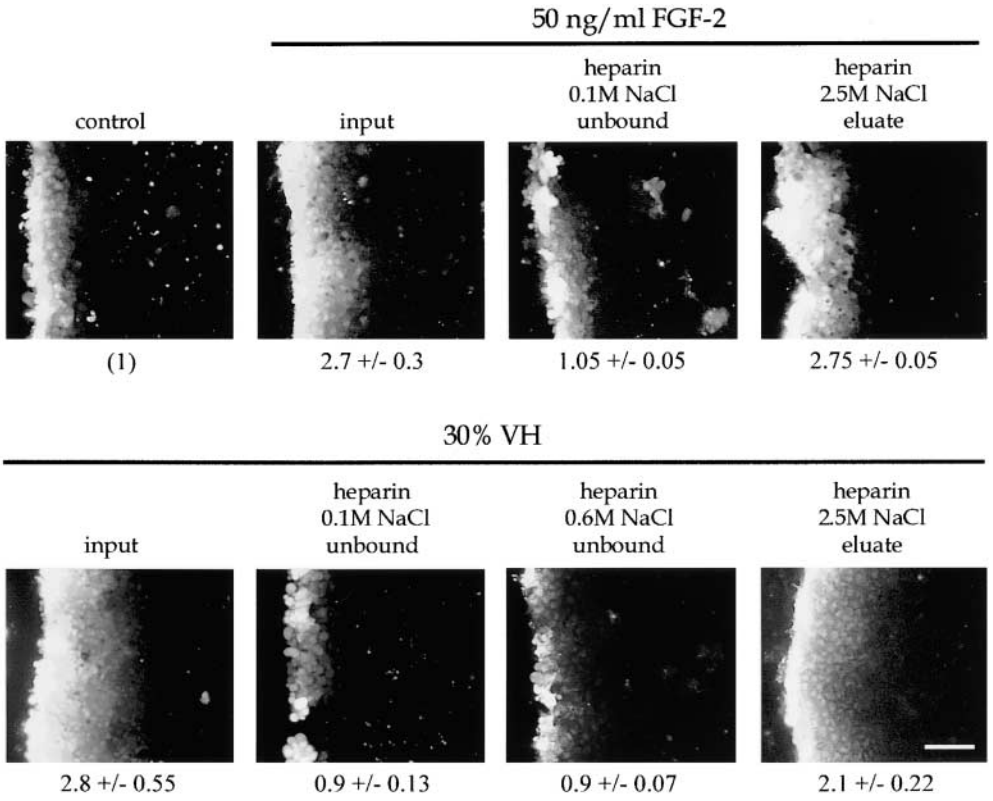


Figure 9. **Vitreous humor contains a gap junctional intercellular communication-promoting activity with properties of an FGF.** 50 ng/ml FGF-2 in M199/BOTS or E10 chick vitreous humor diluted with 2.3 vol of M199/BOTS (30% VH) was incubated with heparin-conjugated Affigel beads in the presence of 0.1 M NaCl or 0.6 M NaCl as described in Materials and methods. The beads were pelleted, and after removal of the supernatant (heparin 0.1 M or 0.6 M NaCl unbound), FGF-like activity was eluted with 2.5 M NaCl (heparin 2.5 M NaCl eluate). The fractions were then brought to 0.15 M NaCl by repeated rounds of concentration and dilution with M199 medium. DCDMLs were cultured for 2 d in M199/BOTS with no additions (control), in M199/BOTS with unfractionated FGF-2 (50 ng/ml FGF-2 input), in unfractionated 30% VH (30% VH input), or with the indicated heparin-Affigel fraction. Gap junctional intercellular communication was assessed as described in the legend to Fig. 1 A; only Lucifer yellow immunofluorescence is presented. In all cases, rhodamine-dextran was confined to a single row of cells immediately bordering the wound. Representative results from three independent experiments; similar results were obtained with vitreous body conditioned medium (not shown). Note that the heparin beads quantitatively bound the communication-promoting activity of even the highest concentration of FGF tolerated by lens cells (50 ng/ml). The values given underneath the micrographs represent the fold Lucifer yellow transfer (± standard deviation) relative to untreated controls within the same experiment. Only the values obtained for the 30% VH input and the heparin 2.5 M NaCl eluate were significantly higher than control ($P < 0.03$). Bar, 50 μm.

