REGULATION OF GAP JUNCTIONAL INTERCELLULAR COMMUNICATION IN THE VERTEBRATE LENS

by

Anh-Chi N. Le

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CERTIFICATE OF APPROVAL

This is to certify that the Ph.D. thesis of

Anh-Chi N. Le

has been approved

[redacted]

Professor in Charge of Thesis - Dr. Linda Musil

[redacted]

Chairperson - Dr. Rae Nishi

[redacted]

Member - Dr. Gail Clinton

[redacted]

Member - Dr. Jan Christian

Dean of Graduate Studies - Dr. Rich Mauer

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List of Abbreviations

18β-GA (or βGA)	18β-glycyrrhetinic acid
BMP-4	bone morphogenetic protein-4
Cx	connexin
DAG	diacylglycerol
DCDML	dissociated cell-derived monolayers
EGF	epidermal growth factor
ERK	extracellular-regulated kinase
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FRS2	FGF receptor substrate 2
GJIC	gap junctional intercellular communication
Grb2	growth factor receptor-bound protein 2
HGF/SF	hepatocyte growth factor/scatter factor
HSPG	heparin sulfate proteoglycan
IGF-1	insulin-like growth factor-1
IP_3	inositol-1,4,5-triphosphate
JNK	c-Jun-N-terminal kinase
MAPK	mitogen-activated protein kinase
MAPKK	MAP kinase kinase
MAPKKK	MAP kinase kinase kinase
MBP	myelin basic protein

MKP-1	map kinase phosphatase-1
MKP-2	map kinase phosphatase-2
NCAM	neural cell adhesion molecule
NGF	nerve growth factor
PDGF	platelet-derived groth factor
PIP_2	phosphatidylinositol-4,5-bisphosphate
PKA	protein kinase A
PKC	protein kinase C
PLCγ	phospholipace Cγ
PP2A	protein phosphatase type 2A
PTB	phosphotyrosine binding domain
RPTK	receptor protein tyrosine kinase
p38/RK	reactivating kinase
SAPK	stress-activated protein kinase
SH2	Src-homology 2
Shc	SH2-containing protein
Sos	son of sevenless
TGFα	transforming growth factor α
TGFβ	transforming growth factor β
VBCM	vitreous body-conditioned medium
VH	vitreous humor

I. Abstract

The ocular lens consists of two cell types: epithelial cells at the anterior surface and the highly elongated and crystallin-rich core of fiber cells which differentiate from the epithelial cells at the lens eqator. The cells of the vertebrate lens are joined into a functional synctium by gap junctions, clusters of intercellualr channels that mediate the direct transfer of low-molecular weight substances between the cytosols of adjoining cells.

The highest levels of gap junctional intercellular communication (GJIC) in the lens are at the equator, where secondary fiber differentiation is initiated. In many organs, gap junctions play an important role in coordinating growth and/or differentiation signals. The possibility that gap junctions are involved in epithelial-to-fiber differentiation was addressed in experiments presented in Chapter One. The experimental system used throughout this thesis was primary cultures of embryonic chick lens epithelial cells because they most closely recapitulate *in vivo* lens differentiation and, unlike cultured rodent lens cells, also develop extensive intercellular junctions and intercellular communication between differentiated cells. It was found that reducing GJIC in these cultures with a known gap junction blocker (18β-glycyrrhetinic acid) had no effect on fiber differentiation. Thus, the high level of GJIC characteristic of the lens equator *in vivo* is not required for secondary fiber formation as assayed in culture.

A related issue is the nature of the growth factor that induces epithelial-to-fiber differentiation in the chick lens. In mammals, fibroblast growth factors (FGFs) are thought to be involved in this process. In contrast, chick lens cells have been reported to be unresponsive to FGF and an insulin/IGF-type factor was proposed to be the physiological effector of differentiation. The data presented in Chapter Two reinvestigate this paradox and demonstrate that FGFs *are* in fact potent determinants of chick lens cell fate when used for periods (>5h) in excess of those used in previous investigations. These

studies also addressed the role of ERK MAP kinases in cultured chick lens epithelial cells. Both FGF and insulin/IGF-type factors were shown to induce cell proliferation in an ERK-independent manner whereas the differentiation-promoting activities of the two factors differed in their requirement for ERK activity.

The asymmetric distribution of GJIC in the lens, with the highest level of GJIC in the equator, is believed to be important in setting up a non-vascular microcirculating system that brings nutrients into the lens and flushes out waste products. Despite its importance for lens function, it it unknown *how* the gradient of GJIC in the lens is established. One possibility was that a factor, or factors, that upregulate lens differentiation in the bow region may also play a role in upregulating GJIC in this same region. Based on the findings presented in Chapter Two, FGF, insulin, and IGF-1 were tested for their effect on GJIC in primary chick lens cultures. The studies reported in Chapter Three demonstrated that FGF, but not insulin or IGF-1, increased GJIC and that this increase required sustained ERK activation. Moreover, sustained ERK activation was shown to be sufficient to increase GJIC. Vitreous humor was identified as an *in vivo* source of an FGF-like intercellular-communication-promoting activity. These results, along with the demonstration that FGF-induced ERK activation in the intact lens is highest in the equatorial region, led us to propose the first model of how the asymmetry in GJIC required for lens clarity is established. This is the first investigation, to our knowledge, of the regulation of gap junctions by growth factors in the lens and the downstream signalling events initiated by these factors.

II. Introduction

A. Structure and Function of the Ocular Lens

The ocular lens is located posterior to the iris and is held in place by its suspensory ligaments (Figure 1). The primary task of the lens is to allow precise focusing of light on the retina from a nearby source, a process called accommodation (Figure 2) (Marieb and Mallatt, 1992). Accommodation requires that the lens be transparent, a property that is dependent on several physical features of the lens. First, there are no blood vessels within the lens. Second, the cells in the center of the lens have no intracellular organelles that could scatter light. Third, the intercellular spaces are smaller than the wavelength of light. Disruption of any of the physical properties described here leads to cataractogenesis, a major health problem in humans, in which the lens becomes clouded and is no longer able to focus light on the retina. This may occur for a number of reasons ranging from hereditary abnormalities to excessive exposure to sunlight (Horwitz and Jaffe, 1992).

The lens consists of only two types of cells - epithelial cells at the anterior surface of the organ and the highly elongated fiber cells that differentiate from the epithelial cells (Figure 3). The lens epithelial cells are polygonal cuboidal cells that contain all the usual intracellular organelles, including mitochondria that are responsible for oxidative phosphorylation (Trukel, 1962). Transport of ions within the lens requires Na^+-K^+-ATPase pumps which are concentrated in the epithelial layer (Kinsey and Reddy, 1965). As a consequence of lens development (see Section 2), the polarity of the lens epithelial cells is reversed such that the apical surface faces the inside of the organ and the basal domain faces the outside environment. This orientation allows the lens epithelial cells to secrete the basal laminal capsule that envelops the organ. The lens capsule is composed primarily of collagen type IV, laminin, entactin, heparan sulfate proteoglycan, and fibronectin (Cammarata *et al.*, 1986; Parmigiani and McAvoy, 1984).

The bulk of the lens is made up of the fiber cells. They lack most cellular

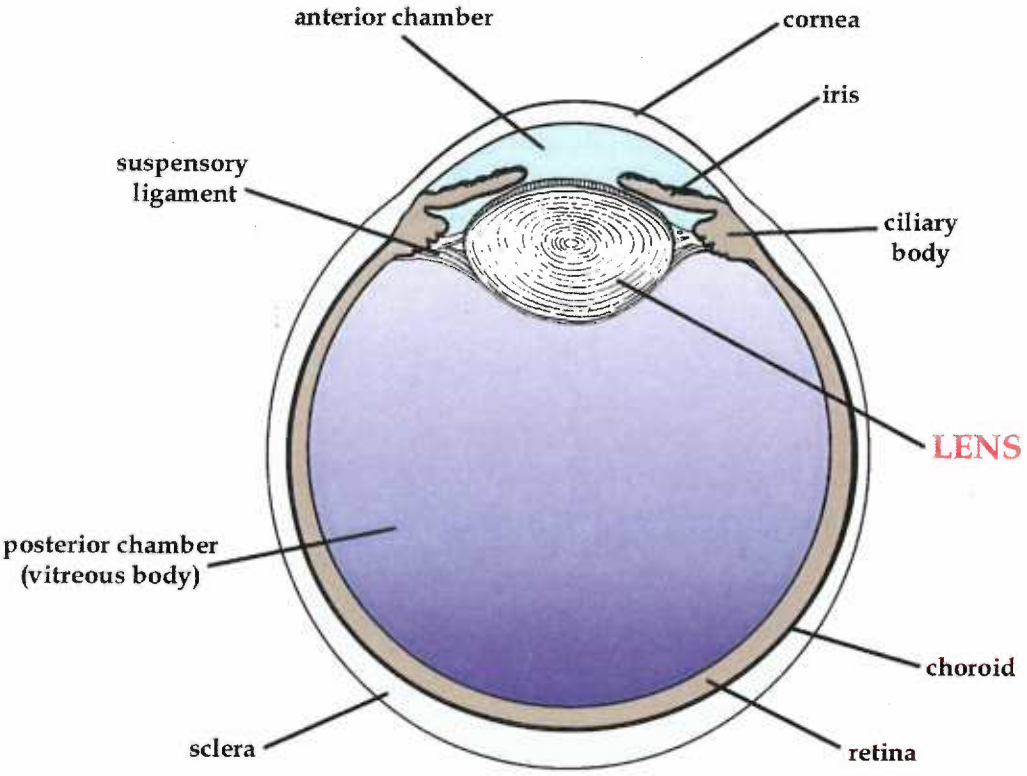

Figure 1. *Location of the lens in the vertebrate eye.* The lens is encased in a thin elastic capsule and is held in place posterior to the iris by its suspensory ligaments.

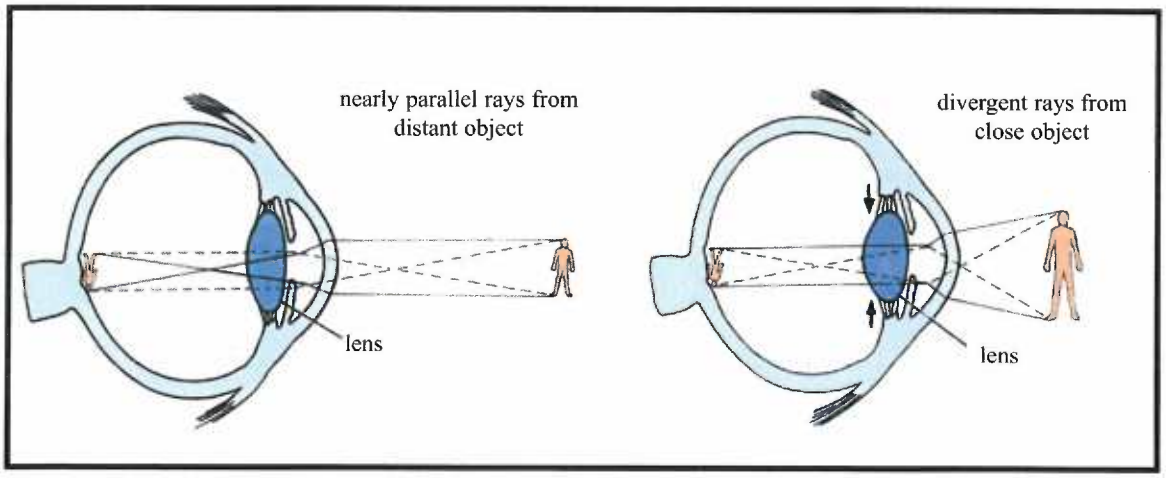

Figure 2. *Lens accomodation.* (A) At rest, the eye is set for distance vision and the parallel light rays from a distant object are focused directly on the central fovea of the retina. (B) To focus on a nearby object, the ciliary muscle contracts, pulling the ciliary body and choroid forward, and rounding the lens which bends the light rays toward the central fovea. This increase in lens curvature is called accomodation.

organelles including endoplasmic reticulum, mitochondria, and nuclei because such structures would scatter light. The two major components of the cytosol of the differentiated lens fiber cells are crystallins and cytoskeletal elements. The crystallins are a somewhat heterogeneous population of low molecular weight, water-soluble proteins that make up more than 90% of the total mass of the lens fibers and that are responsible for producing the high index of refraction necessary for proper lens function. Crystallins are present in extraordinarily high concentration (reaching values greater than 400 mg/ml) in fiber cells and must remain in solution for lens transparency (Horwitz and Jaffe, 1992). The cytoskeletal system of the lens contains actin, myosin, vimentin, α-actinin and microtubules, which are present in both epithelial and fiber cells (Benedetti *et al.*, 1981; Ramaekers and Bloemendal, 1981), as well as cytoskeletal proteins specific to fiber cells such as CP115 (filensin) and CP49 (phakinin) (FitzGerald and Gottlieb, 1989; Hess *et al.*, 1993). Scanning electron microscopy reveals the hexagonal shape of the fiber cells and the presence of "ball and socket" specializations on the plasma membranes (Dickson and Crock, 1972). By interlocking fibers with each other, these structures prevent sliding of fibers and allow the intercellular junctions that join fiber cells to remain intact during the lens shape changes associated with visual accommodation .

The lens is surrounded by two ocular fluids that are thought to be important sources of substances needed for lens development and function (Figure 1). The aqueous humor in the anterior chamber is a clear fluid similar to blood plasma. This fluid forms as a filtrate of the blood from capillaries in the ciliary processes and provides nutrients and oxygen to the lens epithelium (Marieb and Mallatt, 1992). The posterior chamber is filled with vitreous humor, a jelly-like substance containing fine fibrils of collagen that is over 98% water. The vitreous humor supports the posterior surface of the lens and contains growth factors that are thought to be important in lens differentiation (Beebe *et al.*, 1980; Schulz *et al.*, 1993).

B. Development of the Lens

The lens develops as an invagination of surface ectodermal cells overlying the optic vesicle that differentiate into lens cells as the optic vesicle folds in on itself. The surface epithelium then thickens to form the *lens placode*, which invaginates to form the *lens pit*, which subsequently closes to form the *lens vesicle*. Immediately after the formation of the lens vesicle, cells from the posterior wall of the lens vesicle begin to elongate and eventually fill the cavity of the vesicle. These terminally differentiated, crystallin-rich cells are called the *primary fiber cells* and will become the embryonic nucleus. The cells that were not induced to form the primary lens fibers remain a cuboidal monolayer of lens epithelial cells, which (unless injured) will never divide except in a narrowly defined area lying anterior to the lens equator. After primary fiber cell formation is complete, *secondary fiber cells* are formed by differentiation of epithelial cells at the equator of the lens. Proliferation and differentiation occur throughout life, causing layer upon layer of new lens fibers to form on top of each other. As the fibers internalize, the anterior shift of cell nuclei produces a pattern known as the lens *bow*. The development of the lens therefore results in four distinct regions: a nonproliferative central epithelium, a proliferative zone of epithelial cells immediately anterior to the lens equator, an equatorial zone of cellular elongation and differentiation, and a posterior and central zone (nucleus) of crystallin-containing fiber cells (Figure 3) (Kuszak, 1990).

C. Role of growth factors in lens development

In mammals, the major family of growth factors thought to be involved in epithelial-to-fiber differentiation are the fibroblast growth factors (FGF) (McAvoy *et al.*, 1991). The FGF family has about twenty members that share 30-70% identity in their primary sequences. A feature common to all FGFs is their high affinity for the glycosaminoglycan heparin. FGF-1, FGF-2, and several other family members (FGF-9,-11,-12,-13,-14, and-16) lack signal peptides, and their mechanism of release into the

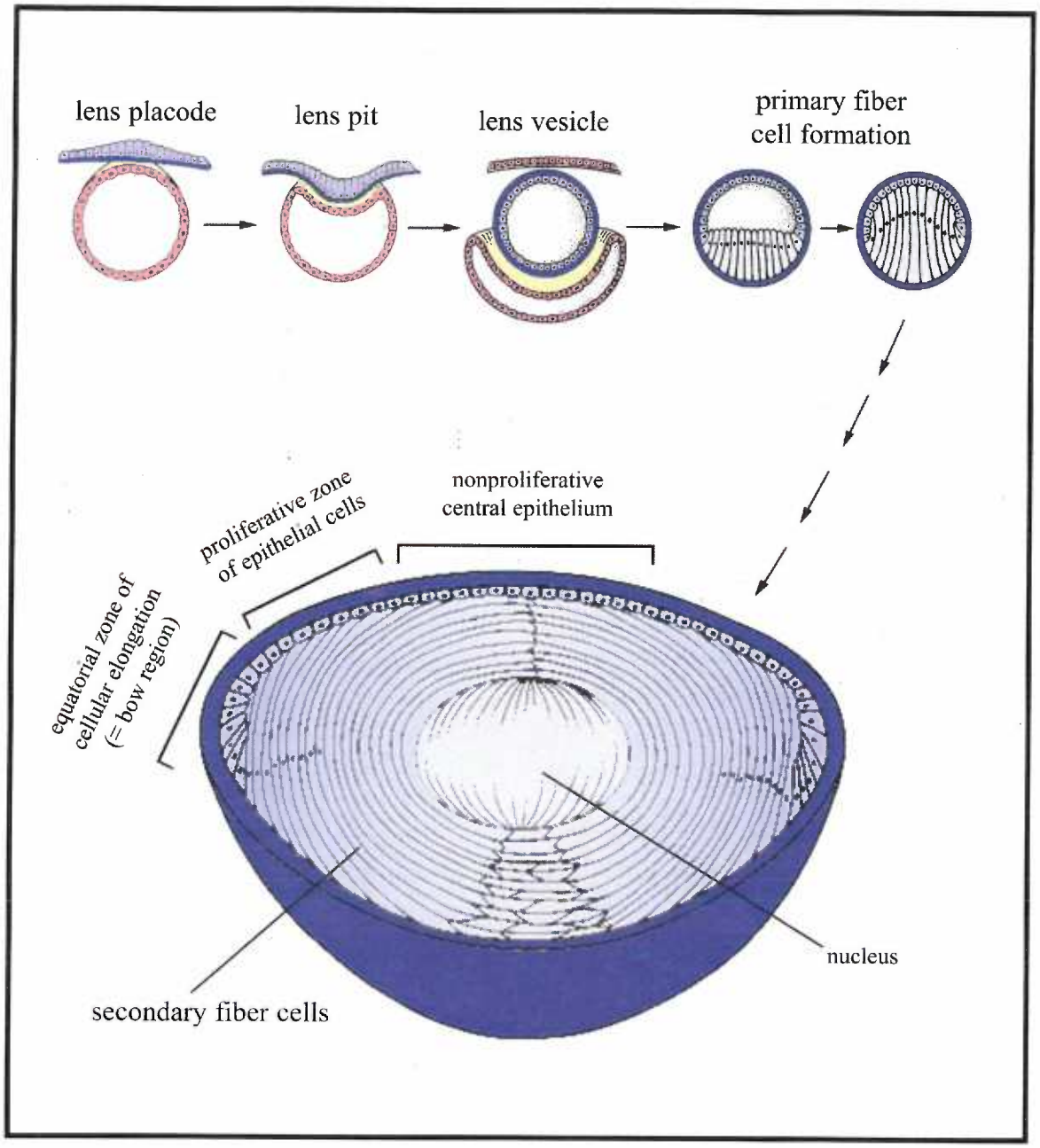

Figure 3. *Lens development.* Development of the lens begins with a thickening of the surface ectoderm overlying the optic vesicle, the *lens placode*, which invaginates to form the *lens pit*, and subsequently pinches off to form the *lens vesicle*. *Primary fiber cells* elongate and fill in the cavity of the vesicle and become the embryonic *nucleus*. All subsequent growth of the lens is due to differentiation of equatorial region epithelial cells into *secondary fiber cells.*

extracellular environment is still not understood. FGF-1 and FGF-2 are, however, abundant in the extracellular matrix and basement membranes of a variety of embryonic and adult tissues (reviewed in Galzie *et al.*, 1997 and Basilico, 1992). Members of the FGF family have been implicated in a number of important biological processes including: tail and inner ear development (FGF-3) (Mansour *et al.*, 1993), postimplantation development (FGF-4) (Feldman *et al.*, 1995), regulation of hair growth (FGF-5) (Hebert *et al.*, 1994), and axial organization and mesodermal patterning (FGFR-1) (Deng *et al.*, 1994; Yamaguchi *et al.*, 1994).

Much of the evidence in support of FGF as a mammalian lens differentiation factor comes from studies initiated about a decade ago by McAvoy and colleagues. These investigators developed a rat lens epithelial explant system in which the intact central epithelial monolayer is cultured capsule-side down after manual removal of the fiber cell mass. They found that increasing concentrations of FGF sequentially induce cellular proliferation, migration, and fiber cell differentiation in these explants at half-maximal doses of 0.15, 3, and 40 ng/ml, respectively (McAvoy and Chamberlain, 1989). These results led the authors to develop the *FGF gradient hypothesis* which proposes that lens development is determined by an anteroposterior gradient of FGF stimulation (McAvoy and Chamberlain, 1989; McAvoy *et al.*, 1991). As described separatedly below, this hypothesis is supported by three lines of evidence: 1) the distribution of FGF in ocular fluids, 2) the expression of FGF receptors in the rodent lens, and 3) the functional consequences of perturbation of FGFs and their receptors in transgenic and knockout mouse model systems (reviewed in McAvoy *et al.*, 1999).

1) Distribution of FGF in ocular fluids. The aqueous humor surrounds the anterior region of the lens whereas the cells in the equatorial and posterior regions are bathed by the vitreous humor (Figure 1). The concentration of FGF in mammalian vitreous humor is considerably higher than in the aqueous humor and is sufficient to induce the differentiation of rat lens central epithelial explants as assessed by

accumulation of the fiber cell marker β-crystallin, cellular elongation, loss of organelles, formation of plasma membrane interdigitations, and denucleation (Lovicu *et al.*, 1995; Schulz *et al.*, 1993). Most importantly, the differentiation-promoting activity of the vitreous is inhibited by antibodies against FGF-1 and FGF-2 (Schulz *et al.*, 1993).