Discussion

Despite recent insights into the structure and physiological role of gap junctions in the lens, the mechanisms that regulate their function in vivo remain obscure. Gap junction–mediated intercellular coupling between outer cortical fiber cells is higher at the equator of the lens than at either the anterior or posterior poles (Baldo and Mathias, 1992). As discussed by Mathias et al. (1997), this asymmetry is believed to be essential for the circulation of substances through the lens and therefore for lenticular homeostasis. In this study, we have demonstrated that FGF upregulates gap junctional communication between cultured chick lens cells and present evidence that FGF signaling may play an important role in establishing and maintaining the gradient of intercellular coupling in the lens in vivo. Although lacking the physiological context of the whole animal, the use of primary lens cell cultures avoids the pleiotropic and deleterious effects of manipulating FGF or gap junction signaling in the lens in vivo (Chow et al., 1995; Robinson et al., 1995a,b; White et al., 1998).

Model of regulation of gap junction–mediated intercellular communication in the lens by FGF

In a previous study, we reported that purified recombinant FGF (either FGF-2 or FGF-1 plus heparin) at ≥10 ng/ml stimulates the expression of fiber differentiation markers in primary cultures of E10 chick lens epithelial cells (Le and Musil, 2001). In the current investigation, we found that these same concentrations of FGF also increased gap junction–mediated intercellular dye transfer in the same system. Upregulation of intercellular coupling required sustained activation of ERK, was not accompanied by a detectable increase in either connexin synthesis or gap junction assembly, and was reversed upon removal of FGF. We also found that vitreous humor contains a factor with heparin-binding properties, ERK activation kinetics, and communication- and differentiation-promoting activity indistinguishable from purified recombinant FGF. Lastly, we have demonstrated that FGF-induced ERK activation in the intact lens is higher in the equatorial region than in polar and core fibers (Fig. 10). Taken together, these results support (although cannot

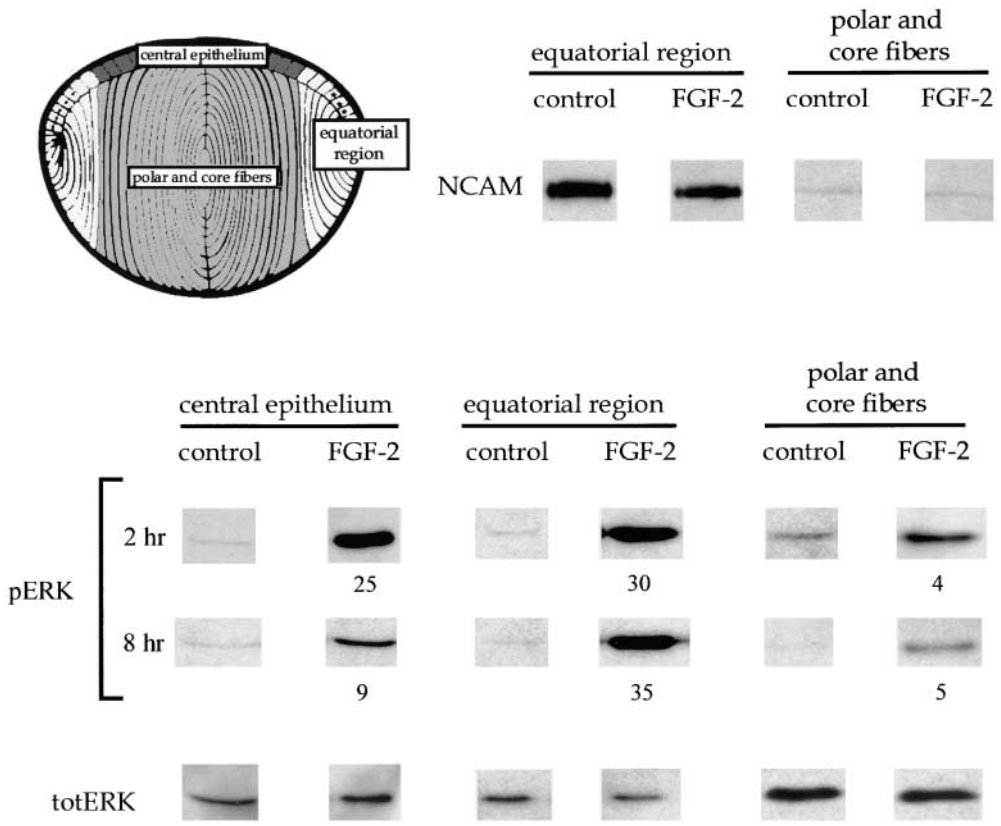


Figure 10. **FGF-induced activation of ERK is higher in the equatorial region than in polar and core fibers.** Intact lenses from embryonic day 13 (E13) chicks were incubated overnight in M199 medium and then treated for either 2 or 8 h at 37°C with no additions (control) or with 25 ng/ml FGF-2. The lenses were then snap-frozen in liquid nitrogen and dissected into three domains: the central epithelium, the equatorial region, and the polar and core fibers (see diagram; core fibers are defined as those in the lens inner cortex and nucleus). The samples were solubilized in SDS and whole cell lysates assessed for expression of NCAM and for activated ERK (pERK) as described in Materials and methods. The accuracy of the dissection was verified by the virtual absence in the polar and core fiber fraction of NCAM, which has previously been demonstrated to be expressed at high levels in equatorial region, but not polar or core, fiber cells in the embryonic chick lens (Watanabe et al., 1989). pERK immunoreactivity was quantitated by densitometry; the numbers in parentheses are the fold increase in pERK levels in FGF-treated lens fractions relative to pERK in the corresponding fraction from untreated control lenses (shown to the left of each FGF-treated blot). The pERK blots were subsequently stripped and reprobed for total (active + inactive) ERK protein (totERK). The experiment was repeated three times, with similar results. When expressed on a per lens basis, the polar/core fibers contained ~1/10 as much FGF-activated pERK as the equatorial region. Even if the polar fibers contained all of the FGF-induced ERK activity in the polar/core fraction, their response would still be much less than that of equatorial region cells.

prove) a model in which regional differences in FGF signaling through the ERK pathway lead to the observed asymmetry of gap junctional intercellular coupling in the lens (Fig. 11). We propose that the low level of FGF in the aqueous humor (Tripathi et al., 1992; Schulz et al., 1993) is inadequate to support either epithelial-to-fiber differentiation or enhanced intercellular coupling in the central epithelium. In contrast, cells in the equatorial region are in close physical proximity to the vitreous humor and respond to the high levels of FGF that diffuse out of the vitreous body by sustained ERK activation that upregulates gap junctional coupling. The posterior aspect of polar and core fiber cells also faces the vitreous body. Unlike equatorial region cells, these fibers do not, however, efficiently activate ERKs in response to FGF (Fig. 10). During the ongoing process of fiber formation, cells once in the equatorial region gradually become displaced towards the lens core as they are buried by newer generations of fiber cells. If exposure of cells to FGF in the equatorial region permanently changed their gap junctional phenotype, then intercellular coupling would be expected to remain high throughout the lifetime of the fiber regardless of its location in the lens. However, our results in cultured lens cells demonstrate that FGF-induced upregulation of gap junction function is reversed within 2 d of FGF withdrawal. We propose that the reduction in FGF signaling in polar fiber cells in vivo leads to a similar drop in intercellular coupling, thereby producing the observed equator-to-pole gradient of lenticular communication.

Several mechanisms could account for the insensitivity of polar/core fiber cells to extralenticular FGF. The first, and most straight-forward, would be a loss of expression of high-affinity FGF receptors in postequatorial fiber cells. This possibility is supported by in situ hybridization and immunolocalization studies in rodents, in which much lower levels of FGF receptor expression were detected in polar/core fibers than in more equatorially located cells (de Iongh et al., 1996, 1997). Alternatively or in addition, the diffusion of vitreous humor–derived FGF through the interfiber space to