2) Expression of FGF receptors in the rodent lens. The biological effects of the FGF family are normally mediated by a family of highly homologous receptors consisting of four distinct genes and their numerous splice variants (reviewed in Galzie *et al.*, 1997). Three FGF receptors are expressed in lens cells: FGFR-1, FGFR-2 (splice forms IIIb and IIIc), and FGFR-3 (de Iongh *et al.*, 1997; de Iongh *et al.*, 1996; Orr-Urtreger *et al.*, 1993; Peters *et al.*, 1993). In general, FGF receptors in the lens exhibit an anteroposterior pattern of expression such that the highest expression of FGF receptors is in the differentiating cells at the lens equator, with a dramatic dropoff in receptor expression in older, more differentiated fiber cells. The one exception to this is FGFR2(IIIc) which exhibits strongest expression in the epithelial cells but is subsequently downregulated in maturing fibers (de Iongh *et al.*, 1997).

3) Functional consequences of perturbation of FGFs and their receptors in transgenic and knockout mouse model systems. Overbeek and his colleagues have generated a number of transgenic mice strains in which the αA-crystallin promoter was used to drive high level expression of various FGFs in transitional zone epithelial cells and mature fibers in embryonic and adult lenses (McAvoy, 1978). They found that several members of the FGF family (FGF-1, FGF-3, FGF-4, FGF-5, FGF-7, FGF-8, and FGF-9) could induce premature differentiation of the lens epithelium (Lovicu and Overbeek, 1998; Robinson *et al.*, 1998; Robinson *et al.*, 1995b). The αA-crystallin promoter was also used to generate transgenic mice expressing a truncated FGF receptor that heterodimerizes with endogenous FGF receptors and inhibits their function. (Chow *et al.*, 1995; Robinson *et al.*, 1995a; Stolen and Griep, 2000). Analysis of the transgenic lenses revealed a disruption of fiber cell differentiation. Although both the FGF overexpression

and FGF receptor dominant negative studies support a role for FGF in lens differentiation, this concept is complicated by the phenotype of knockout mice. Mice in which the genes encoding FGF-1, FGF-2, FGF-3, FGF-5, FGF-7, or FGFR-3 were inactivated have no obvious ocular defects (Colvin *et al.*, 1996; Dono *et al.*, 1998; Guo *et al.*, 1996; Hebert *et al.*, 1994; Mansour *et al.*, 1993; Miller *et al.*, 2000; Ortega *et al.*, 1998). Whether this means that lens development does not involve these proteins or that their loss can be compensated for by other family members is not known. Inactivation of the genes for FGF-4, FGFR-1, and FGFR-2 result in early embryonic lethality, precluding an evaluation of their function in lens development (Deng *et al.*, 1994; Feldman *et al.*, 1995; Yamaguchi *et al.*, 1994). Therefore, although there is strong evidence for a role of FGF in lens differentiation, the particular family members involved in this process *in vivo* have yet to be determined. Although certain other growth factors (BMP-4, PDGF, TGF-α/EGF, IGF) have been proposed to potentially participate in lens differentiation in mammals, *in vivo* evidence of such a function is lacking (Brewitt and Clark, 1988; Furuta and Hogan, 1998; Ireland and Mrock, 2000; Klok *et al.*, 1998; Reneker and Overbeek, 1996).

In contrast to the mammalian lens, FGFs have been reported for over twenty years to have no effect on the chick lens. Instead, an insulin/IGF-type growth factor was proposed to be the physiological lens differentiation factor. Insulin was first reported to stimulate cellular elongation in chick lens epithelial cell explants by Piatigorsky and colleagues (Piatigorsky, 1973). Subsequent studies indicated that insulin-induced differentiation was accompanied by an increase in microtubule assembly and δ-crystallin gene expression, both of which are features of developing fibers in the intact avian lens (Milstone and Piatigorsky, 1977; Piatigorsky *et al.*, 1973). Beebe reported that the ability of chick vitreous humor to stimulate cellular elongation in chick lens central epithelial explants over a 4-5 hour period was partially inhibited by a monoclonal antibody to human IGF-1(Beebe *et al.*, 1987). FGF and other growth factors (EGF, PDGF, NGF) had no effect on explant elongation under the same conditions.

D. FGF-mediated signal transduction

Although very little is known about FGF signaling pathways in the lens, there is much literature on FGF-mediated signal transduction in other systems. FGF family members interact with two classes of receptors, one with low affinity (K_d 2-10 nM) and the other with K_d's of 50-500 pM. The cell surface low-affinity receptors are heparin sulfate proteoglycans (HSPGs) which have high association rates with FGFs but also high dissociation rates. Schlessinger et al. have proposed that binding of FGFs to these HSPGs reduces the dimensionality of ligand diffusion from the 3-dimensional volume of the extracellular space to the two dimensional surface of the plasma membrane (Schlessinger *et al.*, 1995). Once bound, FGF molecules exhibit rapid lateral mobility which allows for frequent encounters with the unoccupied less abundant high-affinity receptors (Figure 4). There are also low affinity HSPG binding sites for FGFs in the extracellular matrix, which have been proposed to stabilize FGF and protect it from degradation or serve as a reservoir of growth factors that can be released by enzymes that degrade the proteoglycan (Damon *et al.*, 1989; Gospodarowicz and Cheng, 1986).

High affinity binding of FGF is mediated by the products of four distinct FGF receptor genes. Each of these genes undergoes alternative splicing, giving rise to multiple FGF receptor species with different ligand-binding specificities and affinities (Houssaint *et al.*, 1990; Johnson *et al.*, 1990; Keegan *et al.*, 1991; Partanen *et al.*, 1991). All of these FGF receptors are Class IV receptor tyrosine kinases and function according to the consensus model of signal transduction by receptor protein tyrosine kinases (RPTKs) (reviewed in Klint and Claesson-Welsh, 1999) (Figure 4). Binding of FGF results in receptor homo- or heterodimerization, leading to intermolecular autophosphorylation of multiple tyrosine residues within the cytoplasmic domain of the receptor. These phosphorylated tyrosine residues then serve as binding sites for proteins containing Src-homology 2 (SH2) domains or phosphotyrosine binding (PTB) domains, which after binding become substrates for the FGF receptor. For example, phosphorylation of Y766

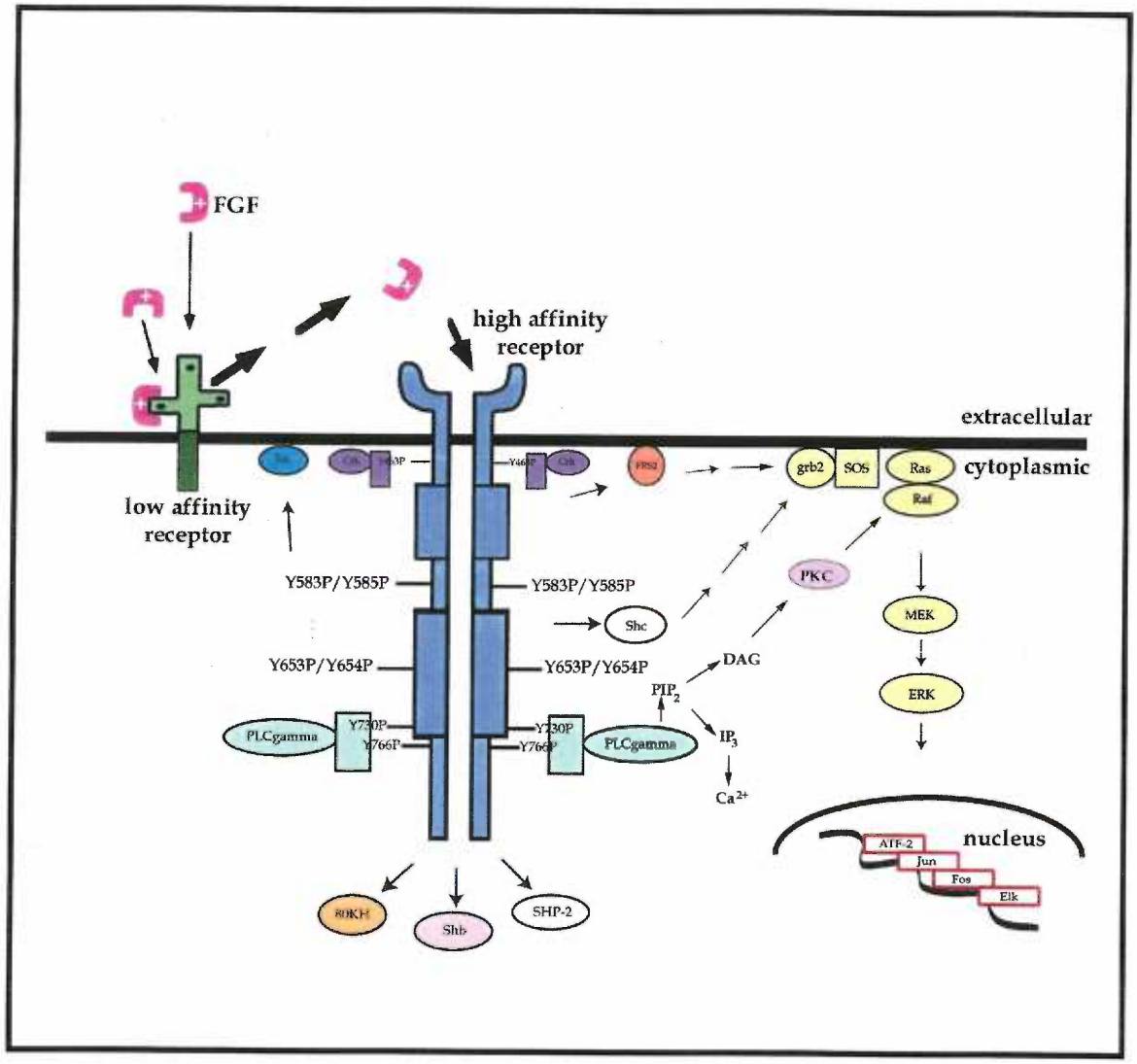

Figure 4. *FGF signalling.* Binding of FGFs to the low-affinity receptors (HSPGs) on the cell surface reduces the dimensionality of ligand diffusion from the three-dimensional volume of the extracellular space to the two-dimensional plasma membrane which allows frequent encounters with the less abundant high-affinity receptors. Subsequent binding of FGF to a high affinity receptor leads to receptor homo- or heterodimerization and intermolecular autophosphorylation of multiple tyrosine residues within the cytoplasmic domain of the receptor.

in FGFR-1 upon receptor dimerization allows the binding of PLCγ, which is then tyrosine phosphorylated by the FGF receptor which increases its ability to hydrolyze phosphatidylinositol 4,5 bisphosphate (PIP_2) to IP_3 and DAG (Berridge, 1993). IP_3 in turn releases Ca^{2+} from internal stores, while DAG accumulation activates members of the PKC family which can then activate the mitogen-activated protein kinase (MAPK) signaling cascade.

MAP kinases are serine/threonine kinases whose activation leads to a variety of cellular responses including proliferation, differentiation, and migration (reviewed in Garrington and Johnson, 1999 and Seger, 1995). Three well-characterized subfamilies of MAP are: 1) p42/44 (ERK-1 and ERK-2, respectively), 2) the stress-activated protein kinases/c-Jun-N-terminal kinases (SAPKs/JNKs), and 3) p38/reactivating kinase (RK). The p44/p42 MAP kinases are primarily activated by mitogens (e.g. FGF, insulin), whereas the SAPK/JNK and p38/RK pathways are highly stimulated by inflammatory cytokines (e.g. tumor necrosis factor a, interleukin 1b) and cellular stresses (e.g. heat shock, UV, ionizing radiation). All MAP kinase signaling pathways contain a central core of three kinases (Figure 5). MAPKs are activated by phosphorylation on threonine and tyrosine by a dual-specificity MAP kinase kinase (MAPKK), and MAPKK is in turn activated by serine/threonine phosphorylation by a MAP kinase kinase kinase (MAPKKK). The ERK signal transduction pathway, once activated, affects a number of downstream targets that include nuclear (e.g. Elk1, Pax6), membrane-associated (e.g. Synapsin I), and cytoskeletal proteins (e.g. myelin basic protein) to effect survival, differentiation, proliferation, gene expression, etc. (reviewed in Grewal *et al.*, 1999).

In most, but not all cases, ERK-dependent FGF signal transduction is meidated by the classic Ras/Raf pathway. The low molecular weight (21 kDa) proteins of the Ras family function as signaling mediators for many types of receptor protein tyrosine kinases and tyrosine kinase-associated receptors (Lowy and Willumsen, 1993; Pronk and Bos, 1994). Ras proteins bind guanine nucleotides, have intrinsic GTPase activity, and

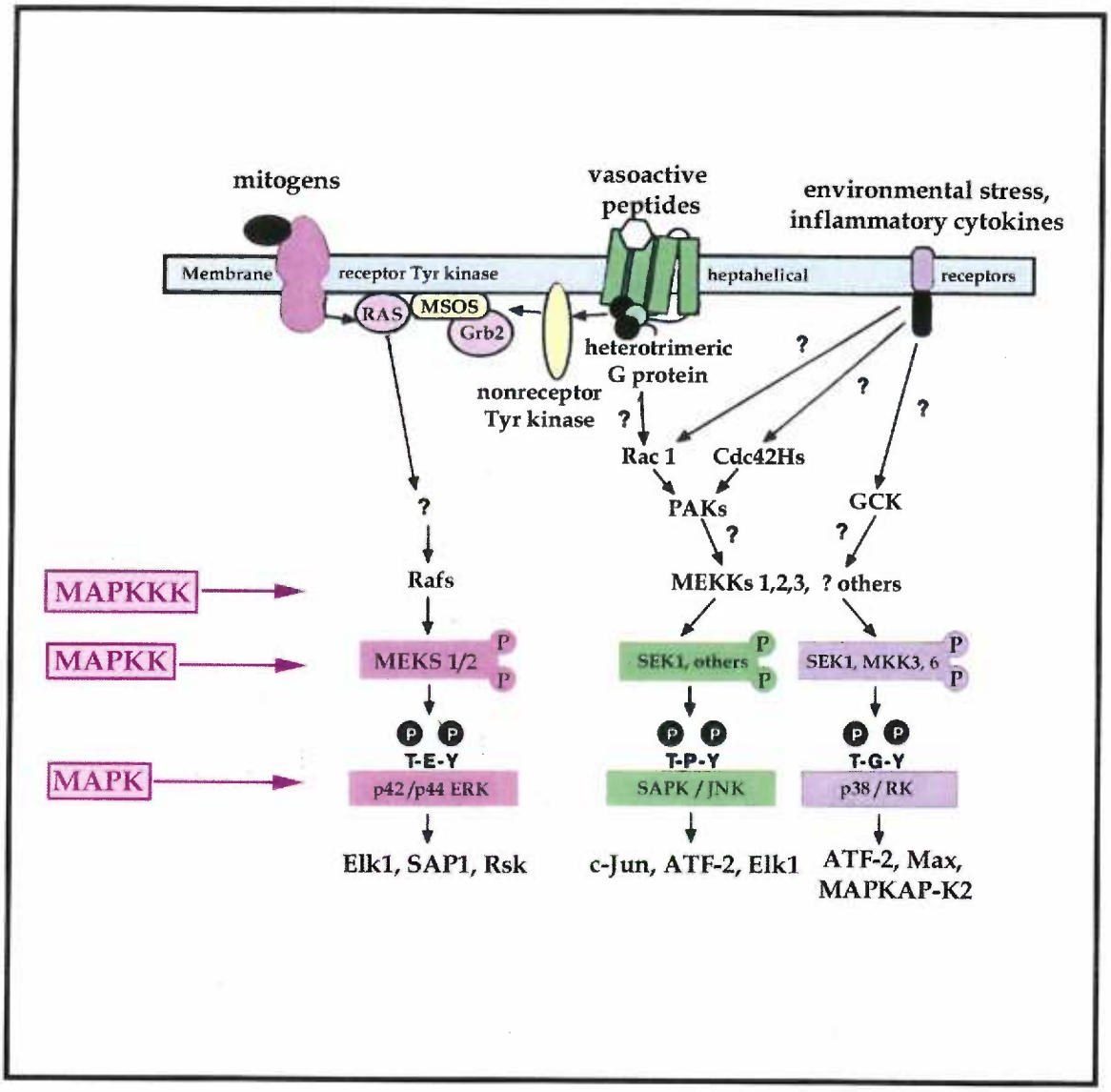

Figure 5. *MAPK pathways.* The principle MAPK subfamilies are: 1) p42/44 (ERK-1 and ERK-2, respectively), 2) the stress-activated protein kinases/c-Jun-N-terminal kinases(SAPKs/JNKs), and 3) p38/reactivating kinase(RK). All three MAP kinase signaling pathways contain a central core of three kinases. Upon activation, a MAP kinase kinase kinase (MAPKKK) phosphorylates a dual-specificity MAP kinase kinase (MAPKK), which subsequently phosphorylates MAP kinase (MAPK). The activated MAPK phosphorylates various proteins in the cell, including other protein kinases and gene regulatory proteins.

alternate between the active (GTP-bound) and inactive (GDP-bound) forms. Most growth factor receptors couple to the Ras pathway via the small adaptor molecule Grb2, which exists in a complex with Sos, a nucleotide exchange factor for Ras. In contrast, all identified FGF receptors lack direct binding sites for Grb2. Instead, FGF stimulation leads to tyrosine phosphorylation of the adaptor proteins FRS2 and/or Shc which then bind the Grb2-Sos complex (Kouhara *et al.*, 1997). Sos then catalyzes the exchange of GDP for GTP on Ras, which in turn binds to and activates a MAPKKK (Raf), which activates a MAPKK (MEK 1/2) that activates MAPK (ERK 1/2) (Figure 4).

One proposed mechanism by which different growth factors can elicit different cellular responses in the same cell type is by regulating the duration of ERK activation (Marshall, 1995). This concept arose from studies initially performed in PC12 cells. PC12 cells, a phaeochromocytoma cell line derived from rat adrenal medullary cells, can differentiate into sympathetic neurons upon treatment with certain growth factors such as FGF or nerve growth factor (NGF) as characterized by neurite outgrowth and eventual cessation of cell division. Treatment with epidermal growth factor (EGF) leads instead to cell proliferation. All three growth factors require the ERK pathway for their effects, but EGF activates ERKs only transiently (up to 10 minutes, with a maximum at 5 minutes), whereas FGF and NGF induce sustained ERK activation (up to 6 hours, with a maximum at 15 minutes) as detected by phosphorylation of myelin basic protein (MBP). These observations led to the proposal that sustained ERK activation leads to differentiation in PC12 cells. This concept was further supported by studies showing that there is no receptor-specific pathway of differentiation and that sustained ERK activation is sufficient for PC12 cell differentiation (Dikic *et al.*, 1994; Heasley and Johnson, 1992; Traverse *et al.*, 1994). Interestingly, Stork and colleagues have shown that the initial early phase of ERK activation by NGF is mediated by Ras whereas sustained ERK activation is due to a PKA-dependent pathway which involves the small GTPase Rap1 acting through B-raf instead of Raf-1 (York *et al.*, 1998). Subsequently, several groups have demonstrated a

correlation between sustained ERK activation and a variety of cellular responses in cell types other than PC12s (Lessor *et al.*, 1998; Pukac *et al.*, 1998; Weber *et al.*, 1997), For example, in SK-N-MC neuroepithelioma cells, cell scattering is characterized by loss of epithelial and gain of fibroblastic features, cell dissociation, and migration of individual cells. EGF, as well as FGF-2, induce both sustained ERK activation (≥24hrs) and cell scattering, whereas PDGF induces only transient ERK activation (5-10 minutes) without effecting cell scattering (van Puijenbroek *et al.*, 1997). Similarly, two different activators of protein kinase C (PKC) have opposing effects in the megakaryocytic differentiation of K562 cells. While 12-O-tetradecanoylphorbol-13-acetate (TPA) induces sustained ERK activation (>24hrs) and differentiation, bryostatin induces more transient activation of ERKs(~6hrs) and blocks differentiation (Racke *et al.*, 1997).

Although the standard FGF-induced signal transduction pathway is through ERKs, there are examples of ERK-independent effects of FGF. Differentiation of the rat neuronal hippocampal H19-7 cell line and expression of a gene (pip92) of unknown function in response to FGF-2 is MEK and ERK-independent, but Ras and Raf-1-dependent (Chung *et al.*, 1998). In SK-N-MC cells, FGF can stimulate a p38-dependent increase in the experssion of genes such as proenkephalin that can be mimicked by exogenous expression of p38 but not of ERK (Tan *et al.*, 1996). In osteoblastic cells, FGF activates expression of MMP1 through Ras without ERKs or MEK (Newberry *et al.*, 1997).

E. Gap junctions in the lens

Metabolic homeostasis in the lens requires the transfer of nutrients into the organ and the passage of waste products in the opposite direction. Since the terminally differentiated fiber cells lack intracellular organelles, they cannot synthesize or maintain the proteins required for active transport. Fiber cells are therefore uniquely dependent on communication with epithelial cells at the lens surface for their metabolic needs. In lieu of a vascular system, metabolic and ionic balance in the lens is maintained by means of a

network of epithelial-to-epithelial, fiber-to-fiber, and epithelial-to-fiber gap junctions which directly link the cytoplasms of adjoining cells (Goodenough *et al.*, 1980). Nutrients produced by the epithelial cells are transferred to the interior fiber cells via this gap junction-mediated intercellular pathway (Figure 6). This pathway is also utilized to extrude metabolic waste products from the lens. The importance of gap junctions in lens homeostasis is underscored by the demonstration that targeted deletion of genes encoding lens gap junction proteins results in the formation of cataracts in mice (Gong *et al.*, 1998; White *et al.*, 1998).

Gap junctions are specialized communicating intercellular junctions, that mediate the passage of chemical and electrical signals from one interacting cell to its adjoining partner(s) (Figure 7). Molecules up to ~1kD are able to pass through gap junctional channels, whereas larger macromolecules are retained in their cell of origin to ensure its genetic and structural individuality (Simpson *et al.*, 1977). Substances proposed or experimentally demonstrated to pass through gap junctions include ions, monosaccharides, amino acids, IP_3, and cyclic nucleotides (Bennett and Goodenough, 1978; Saez *et al.*, 1989). Known or proposed functions of gap junctions include serving as a low-resistance passageway for current-carrying ions in electrically excitable tissues such as myocardium, smooth muscle and nerve, and regulating growth control, glandular secretion, and cellular differentiation in nonexcitable tissues (reviewed in Simon and Goodenough, 1998).

Gap junctions are composed of a family of highly homologous integral membrane proteins called *connexins(Cx)* which are found in virtually all animal tissues. All connexin family members share conserved transmembrane, extracellular, and, to a lesser extent, amino terminus sequences while unique sequences are found in their cytosolic loop and carboxyl tail domains (Figure 8) (Bennett *et al.*, 1991; Kumar and Gilula, 1992). The assembly of connexins into gap junctions is a multistep process that includes: 1) oligomerization of connexin monomers into connexons in the *trans* Golgi network, 2)

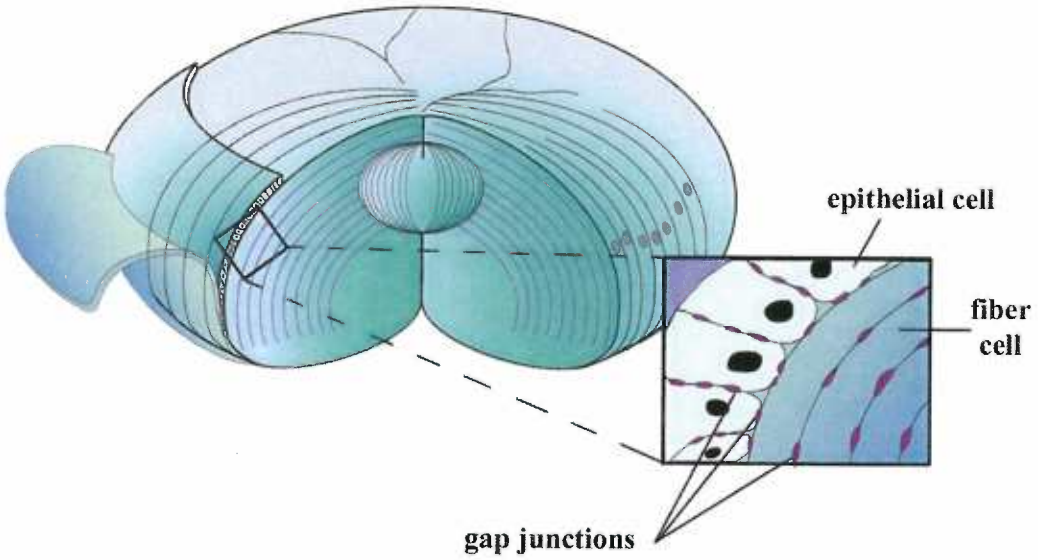

Figure 6. *Location of gap junctions in the lens.* Epithelial-to-epithelial, epithelial-to-fiber, and fiber-to-fiber gap junctions directly link the cytoplasms of adjacent lens cells. This intercellular network allows fiber cells to access nutrients produced by the epithelial cells.

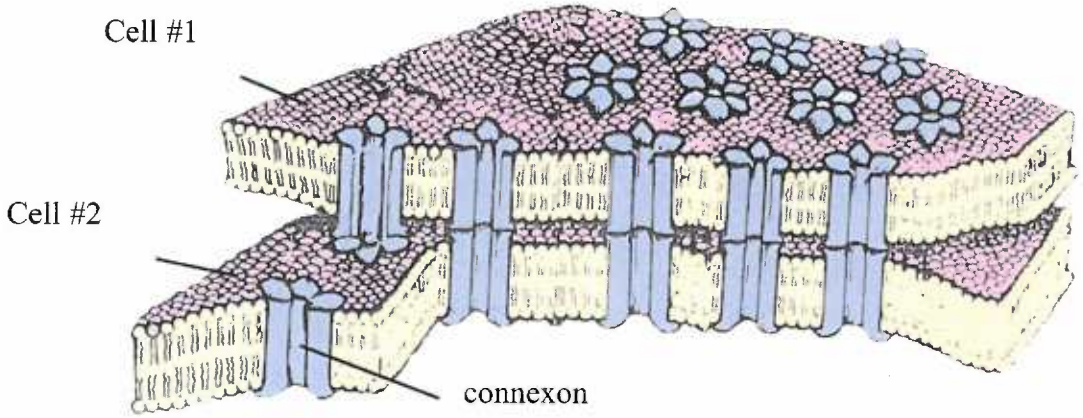

Figure 7. *Gap junctional plaque.* Two half-channels called connexons, one in each cell s plasma membrane, dock head-to-head to form an intercellular pore. A gap junctional plaque is a collection of these tightly packed intercellular channels.

transport of connexons to the cell surface, 3) association of two connexons in adjoining cell surfaces into intercellular channels, and 4) clustering of these channels into gap junctional plaques (Figure 9). At least in the case of Cx43, this last step is closely associated with connexin phosphorylation and acquisition of insolubility in Triton X-100 (Musil and Goodenough, 1991).

Immunohistochemical studies have localized connexin family members to specific anatomical regions of the lens in various species (Gruijters *et al.*, 1987; Jiang *et al.*, 1994; Musil *et al.*, 1990; Paul *et al.*, 1991). In chick, three members of the connexin family, Cx43, Cx45.6, and Cx56, are expressed in the lens epithelium and in the differentiating fibers of the bow (equatorial) region (Figure 10). Cx45.6 and Cx56, but not Cx43, are also present in mature postequatorial fiber cells where their expression levels are greatly increased (Jiang *et al.*, 1994). In mammals, only Cx43 has been detected in the lens epithelium (Beyer *et al.*, 1989). Cx50 and Cx46, orthologs of (respectively) chick Cx45.6 and Cx56, share an overlapping distribution with Cx43 in the bow region and are expressed in high levels in postequatorial fiber cells (Gruijters *et al.*, 1987; White *et al.*, 1992).

Gap junctions are not passive intercellular holes connecting cells. A highly significant property of gap junction-mediated intercellular communication (GJIC) is that it can be *reversibly* regulated within seconds by an enormous variety of effectors. These effectors include: 1) intracellular acidification, 2) elevation of free intracellular Ca^{2+}, 3) transjunctional voltage, 4) cyclic nucleotides, 5) some lipophilic compounds (e.g. heptanol, n-octanol, halothane, arachidonic acid), and 6) growth-controlling substances including phorbol esters, retinoic acid, certain oncogene products (Bennett and Verselis, 1992; Kolb and Somogyi, 1991), and some growth factors. This last category includes epidermal growth factor (EGF), hepatocyte growth factor/scatter factor (HGF/SF), and platelet-derived growth factor (PDGF). Most studies have shown that growth factors transiently decrease gap junction-mediated intercellular communication (GJIC), most

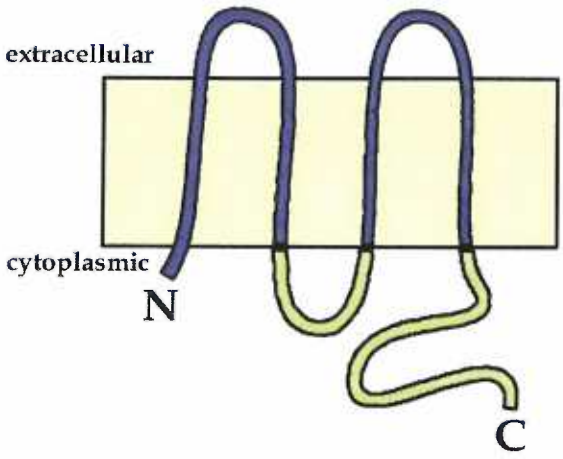

Figure 8. *Connexin topology.* All connexin family members contain four transmembrane domains and are oriented such that their amino and carboxyl termini are cytoplasmic. Regions conserved between different connexins are in blue, whereas the less conserved domains are shown in green.

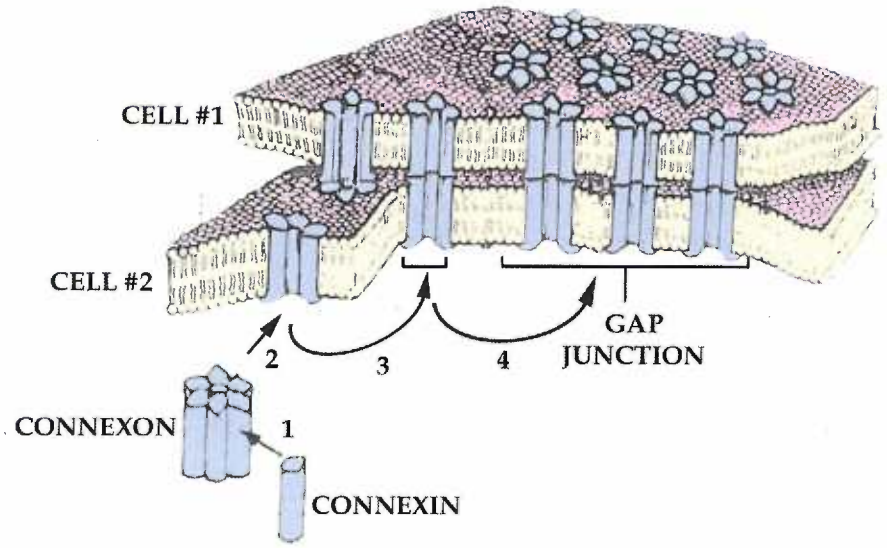

Figure 9. *Gap junction assembly.* Gap junction assembly is a multi-step process involving: 1) oligomerization of connexin monomers into connexons; 2) transport of connexons to the cell surface; 3) association of two connexons in adjoining cell surfaces into intercellular channels; and 4) clustering of these channels into gap junctional plaques.

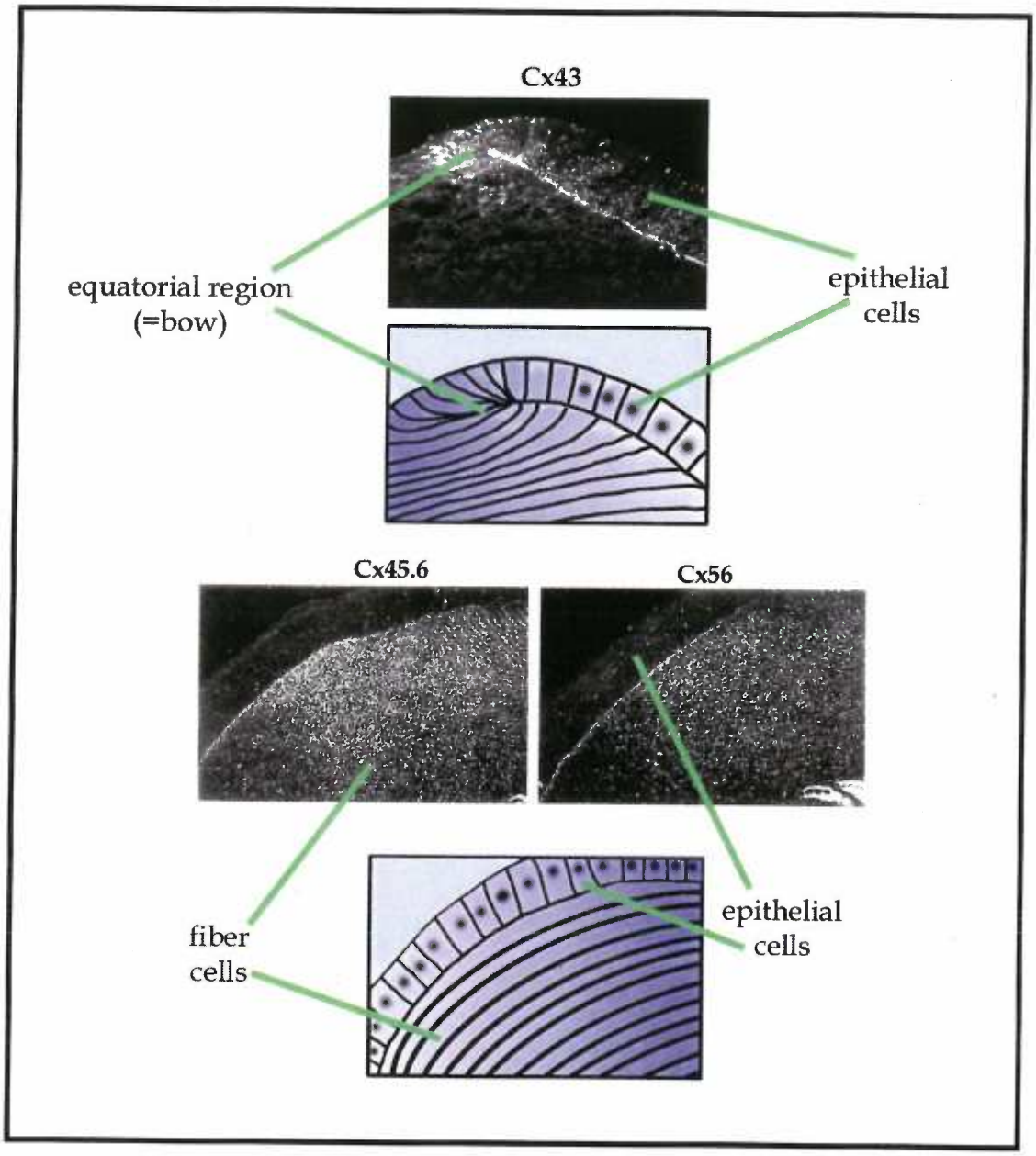

Figure 10. *Connexin expression in the lens.* Three members of the connexin family are expresed in the chick lens, as shown here by immunofluorescence microscopy of frozen sections. Cx43 is expressed in the central epithelial and bow regions, but not the mature fiber cells, whereas Cx45.6 and Cx56 are most highly expressed in fiber cells.

likely at the level of channel gating. For example, addition of HGF/SF to mouse keratinocytes or treatment of T51B rat liver epithelial cells with platelet-derived growth factor (PDGF) rapidly inhibits GJIC (within 5-10 minutes or 15-20 minutes, respectively) (Hossain *et al.*, 1998; Moorby *et al.*, 1995). Similarly, GJIC is reduced by 60% within ten minutes after EGF addition in T51B cells (Lau *et al.*, 1992), and FGF-2 decreased coupling between rat cardiomycytes within 30 minutes (Doble *et al.*, 1996). However, there are also several studies which demonstrate that long-term exposure to growth factors can increase connexin protein expression leading to an increase in GJIC. For example, EGF increases Cx43 protein and GJIC in primary human kidney epithelial cells after a four hour exposure (Rivedal *et al.*, 1996). Addition of FGF to cardiac fibroblasts increased GJIC and Cx43 mRNA and protein after six hours (Doble and Kardami, 1995).

F. Issues addressed in this thesis

A.. Do gap junctions play a role in lens development? Several studies in non-lenticular systems have indicated that gap junctions mediate the intercellular transfer of morphogens and help establish embryonic compartments. Function-blocking anti-connexin antibodies or pharmacological inhibitors disrupt early vertebrate embryogenesis and limb morphogenesis (Lee *et al.*, 1987; Warner *et al.*, 1984). Dominant-negative connexin mutants and antisense mRNA also support a role for gap junctions in the above processes (Bevilacqua *et al.*, 1989; Paul *et al.*, 1995). Gap junctions have also been implicated in the differentiation of specific tissues. For example, gap junction blockers reduce expression of the muscle regulatory factors myogenin and MRF4 as well as myotube fusion in skeletal myoblasts (Constantin *et al.*, 1997; Mege *et al.*, 1994; Proulx *et al.*, 1997). Connexin knockout studies have implicated Cx43 in heart development and Cx37 in oocyte development (Reaume *et al.*, 1995; Simon *et al.*, 1997). A potential role for gap junctions in the development of the lens is supported by the following studies. Gap junctional communication and fiber cell differentiation are both blocked when chick

lens cells are transformed with Rous sarcoma virus (Menko and Boettiger, 1988). Moreover, anti-NCAM Fab fragments inhibit both cellular elongation and the formation of fiber-type gap junctions in chick lens epithelial explants (Watanabe *et al.*, 1989). Although targeted gene disruption of either of the lens fiber connexins (Cx46 or Cx50) resulted in no obvious defects in epithelial-to-fiber differentiation, the continued expression of the undisrupted connexin could have compensated for the missing protein (Gong *et al.*, 1997; White *et al.*, 1998). Chapter One in the Results section of this thesis addresses the role of gap junctions in lens development by directly assessing secondary fiber cell differentiation of primary chick lens epithelial cells after inhibition of gap junctional intercellular communication with a known gap junction blocker, 18β-glycyrrhetinic acid.

B. Does FGF play a role in development of the chick lens? As discussed on pages 6-8, the prevailing concept in the literature has been that an FGF induces epithelial-to-fiber differentiation in the mammalian lens whereas chick lens cells are unresponsive to FGF and are instead induced to differentiate by insulin/IGF-type factors. Since the steps involved in lens development in the chick are very similar to those in rodents, and because chick lens cells express FGF receptors (Ohuchi et al., 1994) and chick vitreous humor contains FGF (Mascarelli et al., 1987), it seems surprising that chick cells should respond to growth factors so differently than mammalian lens cells. Chapter Two in the Results section of this thesis reinvestigates the effect of FGFs on chick lens cells and presents evidence that FGFs are in fact potent determinants of chick lens cell proliferation and differentiation, and that chick vitreous humor is an *in vivo* source of an FGF-like differentiation factor.

C. Are growth factors involved in establishing the asymmetry of gap junction-mediated intercellular communication (GJIC) required for lens function? Several lines of evidence indicate that ions and water enter the lens along the intercellular clefts, cross fiber cell membranes, flow from cell-to-cell through gap junctions toward the surface, and

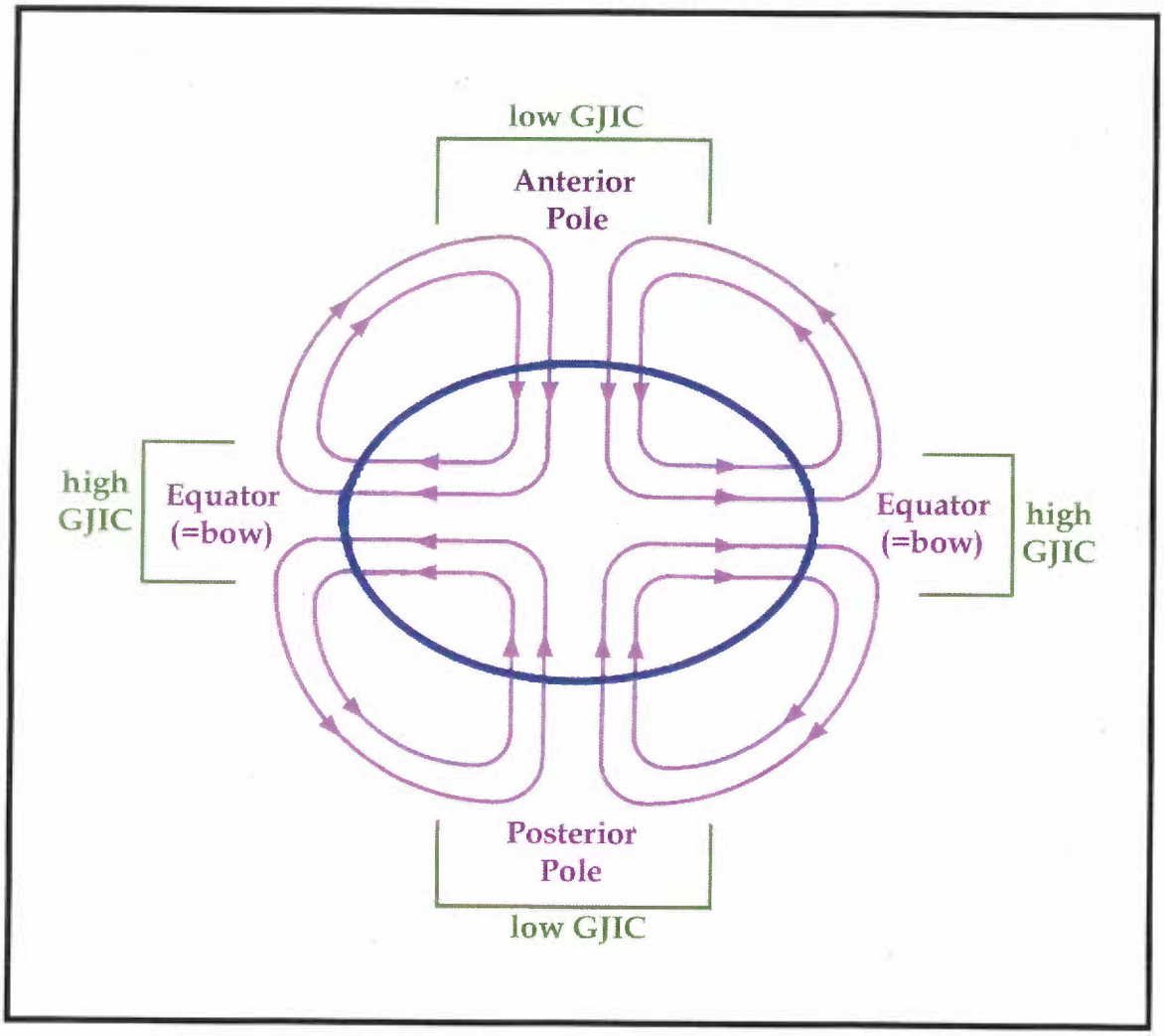

Figure 11. *Lens circulation.* Ions and water enter the lens at the anterior and posterior poles along the intercellular clefts, cross fiber cell membranes, flow intracellularly from cell to cell through gap junctions towards the lens surface, and then cross surface cell membranes to exit the lens at the equator. This angular flow serves to circulate fluids and dissolved solutes through the lens. The assymmetric distribution of gap junctions in the lens is thought to be essential in setting up these circuits and thus for normal lens physiology. (Circulation diagram from Mathias, 1997.)

cross surface cell membranes (Figure 11) (Robinson and Patterson, 1982). This creates an angular flow that serves to carry oxygen, glucose, and other nutrients into the lens via the intercellular space, and to flush waste products through the intracellular space from inner lens fibers to the surface where they can be metabolized and/or extruded. The establishment and maintenance of these circuits is thought to be critically dependent on the fact that GJIC in the lens is higher at the equator than at either pole. Despite its importance for lens function, it is unknown how the gradient of GJIC in the lens is established. Chapter Three in the Results section of this thesis explores the possibility that a factor that upregulates lens differentiation at the equator also plays a role in upregulating GJIC in this same region. The results presented support a model in which regional differences in FGF signaling are responsible for the asymmetry of GJIC in the lens.