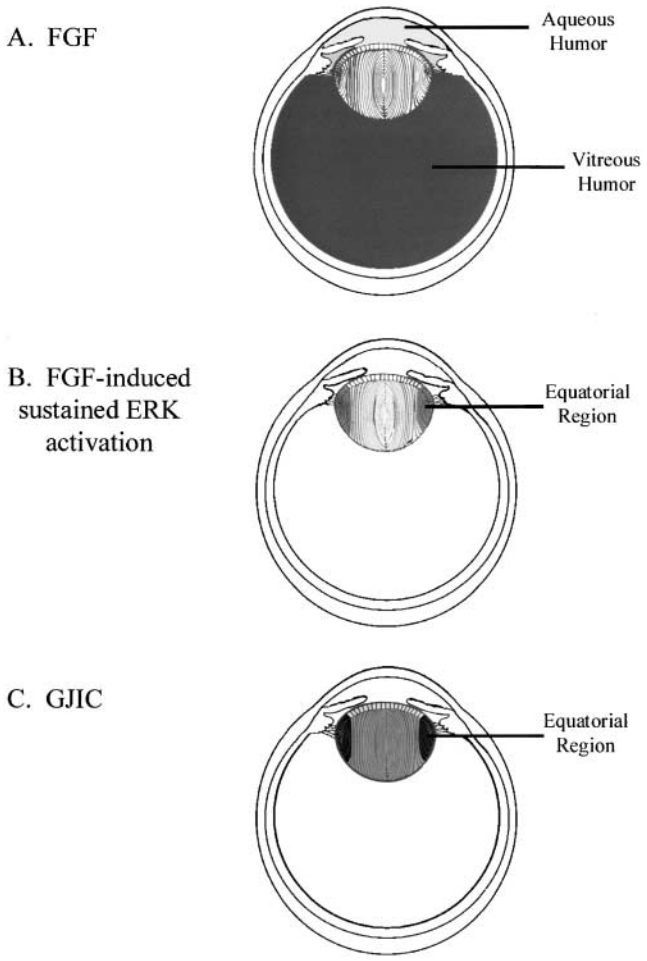


Figure 11. **Model of the role of FGF in establishing regional differences in gap junction–mediated intercellular communication in the lens.** (A) The concentration of FGF is known to be higher in the vitreous than in the aqueous humor. The level of FGF in the aqueous humor is too low to stimulate either gap junctional communication or fiber differentiation in the central epithelium. (B) Both the equatorial region and polar/core fiber cells have access to the high levels of FGF that diffuse out of the vitreous body, but only the former cell population can efficiently respond to this FGF by sustained activation of ERKs. (C) Sustained ERK activation leads to an increase in gap junctional intercellular communication (GJIC) in equatorial region cells. The decreased FGF signaling in polar and core fiber cells results in a reduction in intercellular coupling, thereby producing the observed equator-to-pole gradient of lenticular communication. See Discussion for details.

receptors on lateral cell surfaces may be more restricted at the poles than at the equator. It is also conceivable that polar fibers are deficient in a protein or activity required for transduction of the FGF signal to ERKs.

If the model presented in Fig. 11 is correct, then inhibition of FGF signaling in lens fibers would be predicted to reduce FGF-dependent junctional coupling in the equatorial region and consequently disrupt the lenticular non-vascular microcirculatory system. If so, then the cells at the center of the lens, which are the most dependent on this system, would be expected to be the most severely affected. Consistent with this possibility, expression of a dominant interfering mutant of FGF receptor-1 in equatorial, polar, and core fibers results in the preferential, postnatal death of the latter population without affecting the viability of the central epithelium (Chow et al., 1995). Because of the paucity of FGF receptors in core cells and their restricted access to extralenticular FGF, this effect is likely to be noncell-autonomous and may reflect a requirement for FGF signaling in equatorial region fibers.

Mechanism of FGF-induced upregulation of gap junctional communication

The macroscopic gap junctional conductance between two cells (g_j) is given by:

$$g_j = fn \times P_0 \times \gamma_j$$

in which n represents the total number gap junctional channels, f represents the fraction of those channels that are active at a given time, P_0 represents the single channel open probability, and γ_j is the single channel conductance. As summarized in the Introduction, the increase in g_j in the equatorial region of the lens is not associated with a proportional rise in the number of morphologically detectable gap junctional channels. Likewise, treatment of cultured lens cells with either purified recombinant FGF or FGF-containing vitreous humor enhanced intercellular dye transfer without increasing the expression or gap junction assembly of Cx43, Cx45.6, or Cx56. Upregulation of gap junction function at the lens equator in vivo as well as in FGF-treated lens cells in culture is therefore unlikely to be accomplished by an increase in the total number of gap junctional channels. An increase in any of the other parameters could, however, conceivably participate in raising g_j. A potentially very effective means of enhancing gap junctional coupling would be to increase the fraction of active channels (f). In several systems, junctional conductance between two cells has been reported to be disproportionately small relative to the number of gap junctional channels detected morphologically (Weidmann and Hodgkin, 1966). Gap junctions at club endings on the Mauthner (M−) cell in goldfish provide a particularly dramatic example in which the fraction of active gap junctional channels under resting conditions has been estimated to be ~1% (Lin and Faber, 1988). A more recent study in which the accumulation of GFP-tagged connexin constructs at cell–cell interfaces was correlated with g_j estimated that only between 2–20% of the channels formed were active in each of the three cell types examined (mouse neuroblastoma N2A cells, human cervical carcinoma HeLa cells, and RIN rat insulinoma cells; Bukauskas et al., 2000). If unstimulated lens cells also contain a substantial pool of functionally silent gap junctional channels, than increasing the fraction of active channels (f) would be an efficient means by which FGF could elevate junctional coupling without affecting total channel number. Electrophysiological evidence for such a mechanism has been obtained in some nonlenticular cell types (including HeLa cells) for several connexin species after treatment with effectors such as cAMP, serotonin, and PMA (Moore and Burt, 1995; van Rijen et al., 2000; van Veen et al., 2000).