D. Does the ERK pathway play a role in FGF-induced chick lens epithelial cell proliferation, differentiation, and/or upregulation of GJIC? As discussed on pages 11 and 13, FGF is known to signal by ERK-dependent as well as (less commonly) ERK-independent pathways. To date, the role of the ERK pathway in lens development or function has not been addressed. Chapter Two investigates whether ERK activation is required for stimulation of lens cell proliferatin and/or differentiation by growth factors, and Chapter Three addresses the role of ERKs in FGF-induced upregulation of GJIC.

III. Results

A. Chapter 1

Normal Differentiation of Cultured Lens Cells after Inhibition of Gap Junction-Mediated Intercellular Communication

Anh-Chi N. Le

and Linda S. Musil

Vollum Institute for Advanced Biomedical Research and

Department of Cell and Developmental Biology

Oregon Health Sciences University

3181 SW Sam Jackson Park Road

Portland, OR 97201

1. Abstract

The cells of the vertebrate lens are linked to each other by gap junctions, clusters of intercellular channels that mediate the direct transfer of low molecular weight substances between the cytosols of adjoining cells. Although gap junctions are detectable in the unspecialized epithelial cells that comprise the anterior face of the organ, both their number and size are greatly increased in the secondary fiber cells that differentiate from them at the lens equator. In other organs, gap junctions have been shown to play an important role in tissue development and differentiation. It has been proposed, although not experimentally tested, that this may be true in the lens as well. To investigate the function of gap junctions in the development of the lens, we have examined the effect of the gap junction blocker 18β-glycyrrhetinic acid (βGA) on the differentiation of primary cultures (both dissociated cell-derived monolayers and central epithelium explants) of embryonic chick lens epithelial cells. We found that βGA greatly reduces gap junction-mediated intercellular transfer of Lucifer yellow and biocytin throughout the 8-day culture period. βGA did not, however, affect the differentiation of these cells into MP28-expressing secondary fibers. Furthermore, inhibition of gap junctions had no apparent effect on either of the two other types of intercellular (adherens and tight) junctions present in the lens. We conclude that the high level of gap junctional intercellular communication characteristic of the lens equator *in vivo* is not required for secondary fiber formation as assayed in culture. Up-regulation of gap junctions is therefore likely to be a consequence rather than a cause of lens fiber differentiation and may primarily play a role in lens physiology.

2. Introduction

The lens is a solid cyst composed of only two cell types: a monolayer of epithelial cells that overlies its anterior face and a core of elongated, crystallin-rich fibers that is responsible for the refractive properties of the organ. These cells are physically and functionally linked to each other by gap junctions at their plasma membranes (Goodenough, 1992). Gap junctions are clusters of intercellular channels that link the cytosols of adjoining cells and thereby act as direct pathways for the cell-to-cell transfer of small (under ~1 kDa) nutrients and signaling molecules. In vertebrates, gap junctions are composed of members of a family of structurally homologous integral membrane proteins known as connexins which differ from each other with respect to their channel permeabilities, modes of regulation, and ability to interact with other connexin species (Bruzzone *et al.*, 1996; Goodenough *et al.*, 1996). Lens epithelial cells predominantly express connexin43 (Cx43), a widely distributed connexin that is believed to participate in cell-cell communication in a variety of embryonic and adult organs (Beyer *et al.*, 1987; Musil *et al.*, 1990a). Mature fiber cells lack Cx43 but instead express very high levels of two other connexin species. In the chick, these fiber-type connexins are referred to as Cx45.6 and Cx56 (Jiang *et al.*, 1994; Rup *et al.*, 1993); their orthologs in rodents are termed Cx50 and Cx46 (Paul *et al.*, 1991; White *et al.*, 1992). Because they lack biosynthetic or energy-producing organelles, mature fiber cells are uniquely dependent on the active transport and oxidative phosphorylation capacity of the anterior epithelial cells. Electrical impedance studies (Mathias and Rae, 1985) and analysis of intercellular coupling by diffusion of low molecular weight fluorescent dyes (Miller and Goodenough, 1986) or radiolabeled metabolites (Goodenough *et al.*, 1980) indicate that gap junctions join the lens cells into an ionic and metabolic syncytium. A role for gap junctions in lens transparency is supported by the recent finding that targeted disruption of Cx46

expression in mice results in the postnatal development of a nuclear cataract (Gong *et al.*, 1997).

Gap junctions are not specific to the lens but are instead found in almost all cell types in animals ranging from coelenterates to humans (Bennett *et al.*, 1991; Goodenough *et al.*, 1996). Two broad classes of functions have been ascribed to gap junctions. The first, similar to that described above for the lens, is to help maintain metabolic continuity within, and synchronize the function of, differentiated multicellular tissues. The second arises from numerous studies implicating gap junctions in various developmental processes, in which they have been proposed to mediate the intercellular transfer of morphogens and to help establish embryonic compartments (reviewed in Guthrie and Gilula, 1989; Lo, 1996; Warner, 1992). Reducing gap junctional intercellular communication with either function-blocking anti-connexin antibodies or pharmacological inhibitors of gap junction permeability disrupts early vertebrate embryogenesis (Warner *et al.*, 1984) and limb patterning (Allen *et al.*, 1990). Studies utilizing dominant-negative connexin mutants and anti-sense mRNA constructs have supported a role for connexin-mediated intercellular communication in these processes (Bevilacqua *et al.*, 1989; Paul *et al.*, 1995). With regard to the differentiation of specific tissues, blockers of gap junction-mediated intercellular communication have been shown to reduce the expression of the muscle regulatory factors myogenin and MRF4 in skeletal myoblasts and the fusion of these cells into myotubes (Constantin *et al.*, 1997; Mege *et al.*, 1994; Proulx *et al.*, 1997a). Conversely, introduction of Cx43 into connexin-deficient rhabdomyosarcoma cells increase cell fusion and myosin expression concomitant with acquisition of gap junction-mediated intercellular coupling (Proulx *et al.*, 1997b). Restoration of gap junctional intercellular communication upon transfection with connexin-encoding cDNA has also been reported to increase the expression of tissue-specific genes (thyroglobulin and insulin, respectively) in cell lines derived from the thyroid (Statuto *et al.*, 1997) and from the pancreas (Vozzi *et al.*, 1995). In the mouse,

elimination of Cx43 expression by targeted gene disruption results in lethal developmental malformations in the heart (Reaume *et al.*, 1995), whereas lack of connexin37-mediated oocyte-granulosa cell communication leads to female infertilitiy by arresting oocyte development (Simon *et al.*, 1997).

Despite the presence of gap junctions throughout the lens and the importance of these structures in the development of other organs, the role of gap junctional intercellular communication in lens differentiation is not known. Although the time course and anatomical details differ between species, the general process by which the vertebrate lens develops is remarkably conserved between amphibians, birds, and mammals including humans (reviewed in Piatigorsky, 1981). Following induction, the embryonic ectoderm overlying the optic vesicle thickens to form the lens placode. The lens placode invaginates and eventually pinches off as the lens vesicle, a hollow sphere of epithelial cells. The cells at the posterior of the lens vesicle then differentiate into the primary fiber cells, which elongate to fill the lumen of the lens vesicle. In addition to an increase in cell volume, fiber cell differentiation is characterized by restructuring of the cell surface and cytosol, upregulation of fiber-specific proteins including various crystallins and the plasma membrane protein MP26 (known as MP28 in the chick), and eventual loss of intracellular organelles along with cessation of DNA synthesis and cell division. All subsequent growth of the lens (which continues throughout the life of the organism) is due to differentiation of epithelial cells into so-called "secondary" fiber cells, whose properties are similar to those of the primary fibers.

The process of secondary fiber formation begins with the progressive morphological and biochemical differentiation of a small population of epithelial cells near the equatorial axis of the lens. The equatorial region is also the site of the highest level of gap junctional intercellular coupling within the organ (Baldo and Mathias, 1992; Mathias *et al.*, 1997). The latter observation is likely to be due in part to the up-regulation of fiber-type connexin expression that occurs within this area (Berthoud *et al.*, 1994;

Evans *et al.*, 1993; Gong *et al.*, 1997). In the chick, immunofluorescence and immunoelectron microscopy have demonstrated that the level of Cx43-containing gap junctions in equatorial epithelial cells also increases dramatically relative to the amount of Cx43 detected in the central epithelium at the anterior pole (Musil *et al.*, 1990a). Why synthesis and/or assembly of Cx43 should increase so substantially shortly before being shut off in mature fiber cells is unknown. Early studies of lens differentiation in culture have established the necessity of direct cell-cell contact in epithelial-to-fiber differentiation (Creighton *et al.*, 1976). Given the importance of gap junctions in other development processes, this could conceivably reflect a need for gap junctional intercellular communication in initiating and/or coordinating lens cell differentiation, a possibility raised previously by Menko as well as others (Menko and Boettiger, 1988; Menko *et al.*, 1987; Watanabe *et al.*, 1989). A potential role for gap junctions in lens development is supported by the observation that transformation of cultured chick lens epithelial cells with Rous sarcoma virus blocks both gap junctional intercellular communication and fiber cell differentiation (Menko and Boettiger, 1988). Furthermore, Watanabe *et al.* (1989) have reported that treatment of lens epithelial explants with anti-NCAM Fab fragments, while having no apparent effect on cell-cell contact per se, inhibits the formation of fiber-type gap junctions as well as cell elongation (a marker of initiation of fiber cell differentiation). Histological examination of the lenses of mice lacking Cx46 failed to reveal obvious defects in lens development (Gong *et al.*, 1997). However, the fact that these mice still synthesize Cx43 and Cx50 (both of which are able to form functional gap junctions in the absence of Cx46) and continue to assemble the latter into lens fiber gap junctional plaques makes it very likely that a high level of cell-cell coupling is maintained at the cataract-free lens bow region. Thus, whether there is a causal relationship between gap junctional intercellular communication and lens differentiation has not been directly tested.

To address the role of gap junctions in epithelial-to-fiber maturation, we have used

a derivative of glycyrrhetinic acid (18β-glycyrrhetinic acid; βGA) that has previously been demonstrated to potently inhibit gap junction-mediated intercellular dye and metabolic transfer in a variety of cell types (Davidson *et al.*, 1986; Guan *et al.*, 1996). Our results indicate that the extensive gap junctional intercellular communication characteristic of the equatorial region *in vivo* is not required for secondary fiber formation as assayed in two *in vitro* model systems of lens development. The primary function of gap junctions at the equator and throughout the lens may therefore be to support the metabolic and functional needs of the organ.

3. Materials and Methods

Preparation of embryonic chick lens cultures. Dissociated-cell derived monolayer cultures were prepared using a modification of the procedure of Menko *et al.* (1984) as follows. Lenses were excised from E10 white leghorn chicken embryos and collected into TD buffer (0.14 M NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 5 mM D-glucose, 0.025 M Tris base, pH 7.4). Contaminating ciliary epithelium was removed by incubating the lenses in 0.08% trypsin in TD buffer at 37°C for 30 minutes and subsequent gentle trituration with a fire-polished Pasteur pipet. The cleaned lenses were then broken in M199 medium (Gibco BRL) supplemented with 10% fetal calf serum (Hyclone) and subjected to centrifugation at 1,000 rpm for 10 minutes. The pelleted cells were resuspended in serum-free M199 medium and filtered through three layers of lens paper (A.H. Thomas) to remove capsule material and any cell clumps. The cells were then plated at near confluent density (1.8 $X10^5$ cells/well) onto glass coverslips (Bellco) in a 96-well tissue culture plate in M199 + OTS (25 mg/ml ovotransferrin, 30 nM selenium) with penicillin G and streptomycin and maintained for up to 8 days at 37°C in a 5% CO_2 incubator. Prior to cell plating, each coverslip was treated overnight at 37°C with 0.5 mg/ml poly-D-lysine in 0.15 M borate buffer (pH 8.4), rinsed with distilled H_2O, and then coated with 0.03 mg/ml laminin in Earle's balanced salt solution (Gibco BRL) for 4-5 hours at 37°C. Where indicated, cell were cultured in M199 supplemented with 15% fetal calf serum (Hyclone) instead of OTS.

Embryonic chick central epithelium explant cultures were prepared as described by Piatigorsky *et al.* (1972). E6 chick lenses were excised and placed in a 35-mm tissue culture dish containing M199 medium with the anterior epithelial cells closest to the dish. The posterior capsule was then punctured and the fiber mass was removed through the tear, leaving the epithelium intact. A scalpel was used to cut out a square of approximately 0.5 mm^2 of central epithelium, which was subsequently cultured in M199 medium

supplemented with 15% fetal calf serum, penicillin G and streptomycin for up to 6 days at 37°C with 5% CO_2.

To inhibit gap junction-mediated intercellular communication, 18β-glycyrrhetinic acid (βGA; Sigma) was added to the culture medium at the time of plating from a 100 mM stock in DMSO. Both dissociated cell-derived monolayers and central epithelium explants were fed every two days with fresh medium either with or without βGA. Dissociated cell-derived monolayers treated with ≥40 μM βGA were cultured in the presence of 0.25% bovine serum albumin (Sigma) because concentrations of βGA exceeding 10 μM were toxic to lens cells in its absence.

Scrape-loading/dye transfer assay for gap junctional intercellular communication. Dissociated-cell derived chick lens monolayer cultures grown on laminin-coated glass coverslips in either the absence or presence of βGA were transferred to a 35-mm tissue culture dish and their culture medium saved. The transferred coverslips were rinsed three times with Hank's balanced salt solution containing 1% bovine serum albumin and 1 mM $CaCl_2$ (HBC). The HBC was removed and 2.5 μl of Dulbecco's phosphate buffered saline (DPBS) containing 0.75% rhodamine dextran (Molecular Probes) with either 1% Lucifer Yellow (Sigma) or 1% biocytin (Molecular Probes) was directly applied to the center of the glass coverslip, after which a 27-gauge needle was used to create two longitudinal scratches through the cell monolayer. Cells were incubated in the dye mix for exactly one minute and then quickly rinsed three times with HBC. After removal of the last rinse, the coverslip was encircled with a glass cloning cylinder to which the saved culture medium was added and the cells incubated for five minutes (or, for Figure 2, 15 minutes) at room temperature to allow dye transfer. Saved instead of fresh medium was used because of the rapid reversibility of the βGA-induced block in gap junctional communication upon removal of the compound. The cells were then rinsed three times with PBS and immediately fixed for 30 minutes at room temperature with 2%

paraformaldehyde/DPBS, pH 7.5. LY and rhodamine dextran were subsequently examined by fluorescence microscopy (Leitz DMR) using (respectively) fluorescein and rhodamine filter sets. For biocytin visualization, fixed cells were permeabilized for 15 minutes with DPBS containing 0.1% Triton X-100, 0.2% bovine serum albumin, and 5% normal goat serum followed by incubation with avidin-FITC (Molecular Probes) for two hours at room temperature.

For embryonic chick epithelial explants, a modified scrape-loading/dye transfer assay was used because scratching the explant with a 27-gauge needle resulted in an unacceptable amount of tissue damage. A small punctate wound was created in the center of the explant using a micropipet containing either 1% Lucifer Yellow/0.75% rhodamine dextran in DPBS or 1% biocytin/0.75% rhodamine dextran in DPBS under the control of a micromanipulator. Immediately after the cells were loaded with the dye mixture, the micropipet was removed and transfer allowed to proceed for two minutes. The explants were maintained in their original culture medium (either with or without βGA) throughout the procedure. The explant was then carefully rinsed three times with DPBS and fixed for 30 minutes with 2% paraformaldehyde in DPBS (pH 7.5). The dyes were visualized by fluorescence microscopy as described above for dissociated cell-derived monolayers.

Indirect immunofluorescence. Cells grown on laminin-coated glass coverslips or as epithelial explants were fixed in 2% paraformaldehyde in DPBS (pH 7.5) for 30 minutes at room temperature and rinsed in DPBS for 30 minutes. For Cx43 staining only, cells were then post-fixed for 5 minutes with -20°C acetone followed by a 30 minute rinse with DPBS. All cells were subsequently permeabilized in DPBS containing 0.1%Triton X-100, 0.2% bovine serum albumin, and 5% normal goat serum (PTBN) for 30 minutes at room temperature followed by another rinse with DPBS for 30 minutes at room temperature. The primary antibody diluted in PTBN was applied to the fixed and permeabilized cells overnight at 4°C. After a 30 minute rinse with PTBN at room

temperature, the appropriate secondary antibody diluted in PTBN was added for 2 hours at room temperature. The cells were given a final 30 minute rinse in PTBN and then either mounted cell-side down on a microscope slide (in the case of coverslip-grown cells) or, for explants, topped with a coverslip using MOWIOL (Calbiochem) as a mounting medium.

The following primary antibodies were used in this study. Affinity-purified rabbit antibodies monospecific for Cx43 (AP7298) have previously been characterized (Musil *et al.*, 1990b). Rabbit antibodies directed against Cx45.6 or Cx56 were the kind gifts of Drs. Jean Jiang and Daniel Goodenough, Harvard Medical School (Jiang *et al.*, 1995). Dr. Goodenough also provided polyclonal antibodies against chicken occludin (Chen *et al.*, 1997) and a mouse monoclonal antibody (R40.76) to ZO-1 (Anderson *et al.*, 1988). The anti-chicken MP28 polyclonal serum (No. 6182) and its preimmune control were generously provided by Dr. Ross Johnson, University of Minnesota. This antiserum recognizes chick MP28 in whole lens lysates on Western blots and specifically stains differentiated fiber cells in frozen sections from E10 chick lens in a pattern indistinguishable from that obtained in duplicate sections with a monoclonal anti-MP28 antibody (Sas *et al.*, 1985). Rabbit anti-N-cadherin (C 3678) and anti-β-catenin antibodies (C 2206) were from Sigma. NCAM was detected with monoclonal antibody Mab 5E (Watanabe *et al.*, 1989).

[^{35}S]-methionine metabolic labeling and immunoprecipitation. Intact chick lenses dissected from E10 embryos, dissociated-cell derived monolayer cultures grown in 24-well laminin-coated tissue culture wells, or central epithelial explants were rinsed two times with Dulbecco's minimum essential media (DMEM) without methionine and labeled with [^{35}S]-methionine (EXPRE^{35}S^{35}S, New England Nuclear) in methionine-free DMEM at 37°C in either the absence or presence of the indicated concentration of βGA. Cultures grown in M199/OTS were labeled in the absence of serum; for all other cells, the

labeling medium was supplemented with 5% dialyzed fetal calf serum. After the labeling period, the lenses or cultures were rinsed three times with 4°C TD buffer and then once with ice-cold lysis buffer (5 mM Tris base, 5 mM EDTA, 5 mM EGTA, 10 mM iodoacetamide, 2 mM PMSF, pH 8.0). The buffer was removed and the samples lysed by passage through an 18-gauge needle in 4°C lysis buffer supplemented with 0.6% SDS, 250 μg/ml soybean trypsin inhibitor, and 200 μM leupeptin. The lysates were then boiled for three minutes, followed by repeated passage through a 25-gauge needle to shear DNA released from lysed nuclei. Equal aliquots were removed for analysis of total cell lysates and the remainder diluted with four volumes of immunoprecipitation buffer prior to immunoprecipitation with affinity-purified anti-Cx43 polyclonal antibodies (AP7298) as described in (Musil *et al.*, 1990a). The immunoprecipitates were analyzed on 10% SDS-polyacrylamide gels and quantitated on a Phosphorimager utilizing IPLab Gel software.

4. Results

Differentiation of primary embryonic chick lens cells in dissociated-cell derived monolayer cultures. Although epithelial cells from the lens of many vertebrate species will take on fiber-like characteristics when cultured, secondary fiber formation is most completely recapitulated by cells isolated from embryonic chick (Menko *et al.*, 1984; Menko *et al.*, 1987; TenBroek *et al.*, 1994). We began our study by reproducing the culture conditions of Menko *et al.* (1984). E10 chick lens were dissociated into single cells after removal of any nonlenticular contaminants and plated into tissue culture wells in M199 medium containing 15% fetal calf serum (M199/FCS). As expected, these cells proliferated to form a flattened epithelial monolayer. Beginning ~3-4 days of culture, discrete areas within these sheets differentiated into multilayered clusters of enlarged cells termed lentoids which increased in number and size over the remaining 4-5 days of the culture period (Figure 1). Ultrastructural analysis by Menko *et al.* (1987) has revealed that the cells contained in such lentoids have acquired many of the defining characteristics of fiber cells in the intact embryonic chick lens, including increased cell volume, loss of intracellular organelles, and extensive formation of gap junctions. Thin-section electron micrographs of the cells closest to the substrate are indistinguishable from those obtained from the differentiated region of the embryonic lens, whereas the more peripheral cells closely resemble the elongating cells of the equatorial region (Menko *et al.*, 1987). Lentoids are also biochemically differentiated in that they accumulate high levels of δ-crystallin and of MP28 (also known as MIP) (Figure 1H), an integral plasma membrane protein of as yet unclear function which is expressed *in vivo* only by differentiated primary and secondary fiber cells (Sas *et al.*, 1985; Yancey *et al.*, 1988).

We next defined the requirements for lentoid formation in dissociated cell-derived monolayers. We found that cells cultured in M199/OTS (M199 medium supplemented with 25 μg/ml ovotransferrin and 30 nM selenium) in the absence of serum proliferated

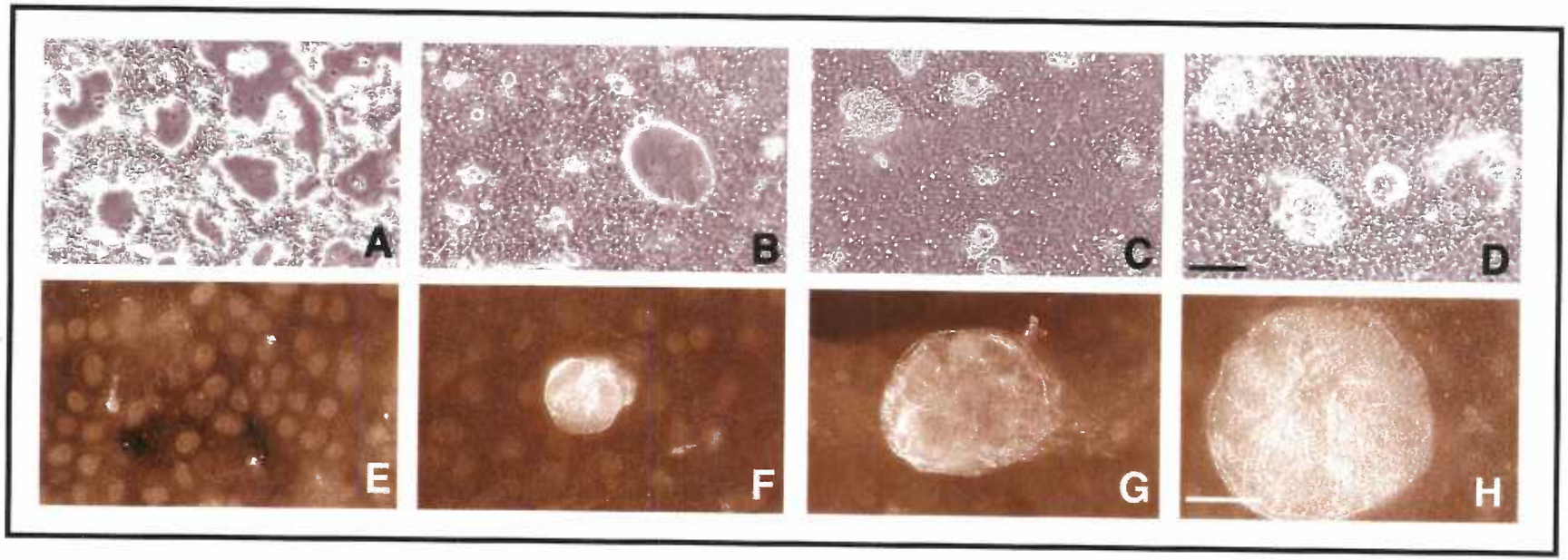

Figure 1. *Differentiation of dissociated cell-derived chick lens cultures.* Primary cultures of embryonic chick lens cells were prepared from dissociated E10 lenses as described under Materials and Methods and plated in M199 with OTS (A-C, E-G) or M199 with 15% fetal calf serum (D, H). Phase-contrast micrographs (A-D) and immunofluorescence staining for the fiber cell-specific marker MP28 (E-H) of cells after 1 day (A, E), 3 days (B, F), or 8 days (C, D, G, H) in culture. The nuclear staining pattern evident in E was also obtained with preimmune serum (not shown); in contrast, staining of lentoid plasma membranes was specific to anti-MP28 immune serum. Bar: 100 μm (A-D); 20 μm (E-H).

and differentiated into MP28-positive lentoids at a rate, and with a frequency, equivalent to serum-supplemented cells although the lentoids tended to be somewhat smaller than in M199/FCS (Figures 1A-1C and 1E-1G; compare 1C with 1D). Serum-independent differentiation was observed for cells plated onto laminin, collagen, uncoated tissue culture plastic, or Lab-tek glass chamber slides (see Figure 5B), although under the latter two conditions the efficiency of initial cell attachment was reduced. The fact that lentoid production does not require the addition of exogenous and potentially non-physiological growth factors to either the medium (in the form of fetal calf serum) or to the substrate (as a contaminant of extracellular matrix preparations) makes it likely that differentiation of these cultures replicates the *in vivo* process of secondary fiber formation as closely as possible. Unless otherwise noted, all experiments utilizing dissociated cell-derived cultures were conducted with cells grown in the absence of serum on coverslips coated with poly-D-lysine and laminin to ensure uniform plating density.

Connexin expression in dissociated-cell derived lens cultures and the effect of βGA on gap junction-mediated intercellular communication and Cx43 processing. Immunofluorescence microscopy using antibodies specific for either Cx43, Cx45.6, or Cx56 demonstrates that the *in vivo* distribution of these connexins was recapitulated in our dissociated cell derived cultures (see Figures 6A-6C). As in the lenses of all avian and mammalian species examined to date, Cx43 was present at cell-cell interfaces throughout the epithelial monolayer during the entire culture period. In contrast, Cx45.6 and Cx56 were most concentrated in lentoids, consistent with their accumulation in fiber cells *in vivo*. These distributions were not, however, mutually exclusive. Epithelial monolayer cells also displayed readily detectable staining for Cx45.6 and Cx56, in keeping with the moderate expression of these connexins throughout the anterior epithelium of embryonic chick lenses *in vivo* (Jiang *et al.*, 1995). Furthermore, many cells within lentoids also stained brightly with the Cx43 antibody. This is likely due to the fact

that the cells within a single lentoid mature asynchronously such that the cells nearest the surface of the structure are the least differentiated and are therefore the most similar to the elongating epithelial cells of the bow region *in vivo* (Menko *et al.*, 1984) in which Cx43 expression is even greater than in the central epithelium (Musil *et al.*, 1990a). Comparable connexin staining patterns and intensities were obtained with cultures maintained in the presence of fetal calf serum (data not shown).

As in intact lenses, the gap junctions expressed by cultured embryonic chick lens cells were functional (Figures 2A-2B). Gap junction-mediated intercellular coupling was assessed using the scrape loading/dye transfer assay, in which a mixture of rhodamine dextran (M_r 10 kDa) and the membrane impermeant, low-molecular-weight fluorescent dye Lucifer yellow (LY) is introduced into cultured cells by scraping the monolayer with a 27-gauge needle (el-Fouly *et al.*, 1987). The bulky dextran remains confined to the wounded cells, whereas LY is able to diffuse via gap junctions to adjacent cells distal to the scratch. As evaluated by the extent of transfer of LY to rhodamine dextran-negative cells, the monolayer lens cells were moderately well-coupled throughout the culture period (Figure 2 shows representative results from cells cultured for 3 days; other days were comparable). Minor local variation in the number of cells receiving LY could be due to uncoupling of cells during mitosis in these proliferating cultures (Stein *et al.*, 1992). In keeping with previous reports (Crow *et al.*, 1994), gap junction-mediated intercellular communication between monolayer epithelial cells was also readily detectable if LY was introduced into a single cell by microinjection (data not shown). The scrape-loading/dye transfer assay was routinely used since it allows simultaneous monitoring of gap junctional intercellular communication within a larger population of cells.

Treatment of dissociated cell-derived chick lens cultures with 10 μM βGA in M199/OTS almost completely abolished the transfer of LY between epithelial monolayer cells, even if transfer of dye was allowed to proceed for 15 minutes instead of the standard 5 minutes (Figure 2). As previously reported for fibroblast cell lines (Davidson *et al.*,

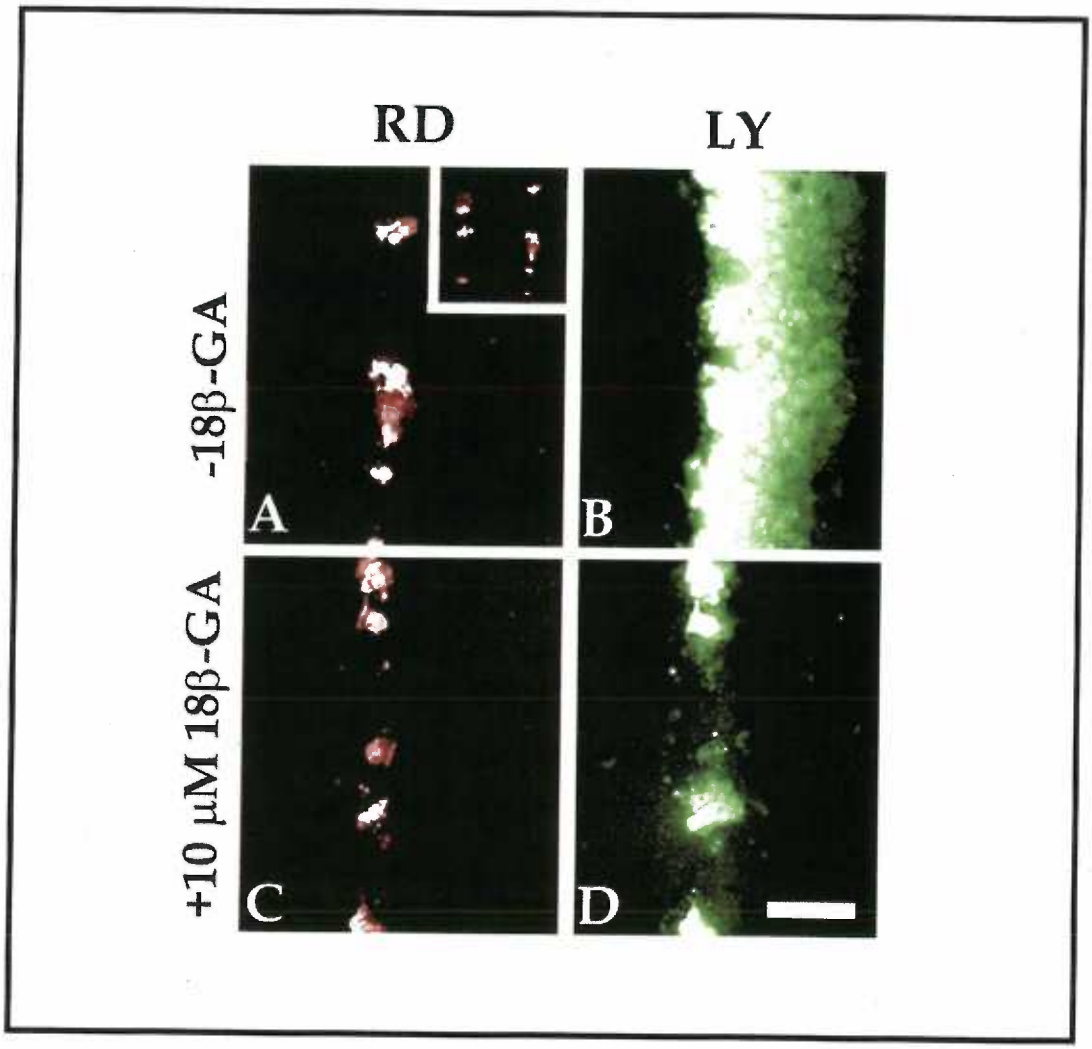

Figure 2. *βGA blocks gap junction-mediated intercellular transfer of Lucifer yellow in dissociated cell-derived chick lens monolayer cultures.* Gap junctional intercellular communication was assessed in dissociated cell-derived chick lens monolayers cultured in M199/OTS in either the absence (A and B) or continuous presence (C and D) of 10 μM 18β-GA using a mixture of rhodamine dextran and Lucifer yellow as described under Material and Methods. The M_r 10 kDa rhodamine dextran (A and C) remains confined to the cells into which dye was directly introduced during the scrape/loading process whereas Lucifer yellow (B and D) can be transferred to additional cells via open gap junctional channels during the 15-min transfer period. Representative results from day 3 of culture are shown. Each panel depicts a portion of the right half of the scrape/load wound (both sides of which are shown in the inset in A) which has been magnified to enhance the resolution of the individual cells. Bar: 100 μm.

1986; Guan *et al.*, 1996), the effect of βGA on lens cell gap junction-mediated intercellular communication was dose-dependent (~50% maximal effect at 5 μM βGA in M199/OTS), complete within 30 minutes, and reversible within one hour of drug removal in the presence of bovine serum albumin or serum (data not shown). Daily assessment of LY transfer in lens cells cultured in the continuous presence of 10 μM βGA (with new βGA added only when the cell medium was changed every 48 hours) revealed efficient inhibition of LY transfer between days 2 and 8 of culture relative to untreated control cells of the same age in 7/7 experiments. The persistence of the communication block throughout the 48-hour period between medium changes demonstrated that βGA was not degraded or otherwise inactivated during this time. Although assessment of dye transfer on the first day of culture was less accurate due to the subconfluent state of the monolayer, βGA also substantially inhibited the number of rhodamine dextran-negative cells receiving LY at this time (data not shown). The fact that dissociated cell-derived chick lens cultures coexpress Cx43, Cx45.6, and Cx56 throughout the culture period indicates that βGA inhibits the function of not only Cx43 but also of the fiber-type connexins. This is an important point because gap junctional intercellular communication in vertebrate lens fibers and in Cx56-expressing transfected tissue culture cells has been reported to be insensitive to certain other treatments (high CO_2; heptanol) that effectively block coupling mediated by other types of connexins (Miller and Goodenough, 1986; Rup *et al.*, 1993).

The limited LY transfer and the small size of the monolayer epithelial cells made it difficult to appreciate the extent to which βGA inhibited gap junctional intercellular communication. We therefore repeated our analysis using biocytin as a tracer of gap junctional coupling (Figure 3). Due in part to its lower molecular weight, ionic charge, and/or more sensitive method of detection (fluorochrome-labeled avidin instead of direct visualization of dye), biocytin has been shown in several cell types to transfer to more cells than simultaneously loaded LY (Teranishi and Negishi, 1994; Umino *et al.*, 1994). We found that this was also the case in untreated lens cells. Despite this increased

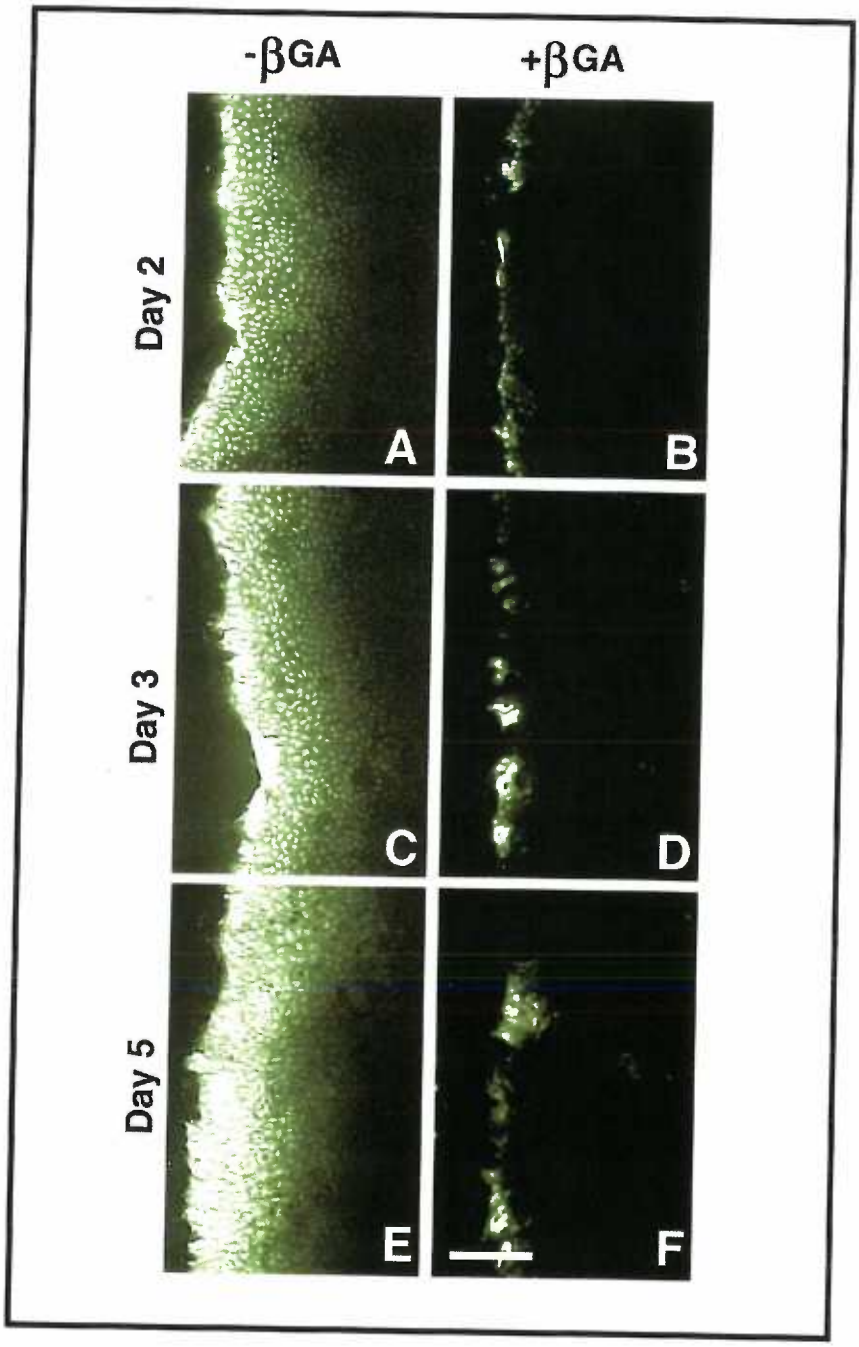

Figure 3. *βGA inhibits gap junction-mediated intercellular transfer of biocytin in dissociated cell-derived chick lens monolayer cultures.* Gap junctional intercellular communication was assessed in dissociated cell-derived chick lens monolayers cultured for up to 8 days in M199/OTS in either the absence (A, C, E) or continuous presence (B, D, F) of 10 μM 18β-GA using a mixture of rhodamine dextran and biocytin as described under Material and Methods (5 min transfer period). Only the FITC-avidin staining for the biocytin is presented; rhodamine dextran (not shown) was confined to a single row of cells immediately bordering the wound (see Figure 2). Representative results from day 2 (A, B), day 3 (C, D) and day 5 (E, F) of culture are presented. Only a portion of the right half of the wound is shown. Bar: 100 μm.

sensitivity, intercellular transfer of biocytin was profoundly reduced by βGA throughout the culture period in 4/4 experiments (Figures 3B, 3D, and 3F). Close inspection of scrape-loading/dye transfer assays did, however, reveal instances in which biocytin was detectable in rhodamine dextran-negative cells in the immediate vicinity of the wound. βGA therefore does not appear to totally abolish all gap junction-mediated intercellular communication in lens cells, in keeping with studies in which an even more sensitive assay of gap junctional permeability (electrical coupling) detected residual intercellular communication in fibroblasts treated with other glycyrrhetinic acid derivatives (Goldberg *et al.*, 1996; Martin *et al*, 1991; see Discussion).

In gap junctional communication-competent cell types including embryonic chick lens epithelial cells, Cx43 is synthesized as a ~42-kDa species that undergoes phosphorylation to two slower-migrating forms, referred to as Cx43-P_1 and P_2, after transport to the cell surface (Musil *et al.*, 1990a; Musil *et al.*, 1990b). It is the P_2 species that is most closely correlated with functional gap junctional plaque formation (Musil and Goodenough, 1991). Guan *et al.* (1996) have reported that when intercellular communication is blocked in WB-F344 rat liver epithelial cells with 40 μM βGA, Cx43 is no longer detectable in the P_2 form and that this species reappears only when cell coupling is restored by removal of the drug. We find that 40 μM βGA similarly abolished phosphorylation of newly synthesized Cx43 to the P_2 form in whole embryonic chick lenses metabolically labeled with [^{35}S]-methionine (Figure 4A, compare lanes 1 and 2). Although the P_1 and P_2 species were not well resolved, 100 μM βGA greatly reduced the amount of phosphorylated (>42-kDa) Cx43 recovered from dissociated cell-derived cultures (Figure 4A, compare lanes 3 and 4). Thus, biochemical as well as functional assays indicate that ≥40 μM βGA affects Cx43 in the lens in a manner similar to that described in other cell types. However, the phosphorylation pattern of newly synthesized Cx43 was unaltered by lower levels of βGA (10 μM) that nonetheless maximally inhibited gap junction-mediated intercellular communication (Figure 4A; compare lanes 5 and 6).

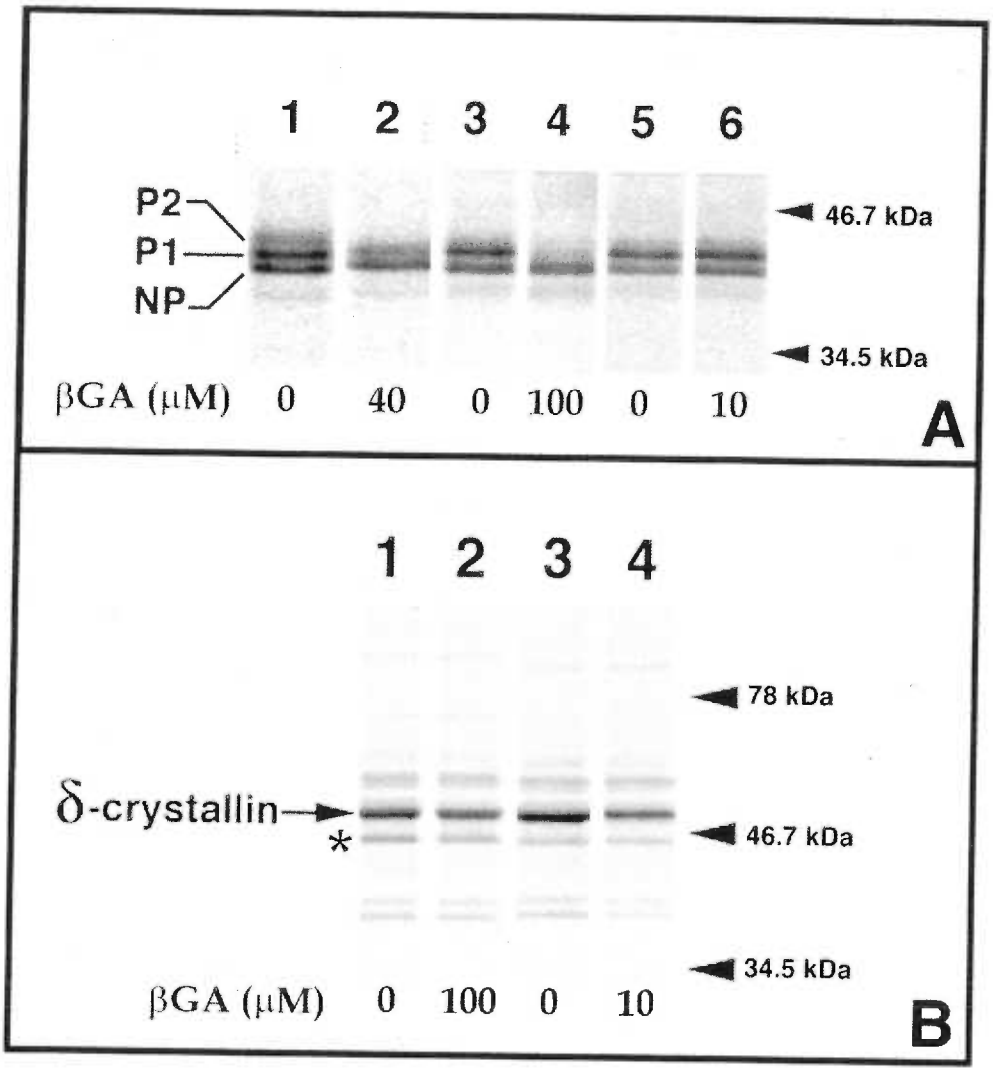

Figure 4. *Effect of βGA on metabolic labeling of connexin43 and δ-crystallin in embryonic chick lenses and dissociated cell-derived cultures.* (A) Disssociated cell-derived chick lens monolayer cultures were propagated in M199/OTS for 8 days with 0 (lanes 3 and 5), 100 μM (lane 4), or 10 μM (lane 6) 18β-GA and then metabolically labeled for 2 hours with [^{35}S]-methionine (0.13 mCi/ml) in the presence of the same concentration of βGA. For comparison, freshly isolated intact E10 chick lenses were labeled with [^{35}S]-methionine for 2 hours in either the absence (lane 1) or presence (lane 2) of 40 μM 18β-GA. The labeled cells were solubilized in SDS and the resulting lysates were immunoprecipitated with affinity-purified rabbit anti-Cx43 antibodies as described under Materials and Methods. The nonphosphorylated (NP) and two phosphorylated (P_1 and P_2) forms of Cx43 were visualized after SDS-PAGE using a PhosphorImager. B shows equal amounts of [^{35}S]-methionine-labeled total cell lysates (2-h labeling period) from dissociated cell-derived chick lens monolayers cultured for 8 days with 0 (lanes 1 and 3), 100 μM (lane 2), or 10 μM (lane 4) 18β-GA. Coomassie blue staining of whole lens lystates confirmed the identity of the d-crystallin band (not shown). When normalized to actin (indicated by the asterisk), synthesis of δ-crystallin in cultures treated with 100 μM βGA was 1X, and with 10 μM βGA 1.3X, of their respective untreated control. In both A and B, cells were cultured with 100 μM 18 β-GA in the presence of 0.25% bovine serum albumin since concentrations of βGA > 10 μM were toxic to lens cells in its absence. Molecular weight markers are indicated on the right.