If FGF influences channel gating instead of connexin expression, then why should its effect on intercellular dye

transfer take more than 12 h to manifest itself? The lengthy lag phase makes it unlikely that FGF upregulates gap junction–mediated intercellular communication by modulating the activity of preexisting cellular components. Instead, FGF may change the expression level of a potential regulator of gap junction function. Because the factors that govern f, P_0, and g_j under basal conditions are largely unknown, we can only speculate as to the identity of such a putative regulatory molecule. One possibility is that FGF relieves a tonic inhibition of gap junction channel activity in lens cells. A "ball and chain" mechanism has been described for certain connexins (including Cx43) in which a domain near the COOH terminus of the connexin molecule (the ball) binds to one or more regions at the opening of the channel (the ball receptor) to gate the pore shut. FGF might enhance the synthesis and/or gap junctional localization of a factor that binds to either the ball or its receptor to prevent them from interacting and closing the channel. It has been demonstrated that "ball" of one connexin molecule can interact with the ball receptor of another connexin species with which it has presumably formed a heteromeric connexon, leading to novel gating properties (Gu et al., 2000). Lens connexins are known to heteroligomerize into mixed connexons (Jiang and Goodenough, 1996), increasing the number of potential ball/receptor combinations that might render channels less active in the absence of FGF signaling. An alternate possibility arises from the finding that activators of protein kinase C increase the macroscopic junctional conductance of Cx45 channels without detectably changing Cx45 expression, subcellular localization, phosphorylation, or single channel conductance (van Veen et al., 2000). Although PKC itself is unlikely to play a comparable role in lens cells (Berthoud et al., 2000), it is conceivable that some other, as yet unidentified kinase or phosphatase behaves analogously in lens cells to increase f and/or P_0 and that FGF regulates its expression, function, and/or subcellular localization. Finally, it has recently been suggested that binding of the scaffolding protein ZO-1 to Cx43 and possibly to certain other connexin species may serve to bring signaling complexes to the vicinity of gap junctional channels (Giepmans and Moolenaar, 1998; Toyofuku et al., 1998). FGF might affect the synthesis or junctional targeting of such accessory proteins, which in turn could influence gap junction permeability.

Novel role of ERK in gap junction–mediated intercellular communication in the lens

The results depicted in Figs. 4 and 5 demonstrate that ERK activation is both necessary and sufficient to upregulate gap junction–mediated intercellular communication in cultured lens cells. This is in contrast to many other cell types, in which ERK activation has been reported to have no effect or to transiently (<1 h) (Hill et al., 1994; Hossain et al., 1998; Warn-Cramer et al., 1998) or more permanently (Zhou et al., 1999) reduce cell–cell coupling. In mammalian cells, such inhibition of communication has been causally linked to direct phosphorylation of Cx43 by ERK on Ser255, Ser279, and Ser282 within ERK consensus motifs. Mutation of these residues, while having no effect on basal gap junction formation and function, prevents cell uncoupling in response to the ERK activator EGF (Warn-Cramer et al., 1998). DCDML lens cultures express high levels of Cx43 which accumulates at cell–cell interfaces as typical gap junctional plaques. Why, then, does activation of ERKs by FGF, insulin, or IGF-1 fail to transiently reduce intercellular dye coupling in these cells? We consider three possibilities. First, chick Cx43 may not be a substrate for ERK. Given that the amino acid sequence of chick Cx43 is very similar (92% identical) to that of rodent Cx43 and contains all three of the aforementioned ERK consensus sequences (Musil et al., 1990a), this appears unlikely. Second, Cx43 in chick lens cells may be phosphorylated by ERK, but be functionally unaffected by this modification due to the absence of some additional component required to transduce connexin phosphorylation into channel closure. This scenario would be inconsistent with recent evidence that ERK phosphorylation induces an intramolecular conformational change within the Cx43 molecule that directly gates the channel shut (Homma et al., 1998; Kim et al., 1999; Zhou et al., 1999; see, however, Hossain et al. [1999] for an opposing view). Lastly, ERK activation may inhibit Cx43 function in DCDML cultures as has been reported in other cell types, but the effect may be masked by enhanced transfer through Cx45.6 and/or Cx56 channels. These possibilities will be experimentally addressed in future studies.

In addition to inducing sustained ERK activation and thereby promoting gap junction function, FGF also stimulates epithelial-to-fiber differentiation. Two findings rule out the possibility that enhanced intercellular coupling in FGF-treated DCDML cultures is a passive downstream consequence of increased differentiation. First, insulin and IGF-1 are as potent as FGF in stimulating fiber differentiation (Le and Musil, 2001), yet have no effect on gap junction function. Second, a detectable increase in the expression of fiber markers in response to FGF requires >3 d, whereas junctional coupling is maximally stimulated within 24 h of FGF addition. Positive regulation of gap junction–mediated intercellular communication is therefore a novel, differentiation-independent function of sustained activation of the MAP kinase cascade.