Reduced phosphorylation of Cx43 is therefore not obligatorily linked to inhibition of gap junctional permeability and may instead reflect a separate effect of higher concentrations of βGA.

Differentiation of dissociated cell-derived chick lens cultures in the presence of βGA. The effect of chronic inhibition of gap junctional intercellular communication was evaluated in dissociated embryonic chick lens cells plated and cultured in the continuous presence of 10 μM βGA in M199/OTS (Figure 5). βGA did not visibly affect the initial attachment or spreading of lens cells onto the substratum nor significantly change the size or the number of cells per well on days 1-3 of culture (data not shown; inefficient trypsinization of lentoids precluded accurate cell counts at later developmental stages). Most importantly, despite the ongoing block of gap junction-mediated intercellular communication, βGA-treated cultures continued to differentiate into MP28-expressing lentoids at a rate equal to untreated cells throughout the 8-day culture period (Figure 5 shows data from late time point). Although the onset and extent of lentoid formation showed slight culture-to-culture variability, in 21/21 experiments the course of lentoid formation was not noticeably affected by the presence of βGA. The [^{35}S]-methionine-labeled (Figure 4B, lane 4) and Coomassie blue-stained (not shown) profiles of total proteins synthesized by treated cultures were very similar to those of control cells and included high levels of δ-crystallin, a major component of differentiated embryonic chick fiber cells (Piatigorsky, 1981). Secondary fiber differentiation also proceeded normally in cells cultured with βGA in the presence of 0.25% bovine serum albumin or 15% fetal calf serum, for which a ten-fold higher concentration of βGA was required to achieve a comparable inhibition of gap junctional communication due to binding of βGA to albumin (data not shown). As previously shown for human lens epithelial cells (Arita *et al.*, 1990), embryonic chick lens cells do not spread efficiently when plated onto less haptotactic substrates and instead show accelerated differentiation into lentoid bodies.

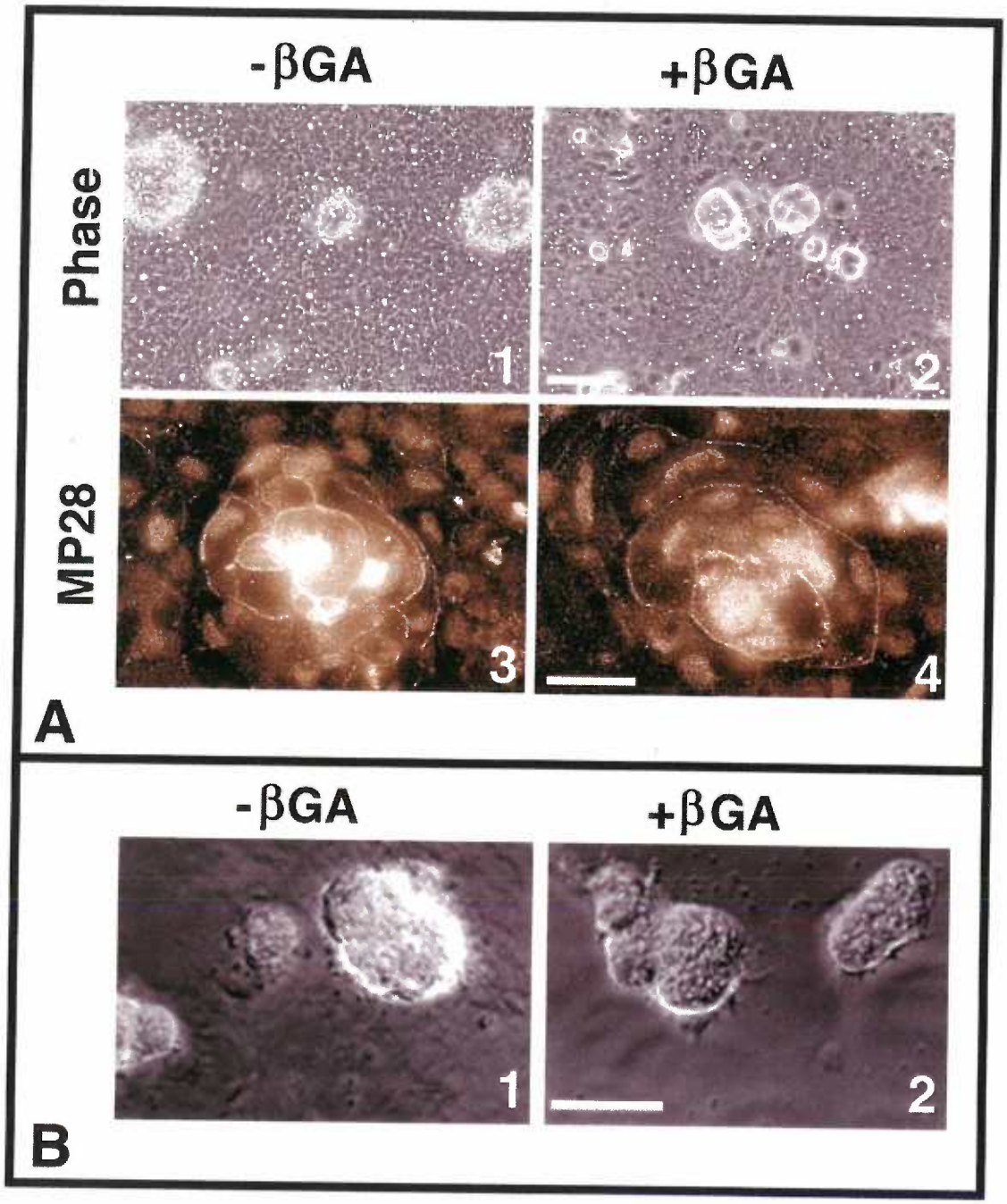

Figure 5. *Inhibition of gap junctional communication with βGA does not affect the differentiation of dissociated cell-derived chick lens monolayer cultuers.* Primary embryonic chick lens cells were cultured in M199/OTS for 8 days either on poly-D-lysine/laminin-coated coverslips (A, 1-4) or on uncoated glass Labtek chamber slides (B, 1 and 2) in either the absence (A1, A3, B1) or continuous presence (A2, A4, B2) of 10 μM 18β-GA. (A, 1 and 2, and B) Phase-contrast micrographs. (A, 3 and 4) Lentoids were visualized with a polyclonal antiserum against chicken MP28 followed by rhodamine-conjugated goat anti-rabbit IgG. Bar: 100 μm (A1, A2); 20 μm (A3, A4); 100 μm (B1, B2).

Even under these extreme conditions, βGA did not inhibit lentoid formation (Figure 5B).

With regard to the expression of connexins, we found that Cx43 in βGA-treated cultures was still localized to what appears at the level of resolution of immunofluorescence light microscopy to be cell-cell interfaces (Figure 6D). Likewise, the distribution of the fiber-type connexins was not notably altered by exposure to βGA and remained detectable throughout the monolayer, with the highest concentration in the lentoids (Figures 6E and 6F). A similar distribution of connexins was observed in cells cultured with 100 μM βGA in the presence of 0.25% bovine serum albumin. In untreated cultures, the staining pattern for each of the three connexins at cell-cell interfaces is often more continuous than (as is typical in most other cell types) concentrated in discrete puncta (especially prominent in Figure 6C). It is perhaps for this reason that we do not observe by immunofluorescence microscopy an apparent disassembly of discrete gap junctional plaques in the presence of βGA as has been reported by Guan *et al.* (1996) in WB-F344 rat liver epithelial cells. Alternatively, the fact that Goldberg *et al.* (1996) could detect changes in gap junction morphology by freeze-fracture electron microscopy but not by light microscopy after exposure of C6 glioma cells to uncoupling levels of the related compound 18a-carbenoxolone suggests that the extent of morphological perturbation of gap junction structure induced by glycyrrhetinic acid derivatives may vary between cell types.

Effect of Inhibition of Gap Junction-Mediated Intercellular Communication on Other Cell-Cell Junctions . As postulated by the "precedence hypothesis" (Edelman, 1988)., several studies have indicated that cadherin-mediated intercellular adhesion is necessary not only for formation of adherens junctions but also for the assembly of other cell-cell specializations including gap junctions, tight junctions, and desmosomes in various cell types (Gumbiner *et al.*, 1988; Musil *et al.*, 1990b). Given that function blocking anti-N-cadherin antibodies have been reported to inhibit the establishment of gap

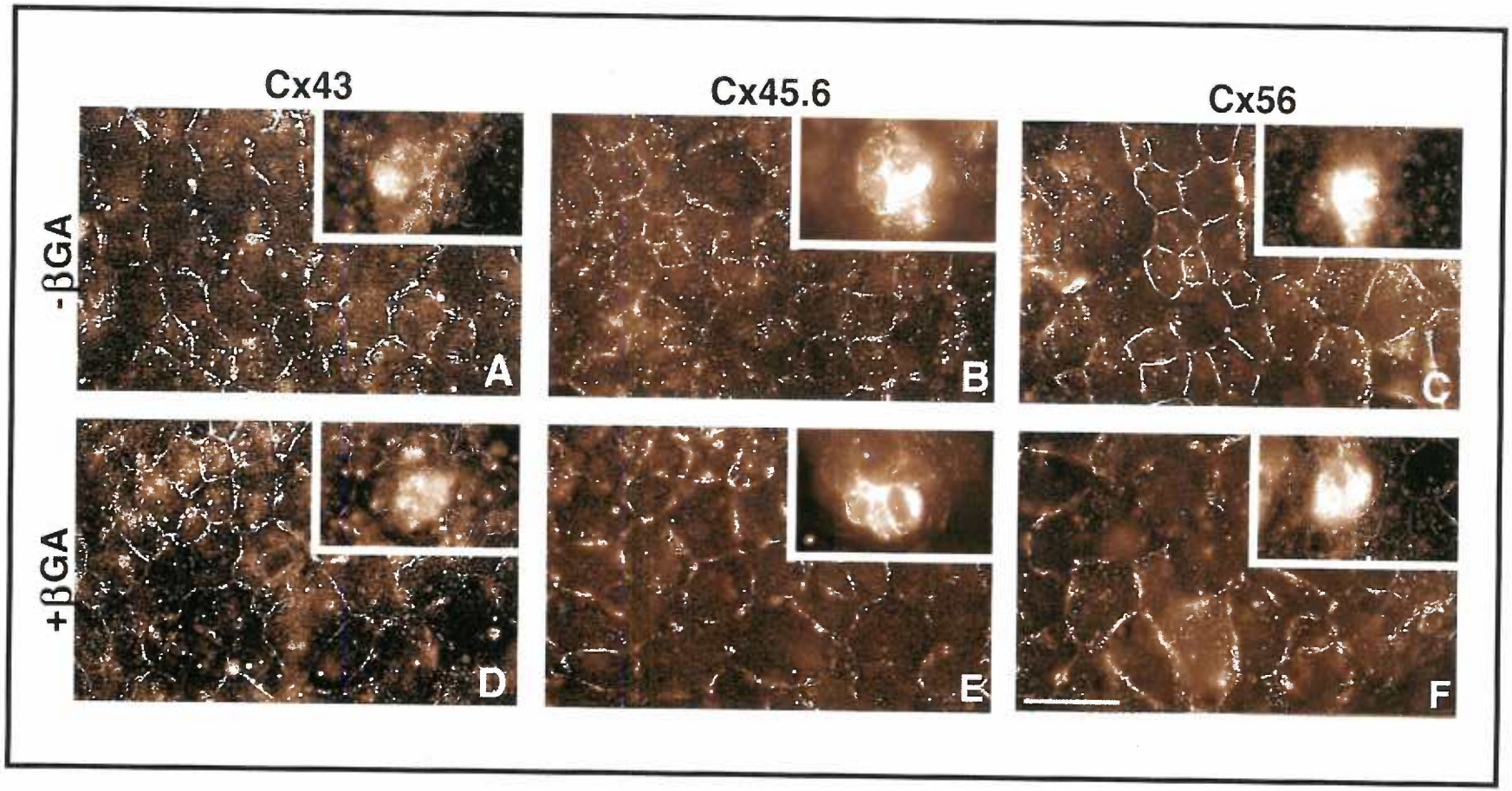

Figure 6. *Immunofluorescence localization of connexins in dissociatedcell-derived chick lens monolayer cultures is not affected by βGA.* Cells cultured in M199/OTS for 8 days in either the absence (A, B, C) or continuous presence (D, E, F) of levels of 18β-GA (10 μM) that inhibit gap junction-mediated intercellular dye transfer were stained with antibodies specific for Cx43 (A, D), Cx45.6, (B,E), or Cx56 (C,F). Bar: 20 μm. As visualized by indirect immunofluorescence microscopy, all three connexins were concentrated at cell-cell interfaces throughout the monolayer of polygonal epithelial cells and were also detectable in differentiating multilayered lentoids (insets), in which the staining of the fiber connexins is so intense that it no longer outlines individual cells (B, C, E, and F). No staining was deteted in cultures incubated with preimmune or normal rabbit serum (data not shown).

junctional intercellular communication between cultured embryonic chick lentoids (Frenzel and Johnson, 1996), this is likely to be true in the lens as well. Evidence is accumulating that cell-cell adhesion may be reciprocally influenced by gap junction-mediated intercellular communication, in that inhibition of gap junctions with either anti-connexin antibodies or via expression of a dominant negative connexin mutant leads to disruption of cell-cell contact in (respectively) tissue culture cells (Meyer *et al.*, 1992) and early *Xenopus* embryos (Paul *et al.*, 1995). We therefore used βGA to investigate the role of gap junctional intercellular communication in the formation of other lens cell-cell junctions.

Adherens junctions. Studies by Geiger and colleagues have established that chick lens epithelial cells both *in vivo* and in culture assemble N-cadherin (A-CAM) into adherens junctions and that this activity is required for close cell-cell apposition (Duband *et al.*, 1988; Volk and Geiger, 1986). As assessed by immunofluorescence microscopy using an antibody raised against chick N-cadherin, βGA treatment has no discernable effect on the expression or distribution of this molecule at cell-cell interfaces (Figures 7A and 7C). Strong cell-cell adhesion is thought to require cadherins to be linked to the actin-based cytoskeleton via catenins, an interaction commonly assessed by resistance to solubilization with the nonionic detergent NP-40 (Hirano *et al.*, 1987; Kreft *et al.*, 1997). In both control and βGA-treated lens cells, the majority of immunofluorescently detectable N-cadherin and β-catenin was not extractable with 5% NP-40 (Figure 7). Control experiments in which the transport blocker brefeldin A was used to accumulate intracellular (and therefore NP-40 soluble) pools of cadherin confirmed that NP-40 efficiently extracted N-cadherin not associated with the cytoskeleton (data not shown). Taken together with the observation that βGA-treated lens cells maintain apparently normal close appositions, we conclude that inhibition of gap junction-mediated intercellular communication does not deleteriously affect cadherin localization or function. As assessed by rhodamine-phalloidin visualization of stress fibers and anti-

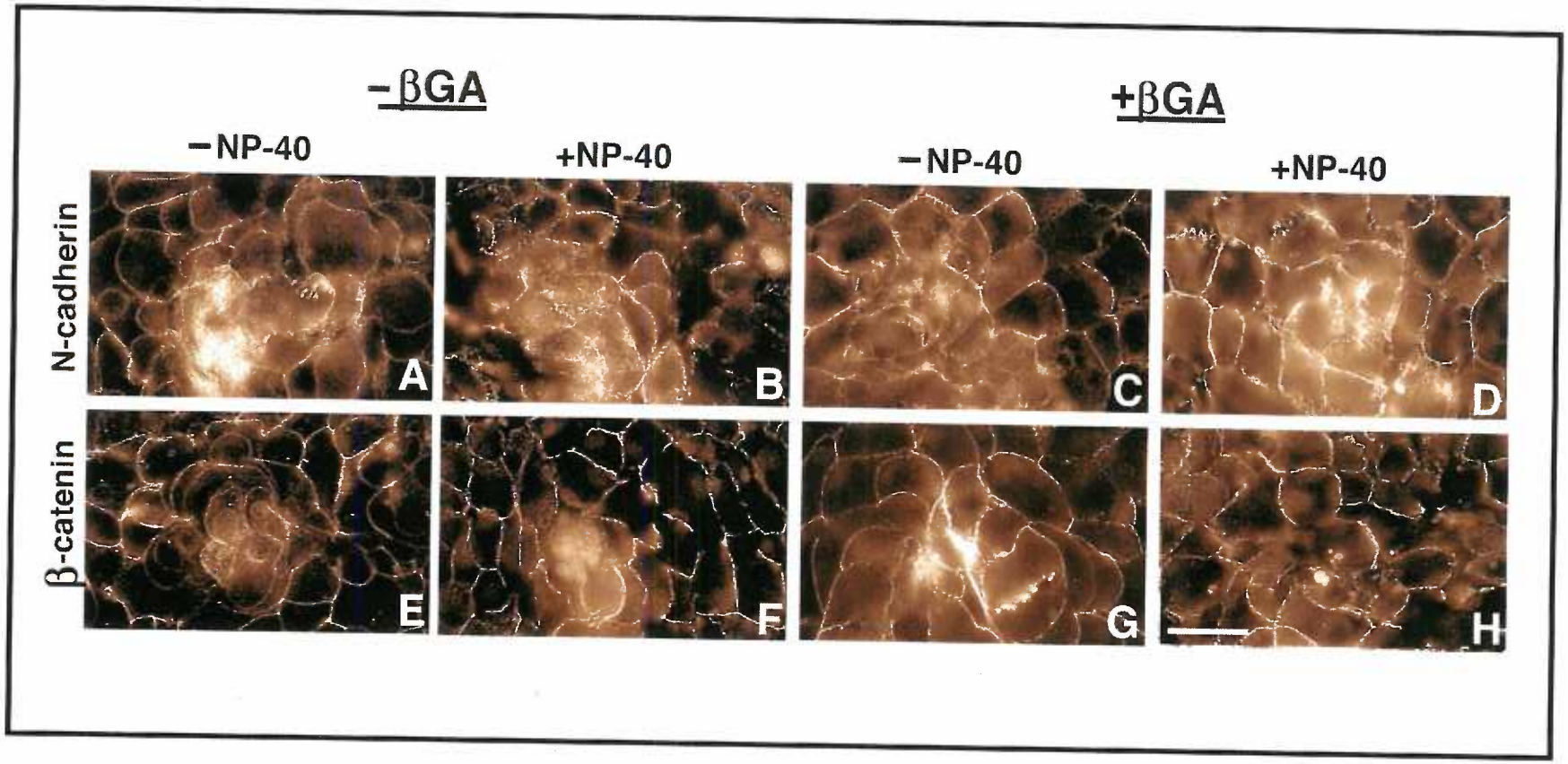

Figure 7. *Expression and detergent extractability of adherens junction proteins in chick lens cell monolayers +/- βGA.* Dissociated cell-derived chick lens monolayer cultures were propagated in M199/OTS for 8 days in either the absence (A, B, E, F) or presence (C, D, G, H) of 10 μM 18β-GA. The cultures were then incubated for 5 minutes at 4°C in calsium-containing PBS either with (B, F, D, H) or without (A, E, C, G) 5% NP-40 detergent. After four gentle rinses with Ca^{2+} PBS, the cells were fixed and stained for either N-cadherin (A-D) or β-catenin (E-H) followed by rhodamine-conjugated goat anti-rabbit IgG. Bar: 20 μM.

vinculin staining, βGA also did not noticeably affect actin-dependent cell-substrate interactions (data not shown).

Tight junctions. Tight junctions have been identified by freeze-fracture electron microscopy in the chick lens (Goodenough *et al.*, 1980). These structures also appear to be assembled by dissociated cell-derived chick lens cultures as assessed by the immunofluorescence localization of the tight junction-specific integral plasma membrane protein occludin and the peripheral membrane protein ZO-1 (Figures 8A and 8C). Both proteins were detected in the "chicken wire" cell interface staining pattern characteristic of tight junctions in epithelial monolayers (Wong and Gumbiner, 1997). Neither the distribution nor amount of these proteins was detectably altered in βGA-treated cells (Figures 8B and 8D). The apparent lack of effect of βGA on lens tight junctions is in keeping with a recent study by (De Sousa *et al.*, 1997) in which epithelial integrity was maintained in Cx43-null early mouse embryos despite their severely reduced capacity to mediate intercellular dye transfer.