Materials and methods

Materials

Recombinant human FGF-1 and bovine FGF-2, gifts from Dr. Felix Eckenstein (Oregon Health Sciences University, Portland, OR), were prepared and assessed for biological activity as described previously (Le and Musil, 2001). Control experiments demonstrated that the recombinant FGF-2 behaved indistinguishably from FGF-2 prepared from bovine pituitary glands (F5392; Sigma-Aldrich) in terms of its effects on chick lens cell proliferation, differentiation, and gap junction–mediated intercellular dye transfer (data not shown). Bovine pancreas insulin and low molecular weight heparin (sodium salt, from porcine intestinal mucosa) were purchased from Sigma-Aldrich, and fetal calf serum was obtained from Hyclone. R^3IGF-1, an analogue of human insulin-like growth factor 1 (IGF-1) with reduced affinity for IGF binding proteins, was from GroPep. The nonhydrolyzable cAMP analogue 8-(4-chlorphenylthio) (8-CPT)-cAMP, forskolin, and RO-20-1724 were purchased from Calbiochem. 18β-glycyrrhetinic acid (18β-GA) and Lucifer yellow were purchased from Sigma, and rhodamine-dextran (M_r = 10,000 Da) and biocytin were obtained from Molecular Probes. UO126, the specific MEK 1/2 inhibitor, was provided by Dr. James Trzaskos (DuPont Pharmaceuticals, Wilmington, DE). The anti-p44/42 MAP kinase polyclonal rabbit antibody (recognizes both activated and inactive forms of ERK) and the antiphospho-p44/42 MAP kinase E10 monoclonal

mouse antibody (specific for activated ERK) were purchased from New England Biolabs, Inc. Cx43 was detected with an affinity-purified rabbit antibody (AP7298) (Musil et al., 1990b), and polyclonal rabbit antisera directed against Cx45.6 or Cx56 were provided by Drs. Jean Jiang (University of Texas Health Science Center, San Antonio, TX) and Daniel Goodenough (Harvard Medical School, Cambridge, MA) (Jiang et al., 1995). Neural cell adhesion molecule (NCAM) was detected with monoclonal antibody Mab 5E (Watanabe et al., 1989).

Cell culture

Dissociated cell-derived monolayer (DCDML) cultures were prepared from E10 chick lenses and plated at 0.9×10^5 cells/well onto laminin-coated 96-well tissue culture plates as described previously in Le and Musil (1998). Cells were cultured for up to 7 d in M199 medium plus BOTS (2.5 mg/ml bovine serum albumin, 25 μg/ml ovotransferrin, 30 nM selenium), penicillin G, and streptomycin (M199/BOTS), with or without additives at 37°C in a 5% CO_2 incubator. Cells were fed every 2 d with fresh medium. For FGF reversal experiments (see Fig. 2 C), cells cultured for 48 h with FGF-2 were washed three times with 10 μg/ml heparin and then three times with M199 medium at room temperature to remove extracellular FGF. The cells were then cultured in fresh M199/BOTS medium with or without FGF for up to two additional days. Similar results were obtained if heparin was omitted from the washing steps.

Plasmids and transient transfection of lens cells

1 d after plating, DCDML cultures were transfected in M199 medium without BOTS or antibiotics using Lipofectamine PLUS (GIBCO BRL) as specified by the manufacturer. Plasmids encoding wild-type MEK1, a constitutively active mutant form of MEK1 (S217E/S221E; Cowley et al., 1994; Yao et al., 1995), or the lacZ gene product β-galactosidase (pCH110; Amersham Pharmacia Biotech) were used at a concentration of 0.1 μg DNA per well of a 96-well tissue culture plate. After incubation with DNA for 3 h, the transfection medium was supplemented with BOTS, penicillin G, and streptomycin and the cells cultured for an additional 48 h before analysis.

Scrape-loading/dye transfer assay for gap junctional intercellular communication

DCDML cultures grown on laminin-coated coverslips were assessed for gap junction–mediated intercellular coupling as described previously (Le and Musil, 1998). In brief, the culture medium from a confluent monolayer of lens cells was removed and saved. The cells were rinsed three times with Hank's balanced salt solution containing 1% bovine serum albumin (HBC), after which a 27-gauge needle was used to create two longitudinal scratches through the cell monolayer in the presence of a solution of Dulbecco's phosphate buffered saline containing 0.75% rhodamine-dextran and either 1% Lucifer yellow or 1% biocytin. After exactly 1 min, the culture was quickly rinsed three times with HBC and then incubated for an additional 8 min (Lucifer yellow) or 2 min (biocytin) in the saved culture medium to allow the loaded dye to transfer to adjoining cells. The culture was then rinsed three times with phosphate-buffered saline and fixed. Rhodamine-dextran, Lucifer yellow, and (after reaction with avidin-FITC) biocytin were visualized by fluorescence microscopy (Leitz DMR). Unless otherwise indicated, cells were assayed on day 3 after plating.