NCAM. In addition to cadherins, lens epithelial and cortical fiber cells express the Ca^{2+}-independent cell-cell adhesion molecule NCAM. Anti-NCAM antibodies have been reported to inhibit the formation of fiber-type gap junctions in embryonic chick lens explants (Watanabe *et al.*, 1989), suggesting that there may be a functional relationship between NCAM and gap junctions. However, βGA inhibition of gap junctional intercellular communication had no apparent effect on NCAM expression or localization as assessed by immunofluorescence (Figure 8E and 8F).

Differentiation of central epithelial explants in the presence or absence of βGA. Studies in rodent lenses have demonstrated regional differences in the inherent capacity of cells derived from different areas of the lens to develop into fibers, with the central epithelium being the most dependent on exogenous growth factors for differentiation (Richardson *et al.*, 1992). The finding that our dissociated cell-derived cultures form

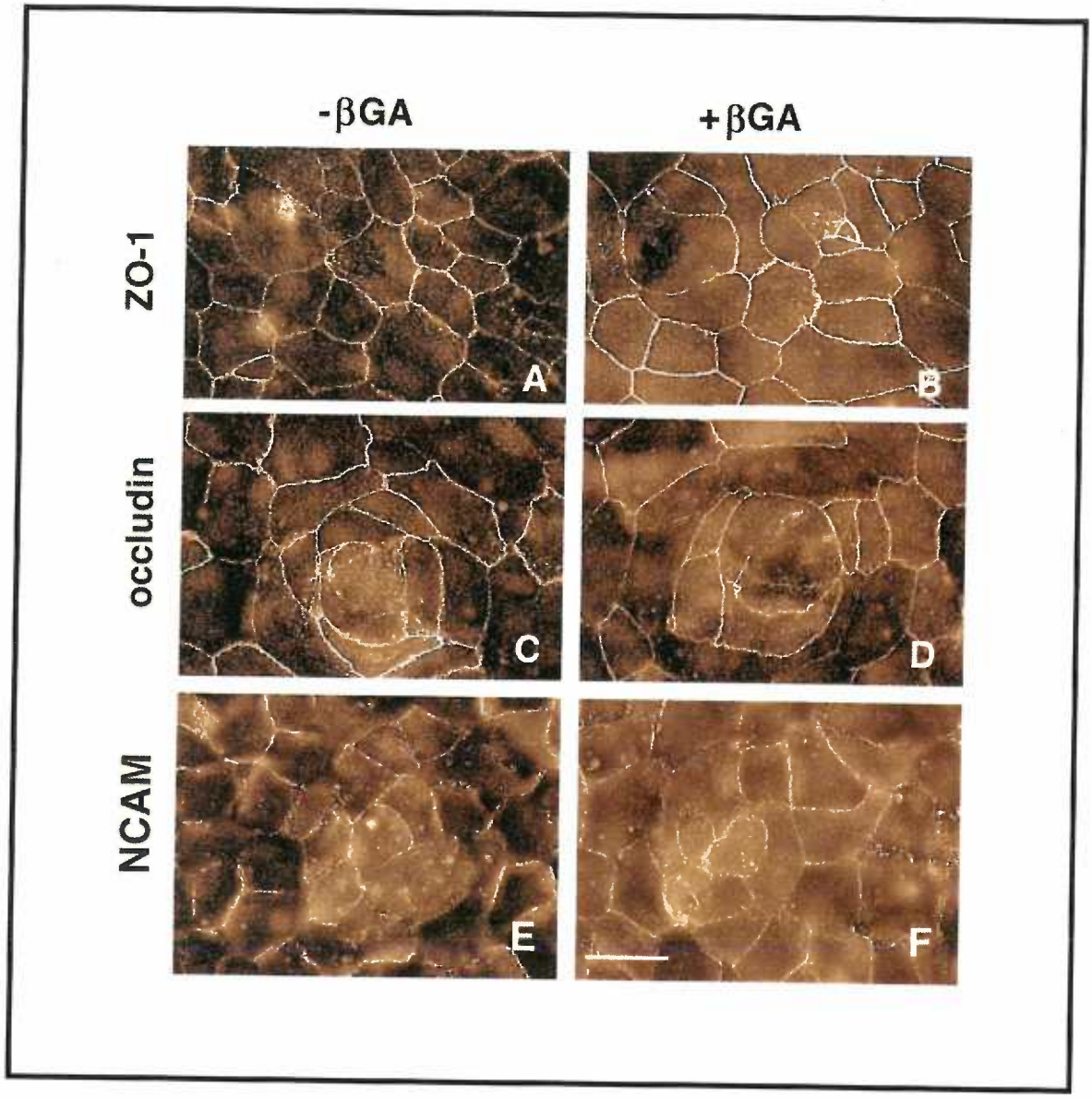

Figure 8. *Localization of tight junction components and NCAM in cultured lens cells is not altered by inhibition of gap junctions.* ZO-1 (A, B), occludin (C, D), and NCAM (E, F) were visualized by indiret immunofluorescence microscopy in chick lens cells cultured for 8 days in the absence (A, C, E) or continuous presence (B, D, F) of 10 μM 18β-GA. Bar: 20 μm.

lentoids even in the absence of added growth factors suggests that many of the cells may have originated from the more peripheral (bow) regions of the lens. It is therefore possible that a requirement for gap junction-mediated intercellular communication, like that for exogenous growth factors, might be greater for differentiation of central epithelial cells than for more peripheral populations and that such a distinction might not be detectable in the heterogenous mixture of cells cultured from dissociated lenses. To address this issue, we prepared lens explants in which the intact central epithelial monolayer is cultured capsule-side down after manual extraction of the fiber cell mass (Philpott and Coulombre, 1968; Piatigorsky *et al.*, 1973; Watanabe *et al.*, 1989). In addition to removing the peripheral epithelial cells, this well-established system (unlike cultures derived from dissociated cells) preserves cell-cell and cell-extracellular matrix interactions established *in vivo*, either of which could conceivably influence the differentiation process.

As expected from the literature (Piatigorsky *et al.*, 1973), central epithelial explants were viable but remained as an undifferentiated, flat epithelial sheet when maintained in the absence of serum or another source of exogenous growth factors. If cultured for more than three days in the presence of 15% fetal calf serum, however, the cells, especially those at the periphery of the explant, became elongated. Although this thickening made it difficult to resolve individual cells by conventional light microscopy, it is clear that the elongated cells expressed high levels of the fiber cell-specific marker MP28 (Figures 9B and 9E). Moreover, as reported by Piatigorsky (Piatigorsky *et al.*, 1973) and others, such explants increased their synthesis of δ-crystallin, another protein whose expression is up-regulated during fiber formation (Figure 11). As assessed by a modified scrape-loading/dye transfer assay, the cells in explants cultured for one day were moderately well coupled by gap junctions (Figures 10A, panels 1-3 and 10B, panels 1-3). Treatment of explants with 100 μM βGA in the presence of 15% fetal calf serum reduced the amount of intercellular transfer of Lucifer yellow (Figure 10A, panels 4-6) or biocytin (Figure 10B, panels 4-6) to an extent comparable to that observed in βGA-treated

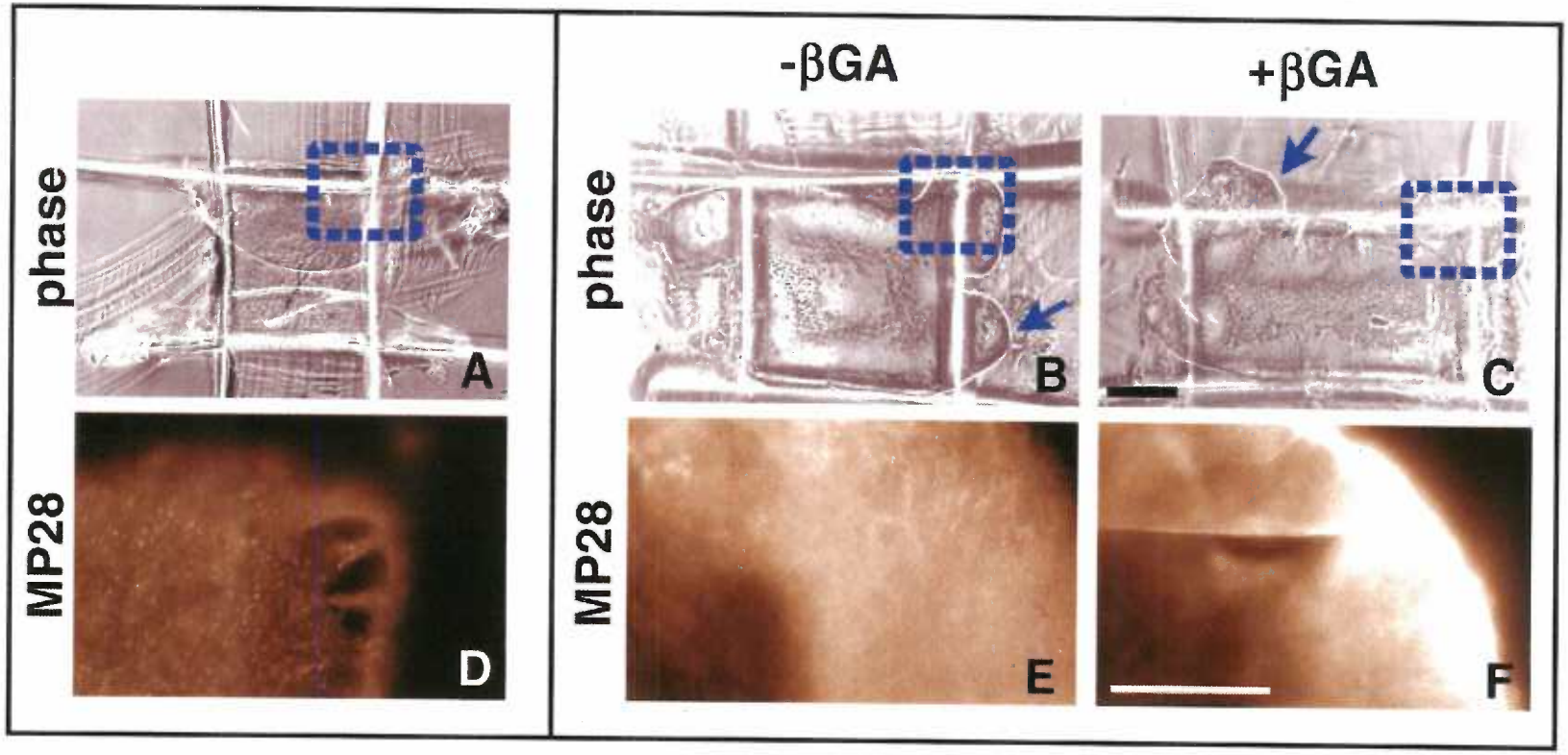

Figure 9. *Inhibition of gap junctional communication with βGA does not affect the differentiation of embryonic chick lens central epithelium explants.* Explants were prepared from E6 lenses as described under Materials and Methods. Immediately after dissection, the explants consisted of a monolayer of flat epithelial cells completely contained within the central square of the grid pattern caused by gouging of the tissue culture plastic during explant preparation (A). When cultured in M199/15% fetal calf serum for 6 days in either the absence (B and E) or the continuous presence (C and F) of gap junction-blocking levels of 18β-GA (100 μM; see Figure 10), these cells differentiated into elongated, fiber-like cells that extend over the original boundaries of the explant (most evident in the region indicated by the arrow in B and C). These peripheral regions expresed very high levels of the fiber-specific marker MP28 as assessed by immuonfluorescence (E and F); the much lower, patchy staining for MP28 observed in day 0 explants (D) most likely originates from fragments of fiber cell membranes that remain adhered to the epithelial monolayer during explant preparation (see Figure 3B in Jiang *et al.*, 1995). The area boxed in A, B, and C denotes the perimeter of the field shown in D, E, and F, respectively. Bar: 10 μm (A, B, C); 20 μm (D, E, F).

dissociated cell-derived cultures (compare Figure 10 with Figures 2 and 3) in 5/5 experiments. Neither the morphological (Figure 9) nor biochemical (as assessed by expression of MP28 and δ-crystallin; Figures 9 and 11) differentiation of central epithelial explants into fiber-like cells was detectably inhibited by the continuous inhibition of gap junctional intercellular communication by 100 μM βGA in 8/8 separate experiments.

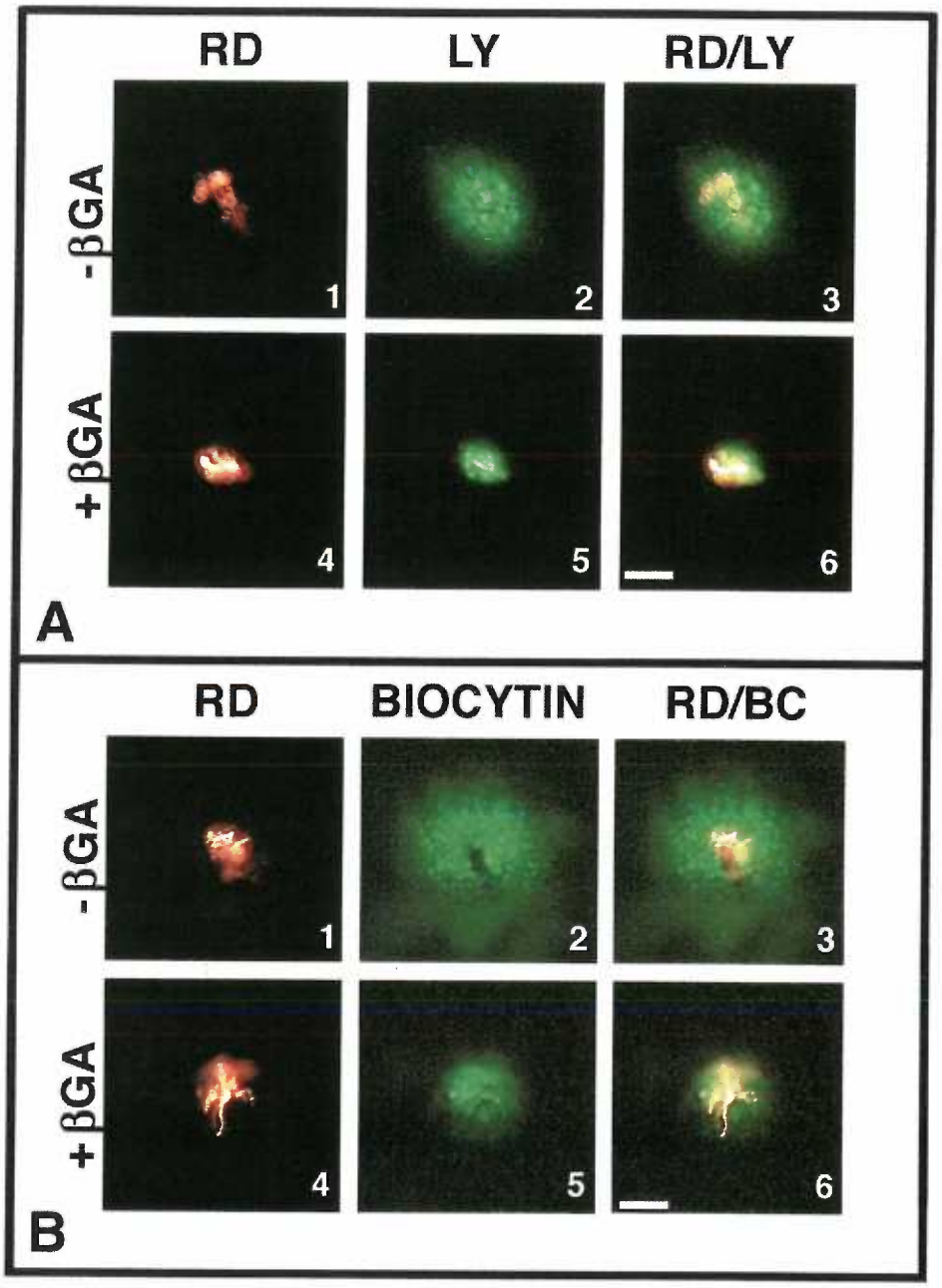

Figure 10. *βGA inhibits gap junction-mediatd intercellular transfer of Lucifer yellow and biocytin in embryonic chick lens central epithelium explants.* Lens epithelium explants cultured for one day in M199/15% fetal calf serum in either the absence (A1-A3; B1-B3) or presence (A4-A6; B4-B6) of 100 μM 18β-GA were scrape-loaded with rhodamine dextran mixed with either Lucifer yellow (A1-A6) or biocytin (B1-B6) as described under Materials and Methods. After two minutes, the cells were fixed and the dyes visualized by fluorescence microscopy. Gap junction-impermeant rhodamine dextran remained confined to the loaded cells immediately bordering the punctate wound (A1, A4; B1, B4) The extent to which Lucifer yellow (A2, A5) and the more sensitive gap junction tracer biocytin (B2, B5) are transferred to rhodamine dextran-negative cells is a measure of intercellular coupling. Superposition of the staining pattern of the two dyes clearly demonstrates the βGA-induced block of gap junction channels (compare A3 with A6; B3 with B6). Bar: 50 μm.

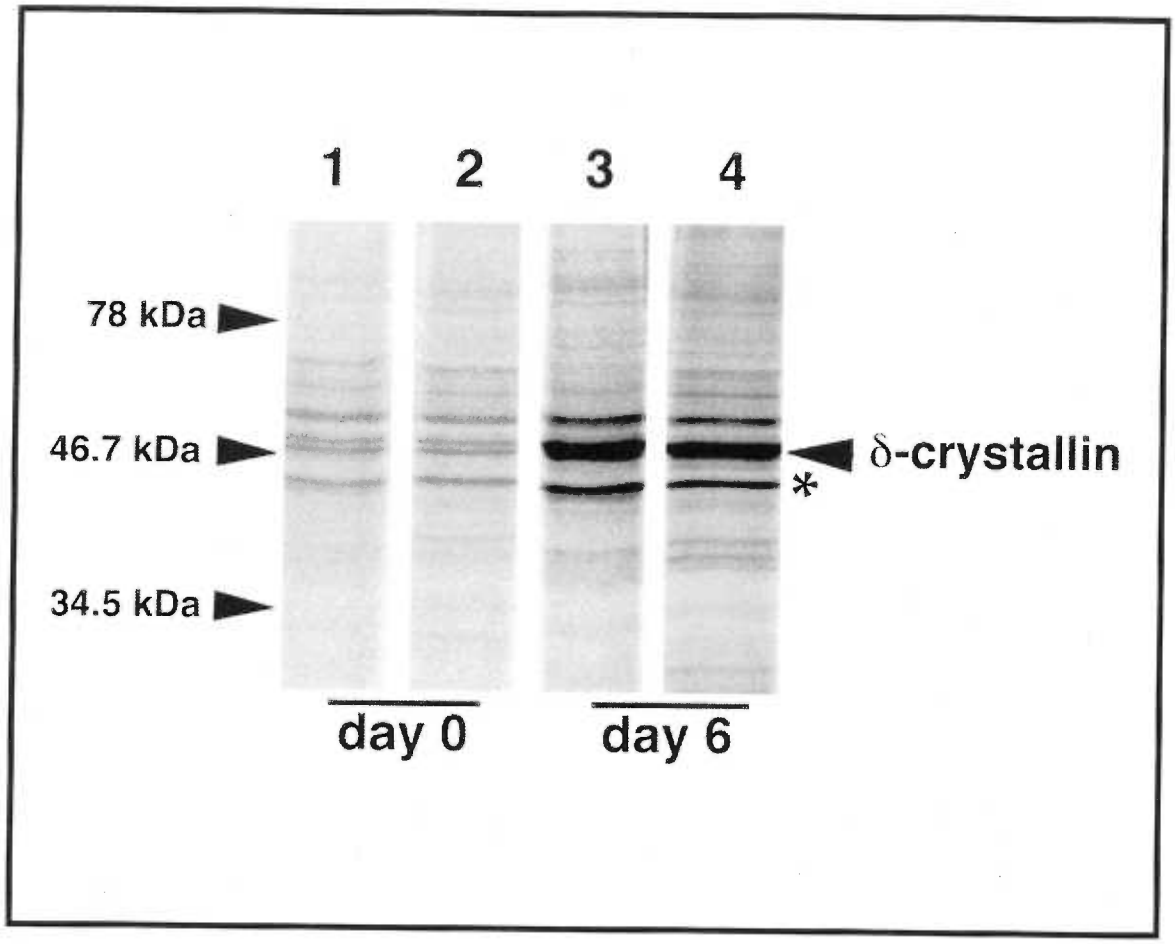

Figure 11. *βGA does not inhibit δ-crystallin expression in differentiating lens explants.* Lens epithelium explants were metabolically labeled with 0.1 mCi/ml [^{35}S]-methionine for one hour in either the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 100 μM 18β-GA, either immediately following preparation (lanes 1 and 2) or after 6 days of culture in M199/15% fetal calf serum with (lane 4) or without (lane 3) 100 μM 18β-GA. The labeled cells were solubilized in SDS and equal amounts of total cell lystates were analyzed on SDS-PAGE. When normalized to actin (indicated by the asterisk) and quantitated with a PhosphorImager, synthesis of δ-crystallin in explants cultured in the continuous presence of βGA (lane 4) was ~1.3X of the untreated control (lane 3). Molecular weight markers are indicated on the left.