Scrape-loading/dye transfer assays were quantitated using the averaged fluorescence plot profile method essentially as described by Giaume et al. (1991) and Opsahl and Rivedal (2000). First, a Leica DM LD photomicrography system and Scion Image 1.60 software were used to generate digital images of the scrape-loaded and fixed monolayers (exposure time 2 s; gain = 0). Next, the images were imported into the graphics program Adobe Photoshop® and converted to greyscale. The fluorescence intensity of the cell-free area in the middle of the scrape was defined as background, and the data from cells labeled with rhodamine-dextran replaced with this background value. This procedure eliminated the signal from cells that had been permeabilized during the scrape-load process and insured that the remaining image contained only cells that had received Lucifer yellow (or biocytin) via gap junction–mediated intercellular coupling. Background for Lucifer yellow-loaded samples was ~50 pixels; for biocytin (assessed after avidin-FITC staining), 0 pixels. Finally, the images were analyzed using the public domain program NIH Image 1.62 (http://rsb.info.nih.gov/nih-image) and Microsoft Excel as follows. The images were scanned along lines perpendicular to the scrape, taking care to avoid regions where the cell monolayer was incomplete or contained enlarged cells undergoing epithelial-to-fiber differentiation. At least three lines were scanned per image. The data obtained from each image were averaged and the mean fluorescence intensity of each pixel (ordinate) plotted as a function of its distance (in μm) (abscissa) to generate an averaged fluorescence plot profile. The proximal edge of intercellular dye transfer was defined as the point nearest to the scrape edge with a mean pixel value >10 U above background, and the distal edge of transfer as the point at which the mean pixel value returned to this level. Because none of the compounds tested significantly altered the size of epithelial cells within the monolayer (data not shown), the distance of intercellular dye transfer was directly proportional to the number of coupled cells. Similar results were obtained if dye transfer was quantitated by measuring the size of the total fluorescent surface area as described by Giaume et al. (1991) using the area measurement function of NIH Image 1.62 (data not shown). The scrape-loading dye transfer assay has the advantage over microinjection techniques of allowing simultaneous monitoring of dye coupling in a large population of cells; its utility in the assessment of gap junction–mediated intercellular communication has been well documented (el-Fouly et al., 1987; Venance et al., 1995). Opsahl and Rivedal (2000) have reported that scrape-loading of Lucifer yellow followed by image analysis (by either the averaged fluorescence plot profile or the total fluorescent surface area method) leads to the same conclusions as dye microinjection, with less data variation.

Paracellular permeability assays

1.8×10^5 embryonic chick lens cells were plated in 100 μl M199/BOTS per laminin-coated 12 mm-diameter Transwell filter (polycarbonate, pore size = 0.4 mm; Corning Costar Corp.) inserted into a 22 mm-diameter tissue culture plate well containing 600 μl of M199/BOTS. Cells were cultured at 37°C in 5% CO_2 until confluent. To assess paracellular permeability to FITC-dextran, 1 mg/ml FITC-dextran (average molecular mass 38,900; Sigma-Aldrich) was added to the upper chamber of the Transwell unit. At 2 h, a 0.1-ml aliquot was collected from the lower chamber and assayed for FITC-dextran content by fluorimetry (Fluostar Galaxy; BMG Lab Technologies) as described by Martin-Padura et al. (1998). Transepithelial electrical resistance was assessed using the Endohm-12 electrode chamber and the EVOM epithelial voltohmmeter (World Precision Instruments, Inc.).

Immunoblot analysis of DCDML cultures

For detection of activated ERK, DCDML cultures were solubilized in SDS-PAGE sample buffer (7.1% glycerol, 1.92 mM Tris, pH 6.8, 2.5% SDS, 2% 2-mercaptoethanol), boiled for 3 min, and the entire cell lysate from each well of a 96-well culture plate analyzed per lane of a 10% SDS-polyacrylamide gel. Electrophoresed proteins were transferred to PVDF membranes (Immobilon), and the blots probed with the antiphospho-p44/42 MAP kinase E10 monoclonal mouse antibody followed by alkaline phosphatase–conjugated goat anti–mouse IgG as described by Le and Musil (2001). For detection of total ERK, the blots were stripped by incubation in 2% SDS, 100 mM β-mercaptoethanol, 62.5 mM Tris-HCl, pH 6.8, for 30 min at 70°C and then reprobed with an anti-p44/42 MAP kinase polyclonal antibody (recognizing both activated and inactive forms of ERK) followed by alkaline phosphatase–conjugated goat anti–rabbit IgG (Promega). Immunoreactive protein bands were detected using the chemiluminescent CSPD substrate and Kodak XAR-5 film as described by the manufacturer (Tropix, Inc.) and quantitated by densitometry using IPLab Gel software.

For immunoblot analysis of Cx43, Cx45.6, and Cx56, 3-d-old DCDML cultures were solubilized in lysis buffer (1 mM Tris base, 1 mM EGTA, 1 mM EDTA, 0.6% SDS, 10 mM iodoacetamide, 2 mM PMSF, pH 8.0) as described previously (Le and Musil, 1998) and boiled for 3 min. One microgram of total cell lysate protein per sample was resolved on 10% SDS-polyacrylamide gels and transferred to PVDF membranes. The blots were blocked for 1 h at room temperature with 5% nonfat dry milk/0.2% Tween-20/1% normal goat serum/phosphate-buffered saline (BLOTTO/NGS), after which they were incubated overnight at 4°C with the appropriate primary antibody diluted in BLOTTO/NGS. Blots were subjected to three rinses (10 min each), incubated for 2 h at room temperature with alkaline phosphatase–conjugated goat anti–rabbit IgG diluted in BLOTTO/NGS, and then rinsed three more times. Protein bands were detected by reaction with 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich) and nitro blue tetrazolium (Sigma-Aldrich).

Microdissection and analysis of embryonic chick lenses

Lenses were excised from E13 chick embryos and incubated in unsupplemented M199 medium for 16 h at 37°C in a 5% CO_2 environment to reduce the level of endogenous ERK activation. The lenses were then incubated for an additional 2 or 8 h in either the absence or presence of FGF-2. Thereafter, the lenses were carefully rolled on a piece of Kimwipe to remove nonlenticular tissue and plunged into liquid nitrogen. Control experiments demonstrated that the freezing step did not affect the level of ERK activation. While still frozen, the lenses were manually dissected into three

domains (central epithelium, equatorial region, and polar and core fibers). Immediately after dissection, the fractions were boiled for 3 min in lysis buffer (see above). 10 μg of protein from the equatorial region and from the polar and core fibers (determined by the Folin phenol assay; Peterson, 1983) were analyzed per lane of a 10% SDS-polyacrylamide gel. Central epithelium fractions (which contain much less protein than the other two fractions) were concentrated by the methanol precipitation method (Wessel and Flugge, 1984), resuspended in 2× sample buffer (see above), and the protein originating from an entire lens analyzed per gel lane. After electrophoresis, the proteins were transferred to PVDF membranes and the blots probed with mouse monoclonal antibodies directed against either phospho-p44/42 MAP kinase or NCAM followed by alkaline phosphatase–conjugated goat anti–mouse IgG as described above. Total ERK was detected after stripping anti-phosphoERK blots as described above.

Indirect immunofluorescence

DCDML cells cultured for 3 d in either the absence or presence of FGF were fixed and stained for Cx43, Cx45.6, or Cx56 as described previously in Le and Musil (1998).

Preparation of Vitreous Humor and Vitreous Body Conditioned Medium

Vitreous bodies were dissected from the eyes of either E10 chicks or adult mice. As described in Le and Musil (2001), vitreous humor was prepared by subjecting vitreous bodies to centrifugation for 10 min at 4°C at 12,000 *g* to remove cells and fibrous elements. For vitreous body conditioned medium, intact vitreous bodies were transferred to the upper chamber of Transwell filter unit containing M199 medium in the upper and lower compartments. After an overnight incubation at 37°C in a 5% CO_2 incubator, the lower compartment medium was collected.

Fractionation of Vitreous Humor and FGF on Heparin Beads

1 ml of either vitreous humor diluted with 2.3 vol of M199 (30% VH/M199) or M199 containing 50 ng/ml of FGF-2 was mixed end-over-end with 0.1 ml of heparin-conjugated Affigel beads (Bio-Rad Laboratories) in the presence of either 0.1 M NaCl or 0.6 M NaCl for 2 h at 4°C. The beads were pelleted by centrifugation, the unbound supernatant collected, and the beads resuspended in 1 ml M199 medium supplemented with 2.5 M NaCl for 1 min at room temperature to elute FGF-like activity. The unbound and eluate fractions were subjected to repeated rounds of concentration by ultrafiltration (Centricon YM-3 filters; 3,000 Da mol wt cut-off) and dilution with M199 medium to lower the salt concentration to 0.15 M NaCl and return the fractions to their original (1 ml) volume.

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