5. Discussion

The abundance and likely functional significance of gap junctions in the lens have made this organ the focus of extensive study of gap junction structure, biochemistry, and physiology (Goodenough, 1992; Zampighi *et al.*, 1992). Despite this level of investigation and precedence in other tissues, the role of gap junctions in the development of the lens is unknown. Given the lack of a specific blocker of gap junction function that is suitable for long-term use in whole animals, there are currently two approaches to this question. The first is to use targeted gene disruption to abolish connexin expression. Because there are three known functional connexins in the vertebrate lens, this would require the generation of a "triple knock-out" in which extralenticular developmental defects might complicate the interpretation of any phenotype. It is also possible that additional, as yet uncharacterized connexin species may be expressed in the lens, necessitating the direct functional evaluation of intercellular communication in the lenses of such mice. Precedence for unanticipated connexin species functionally coupling cells in the absence of the predominant connexin is provided by the observation that targeted gene disruption of Cx43 reduces, but does not eliminate, gap junctional communication in preimplantation mouse embryos due to the presence of connexin45 (De Sousa *et al.*, 1997). Furthermore, the fact that Cx43 expression begins at the initial stages of lens development and that of the fiber-type connexins shortly thereafter (Evans *et al.*, 1993; Jiang *et al.*, 1995) raises the possibility that any observed abnormality in secondary fiber formation could be an indirect consequence of defects in a preceding step in lens development or be due to general cellular dysfunction arising from chronic disruption of gap junction-dependent lens metabolism. For these reasons, we believe that an *in vitro* approach, in which the level of gap junctional intercellular communication can be acutely manipulated and is unlikely to dictate nutrient access, provides valuable insights into the role of gap junctions in lens development that are not obtainable *in vivo*.

For as yet unknown reasons, *in vitro* epithelial-to-fiber maturation is most complete in chick cells, with the most differentiated cells obtained in culture indistinguishable on the ultrastructural level from secondary fiber cells in whole lens cortex (Menko *et al.*, 1984; Menko *et al.*, 1987). We therefore chose to use primary embryonic chick lens cells for our study. Given the morphological, functional, and biochemical similarities in the development of the lens between birds and mammals (Piatigorsky, 1981), it is very likely that our findings will extend to higher vertebrates as well.

We examined the role of gap junctions in lens development using βGA, one of several glycyrrhetinic acid derivatives that have been used to block gap junction permeability in cultured cells (Davidson *et al.*, 1986; Goldberg *et al.*, 1996; Guan *et al.*, 1996). βGA has been shown (as were two other unrelated inhibitors of gap junction function) to inhibit cell-cell coupling and myogenesis of cultured rat L6 myoblasts and of primary embryonic chick myoblasts, illustrating the utility of this compound for addressing the role of gap junctions in differentiation processes *in vitro* (Mege *et al.*, 1994; Proulx *et al.*, 1997a). As documented for other cell types, βGA rapidly (within 30 minutes) and continuously (if replaced every two days) suppressed gap junction-mediated intercellular transfer of Lucifer yellow and biocytin in cultured embryonic chick lens cells without noticeable toxic effects. We found that in the absence of serum, maximum inhibition of gap junctional communication in dissociated cell-derived lens cultures was achieved using 4-10 times lower βGA concentrations than those generally used in its presence, reducing the possibility of non-specific effects of the drug. Unlike βGA, other reported gap junction blockers (heptanol, 18α-carbenoxolone, oleamide, anandamide) eventually killed lens cells under our culture conditions when used at concentrations required to inhibit junctional permeability (data not shown). We found that inhibition of gap junction-mediated intercellular communication by βGA did not appreciably disrupt either the plating or (as assessed by the formation of MP28-expressing lentoids) the

epithelial-to-secondary fiber differentiation of dissociated cell-derived chick primary lens cultures throughout the 8-day culture period. Furthermore, βGA did not alter the expression of adherens or tight junctions as detected by light microscopy.

Interpretation of these results requires consideration of two properties of glycyrrhetinic acid derivatives. First, βGA (like all other known gap junction uncouplers) is not completely specific for connexins and has been reported to affect certain other proteins as well (Davidson *et al.*, 1986). Although this could have been problematic had βGA been shown to perturb fiber differentiation, it seems unlikely that a gap junction-independent activity of glycyrrhetinic acid somehow compensates for a requirement for gap junction-mediated intercellular communication in fiber differentiation. Secondly, as judged from the very low but detectable intercellular transfer of biocytin in βGA-treated lens cells, the inhibition of gap junctional intercellular communication achieved by non-toxic doses of βGA is not absolute. This was expected given that the βGA-related compound 18α-carbenoxolone has been shown to reduce gap junction-mediated electrical coupling between tissue culture cells by only ~75% (Goldberg *et al.*, 1996; Martin *et al.*, 1991). Our experiments therefore do not rule out the possibility that a minimal level of gap junction-mediated communication is necessary for fiber differentiation, but do establish that the extensive up-regulation of gap junction formation and function that takes place in the bow region *in vivo* is not required for rapid and efficient epithelial-to-fiber differentiation in culture. Furthermore, our study does not address whether gap junctions play a role in stages of lens development that precede the time of lens harvest (E6 for explants and E10 for dissociated monolayer cultures) such as lens vesicle formation or primary fiber elongation, nor the function of gap junctions in very late developmental events (e.g., loss of nuclei) that are not recapitulated in our cultures.

A large number of studies have demonstrated that lens cells express receptors for a variety of growth factors and that their developmental fate is sensitive to the balance between proliferation-promoting and differentiating-promoting factors within the eye

(Hyatt and Beebe, 1993; Lovicu *et al.*, 1995). Given the importance of extralenticular growth factors for lens development *in vivo*, it initially seemed surprising that our dissociated cell-derived cultures underwent epithelial-to-fiber differentiation in the absence of any exogenously added soluble or substrate-associated growth factors. As discussed by Menko *et al.* (1984), the cells that are propagated in such cultures apparently originate from multiple areas of the lens, including the bow and annular pad regions. It therefore seems likely that at least some of the cells that developed into fibers in the absence of added growth factors were from these more peripheral zones which had already been "programmed" for fiber differentiation prior to the time (E9-10) of lens harvest. If so, then the possibility remained that the initial specification of epithelial cells for secondary fiber differentiation might have a requirement for high levels of gap junction-mediated intercellular communication. To address this issue, we used central epithelium explants comprised of the least differentiated population of cells within the lens. As expected from the literature (Piatigorsky *et al.*, 1973), these explants survived in the absence of added growth factors but remained undifferentiated unless supplemented with 15% fetal calf serum, indicating that their developmental fate had not been predetermined *in vivo*. Gap junction-blocking levels of βGA did not, however, inhibit serum-mediated differentiation of these explants into MP28- and δ-crystallin-expressing fiber cells. Taken together, our results make it very unlikely that extensive gap junctional intercellular communication plays a role in either the induction or the execution of epithelial-to-secondary fiber differentiation.

Based on our findings, we predict that the concerted differentiation of adjacent cells that occurs in the bow region in the intact lens is coordinated by some mechanism other than increased gap junctional intercellular communication. One possibility consistent with the requirement for cell-cell contact would be paracrine signaling stimulated by an increase in the local concentration of a differentiation-inducing growth factor. The massive up-regulation of gap junction formation observed in the lens bow

might instead be a consequence rather than a cause of fiber initiation and serve primarily to facilitate the intercellular transfer of metabolites involved in the burst of new protein synthesis that is essential for fiber formation. In addition, Mathias and coworkers (Mathias and Rae, 1985; Mathias *et al.*, 1997) have presented a compelling argument that the high level of gap junctional coupling at the equatorial region is required for the "microcirculation" of ions and fluids around and through the lens. A critical role for gap junctions in lens homeostasis is supported by the recent demonstration that elimination of Cx46 expression by targeted gene disruption results in the postnatal formation of nuclear cataracts in mice (Gong *et al.*, 1997).

6. Acknowledgments

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B. Chapter 2

FGF Signalling in Chick Lens Development

Anh-Chi N. Le

and Linda S. Musil

Vollum Institute for Advanced Biomedical Research and

Department of Cell and Developmental Biology

Oregon Health Sciences University

3181 SW Sam Jackson Park Road

Portland, OR 97201

1. Abstract

The prevailing concept has been that an FGF induces epithelial-to-fiber differentiation in the mammalian lens whereas chick lens cells are unresponsive to FGF and are instead induced to differentiate by IGF/insulin-type factors. Mammalian and avian lenses express receptors for both types of molecules and develop by very similar processes. It has therefore been an unexplained paradox why they should respond to growth factors so differently. When treated for periods in excess of those used in previous investigations (>5 h), we find that purified recombinant FGFs (FGF-2 or FGF-1 plus heparin) stimulate proliferation of primary cultures of embryonic chick lens epithelial cells and (at higher concentrations) expression of the fiber cell differentiation markers d-crystallin and CP49. Surprisingly, upregulation of cell proliferation and δ-crystallin synthesis by FGF does not require activation of ERK kinases. ERK function is, however, essential for stimulation of δ-crystallin expression in response to either insulin or IGF-1. Vitreous humor, the presumptive *in vivo* source of differentiation-promoting activity, contains a factor that is capable of diffusing out of the vitreous body and inducing d-crystallin and CP49 expression in cultured chick lens cells. This factor binds to heparin with high affinity and increases δ-crystallin expression in an ERK-insensitive manner, properties consistent with an FGF but not insulin or an IGF. Our findings indicate that differentiation in the chick lens is likely to be mediated by an FGF and provide the first insights into the role of the ERK pathway in growth factor-induced signal transduction in the lens.

2. Introduction

The vertebrate lens is composed of only two cell types: a monolayer of epithelial cells that overlie its anterior face and a core of elongated, crystallin-rich fiber cells that are responsible for the refractive properties of the organ. Following the differentiation and elongation of cells at the posterior of the lens vesicle into primary fiber cells during early embryogenesis, all further growth of the lens is due to proliferation of epithelial cells and their subsequent differentiation into secondary fiber cells at the equatorial (bow) region of the organ (reviewed in Piatigorsky, 1981; Wride, 1996). Epithelial-to-fiber differentiation is characterized by a very large increase in cell length, restructuring of the cell surface and cytosol, and upregulation of fiber-specific proteins including various crystallins, the intermediate filament protein CP49, and the plasma membrane protein MP26 (known as MP28 in the chick). Eventually, intracellular organelles are lost and both DNA and protein synthesis ceases. Proliferation of epithelial cells and their differentiation into secondary fibers continues throughout the lifetime of the organism, albeit more slowly postnatally than during embryogenesis (Harding *et al.*, 1977). Environmental or genetic factors that perturb either process cause vision-destroying cataracts and/or microphthalmia (Lovicu and Overbeek, 1998; Nishiguchi *et al.*, 1998; Reneker and Overbeek, 1996).

In both the mouse and chick, surgically rotating the lens 180 degrees causes the formerly anterior epithelium to differentiate into secondary fiber cells and induces the formation of a partial monolayer of epithelial cells on the opposite pole of the organ (Coulombre and Coulombre, 1963; Yamamoto, 1976). These observations were among the first to support the now widely accepted concept that the fate of lens cells is dependent on gradients of diffusible factors in the ocular environment such that the anterior chamber favors proliferation and the posterior of the eye promotes epithelial-to-fiber differentiation (Hyatt and Beebe, 1993; Mascarelli *et al.*, 1987; Schulz *et al.*, 1993). In mammals, several

lines of evidence indicate that one or more members of the FGF family are critical determinants of the polarity of lens development. First, FGFs are the only growth factors known to be sufficient for epithelial-to-fiber differentiation of cultured rodent cells. Purified FGF-2 at > 10 ng/ml induces fiber differentiation in central epithelial explants prepared from neonatal rat, whereas lower concentrations stimulate cell proliferation (McAvoy and Chamberlain, 1989). In contrast, insulin and IGF-1, both mitogenic for lens cells (Liu *et al.*, 1996; Reddan and Wilson-Dziedzic, 1983), can synergize with, but not substitute for, FGF in fiber induction in this system (Chamberlain *et al.*, 1991). Second, eye-derived FGFs (including, but not limited to, FGF-1 and FGF-2 produced by the retina) are found in higher concentrations in the posterior of the eye in the vitreous body than in the aqueous humor of the anterior chamber (Caruelle *et al.*, 1989; Schulz *et al.*, 1993). Vitreous humor, but not aqueous humor, induces fiber differentiation of rodent central epithelial explants (Lovicu *et al.*, 1995), and the fiber-promoting activity copurifies with FGF-2 and FGF-1 on heparin-Sepharose and is blocked by a mixture of anti-FGF antibodies (Schulz *et al.*, 1993). Third, the highest concentration of immunohistochemically detectable membrane-associated FGF-1 in the lens is in the equatorial region, where fiber differentiation is initiated (de Iongh and McAvoy, 1992; de Iongh and McAvoy, 1993; Lovicu and McAvoy, 1993). Fourth, truncated forms of FGFR-1 with dominant-negative activity inhibit epithelial-to-fiber differentiation when expressed in transgenic mice under the control of a lens-specific promoter (Chow *et al.*, 1995; Robinson *et al.*, 1995; Stolen and Griep, 2000). Because expression of the transgene in the central epithelium is relatively low, this approach cannot be utilized to address the physiological role of FGFs in lens cell proliferation. It is also unknown which member or members of the FGF family (composed of 18 or more different gene products, at least five of which are known to be expressed in the eye) are involved in lens development *in vivo*. To date, five naturally occurring FGFs have been reported to induce fiber differentiation when exogenously overexpressed in the lenses of transgenic mice

(reviewed in Lovicu and Overbeek, 1998; Miller *et al.*, 2000). *In vitro* studies have also implicated FGFs in lens development in other mammalian species (Uhlrich *et al.*, 1986; Wong *et al.*, 1996), including cow in which the concentration of FGF-1 in the vitreous humor has been reported to be 295 ng/ml (Caruelle *et al.*, 1989). The signaling mechanisms by which FGFs exert their effects on lens cell proliferation and differentiation have not been elucidated.

Although minor species-specific differences in timing and anatomy exist, the general process by which the lens develops is remarkably conserved between birds and mammals (reviewed in Piatigorsky, 1981; Wride, 1996). FGFs have been purified from chick vitreous humor (Mascarelli *et al.*, 1987), and avian lenses express FGFR-1-encoding mRNA (Ohuchi *et al.*, 1994; Potts *et al.*, 1993) and contain immunodetectable FGF-2 (Consigli *et al.*, 1993). FGF-2 has been shown to stimulate transdifferentiation of cultured embryonic chick retinal cells into lens-like cells (Hyuga *et al.*, 1993; Karim and de Pomerai, 1990). It is therefore remarkable that the prevailing view in the literature for over a decade has been that chick lens epithelial cells are unresponsive to FGF and are instead induced to differentiate by IGF-1 or an IGF-like growth factor (Beebe *et al.*, 1987; Caldes *et al.*, 1991; Lang, 1999; Lovicu *et al.*, 1995). This concept arose from studies in which purified IGF-1 or insulin, both most likely acting by binding to IGF-1 receptors, were shown to induce cell elongation and to upregulate expression of the fiber cell marker d-crystallin in central epithelial explants prepared from E6 chick lenses (Milstone and Piatigorsky, 1977). Preincubation with anti-IGF-1 antibodies partially neutralized the cell elongation activity of medium containing 20% chicken embryo vitreous humor (Beebe *et al.*, 1987). Although δ-crystallin levels were not assessed, neither purified FGF-1 nor FGF-2 significantly affected cell length under the conditions tested (Beebe *et al.*, 1987). Collectively, these findings have led to the view that there is a fundamental mechanistic dichotomy in vertebrate lens development in which epithelial-to-fiber differentiation is regulated by FGFs in mammals and by an IGF/insulin-type factor in birds.

In the aforementioned studies, the mitogenic and cell elongation-promoting effects of FGF were assessed after (respectively) 3 h (Hyatt and Beebe, 1993) and 5 h (Beebe *et al.*, 1987) of treatment. Because the lens is constantly exposed to growth factors in the ocular environment *in vivo*, we examined the effect of FGF and IGF/insulin on avian lens cells after longer-term incubations. We found that after 12 h of exposure, FGF (similar to insulin or an IGF-1 analogue) upregulated cell proliferation in central epithelial explants as well as in dissociated cell-derived monolayer cultures prepared from embryonic chick lenses. Moreover, both classes of growth factors also stimulated the expression of the fiber differentiation markers δ-crystallin and CP49 in each culture system. Using inhibitors that specifically block the ERK pathway, we show that the two classes of growth factors affect chick lens cell fate via partially independent mechanisms. Expression of fiber markers was also induced when chick epithelial cells were incubated in medium supplemented with chick vitreous humor or conditioned with intact vitreous bodies. The vitreous humor-derived differentiation activity behaved indistinguishably from FGF and very differently from either insulin or an IGF. Taken together, our studies demonstrate that the effect of FGFs on lens epithelial cells is evolutionarily conserved between mammals and birds and support the concept that FGF is an important determinant of fiber differentiation in the vertebrate lens *in vivo*.

3. Materials and Methods

Materials. Recombinant human FGF-1 and bovine FGF-2, generous gifts of Felix Eckenstein (Oregon Health Sciences University), were prepared as described by Stock *et al.* (1992). Bioactivity was assessed by measuring stimulation of DNA synthesis in AKR-2B cells (Shipley, 1986). Bovine pancreas insulin and low molecular weight heparin (sodium salt, from porcine intestinal mucosa) were purchased from Sigma-Aldrich, and fetal calf serum was from Hyclone. The IGF-1 used throughout this study was R^3IGF-1(*GroPep*; Adelaide, Australia). Because of its low affinity for IGF binding proteins, this analogue (also referred to as des [1-3] IGF-1) is more potent than wild-type IGF-1 in biological assays and has been extensively used in the study of IGF-induced cell proliferation and differentiation (Coolican *et al.*, 1997; Stewart and Rotwein, 1996). UO126, the specific MEK 1/2 inhibitor, was generously provided by Dr. James Trzaskos (DuPont Pharmaceuticals). The nonhydrolyzable cAMP analog 8-CPT-cAMP (8-(4-chlorphenylthio)-cyclic AMP) was from CalBiochem. The anti-chicken MP28 polyclonal rabbit serum (#6182) was provided by Ross Johnson, University of Minnesota (Le and Musil, 1998), and the rabbit anti-mouse CP49 polyclonal serum (#900) was a generous gift of Paul FitzGerald, University of California, Davis. The phospho-p44/42 MAP Kinase E10 monoclonal mouse antibody was purchased from New England Biolabs, Inc.

Cell Culture. Central epithelial explants were prepared from embryonic day 6 (E6) chick lenses as previously described (Chepelinsky *et al.*, 1985; Le and Musil, 1998) and cultured in M199 medium (Gibco BRL) supplemented with 0.1% bovine serum albumin, penicillin G, and streptomycin in either the absence or presence of additives (FGF, IGF-1, insulin, vitreous humor, or fetal calf serum) as indicated in Figures 1 and 2. Explants were cultured for up to 6 days at 37°C in 5% CO_2, with one change of medium 24 hours after preparation. Dissociated cell-derived monolayer (DCDML) cultures were

prepared from E10 chick lenses as previously described (Le and Musil, 1998). A single-cell suspension (consisting mainly of cells from the peripheral regions of the epithelium; Menko *et al.*, 1984) was plated at ~40% confluency in a 96-well tissue culture plate (0.9 x105 cells/well) 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. Cells were cultured for up to 6 days at 37°C/ 5% CO_2 and fed every two days with fresh medium. When desired (Figure 4A, d3 washout), cells cultured for 3 days with FGF-2 were rinsed three times with 10 µg/ml heparin and then three times with M199 to remove extracellular FGF, and the incubation continued in the absence of exogenous growth factor.

Plasmids and transient transfection of lens cells. One day after plating, DCDML cultures were transfected in M199 medium without BOTS or antibiotics using Lipofectamine PLUS (GibcoBRL) 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; Pharmacia) 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 hours, the transfection medium was supplemented with BOTS, penicillin G, and streptomycin and the cells cultured for an additional 48 hours prior to analysis.

[^{3}H]-thymidine incorporation. One day after preparation, central epithelial explants or DCDML cultures were labeled with 0.1 mCi/ml [^{3}H]-thymidine (New England Nuclear) for 12 hours at 37°C in M199 medium plus BOTS, penicillin G, and streptomycin, with or without added growth factor. Each condition was tested in triplicate in each experiment. The labeled cultures were incubated at room temperature three times for 10 minutes each with 10% trichloroacetic acid (Mallinckrodt AR) and then solubilized

with 0.2 M NaOH (Shipley, 1986). Radioactivity was determined by liquid scintillation counting using Ecolite (ICN).

[^{35}S]-methionine metabolic labeling. Central epithelial explants or DCDML cultures were labeled at 37°C with 0.1 mCi/ml [^{35}S]-methionine (EXPRE^{35}S^{35}S, New England Nuclear) for 2 or 4 hours, respectively, in methionine- and serum-free Dulbecco's minimum essential medium (DMEM) (GibcoBRL) and then 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 previously described (Le and Musil, 1998). After addition of SDS-PAGE sample buffer (7.1% glycerol, 1.92 mM Tris pH 6.8, 2.5% SDS, 2% 2-mercaptoethanol), the whole cell lysates were boiled for 3 min and electrophoresed on 11% SDS-polyacrylamide gels. [^{35}S]-methionine incorporation into total cellular protein and into δ-crystallin was quantitated on a PhosphorImager (Molecular Dynamics) utilizing IPLab Gel software (Signal Analytics Corp.).

Immunoblot analysis of CP49 and phospho-ERK. For CP49, DCDML cultures and central epithelial explants were solubilized in lysis buffer (see above) and boiled for 3 minutes. The protein concentration of DCDML-derived lysates was determined using the Folin phenol protein microassay (Peterson, 1983), and 1 μg of protein loaded onto each lane of a 10% SDS-polyacrylamide gel. Samples were prepared for analysis of phospho-ERK (pERK) as follows. For DCDML cultures, cells were solubilized directly into SDS-PAGE sample buffer (see above), boiled for 3 minutes, and the entire whole cell lysate from each well of a 96-well culture plate analyzed per lane of a 10% SDS-polyacrylamide gel. For intact lenses, lenses dissected from E10 chicks or P3 rats were carefully rolled on a piece of Kimwipe to remove non-lenticular tissue immediately prior to solubilization in boiling lysis buffer. Ten micrograms of total lens protein (determined by the Folin phenol assay) was analyzed per gel lane. After electrophoresis, samples were

transferred to PVDF membranes (Immobilon) for 45 minutes at 65 mV. Blots were blocked for 1 hour at room temperature with 0.4% I-Block (Tropix, Inc.) in 0.1% Tween-20/PBS, after which the appropriate primary antibody (anti-CP49 serum or phospho-p44/42 MAP Kinase E10 monoclonal mouse antibody) was added in 0.2% I-Block, 0.1% Tween-20/PBS. After overnight incubation at 4°C, the blots were rinsed two times for 5 minutes each in 0.1% Tween-20/PBS and incubated for 1 hour at room temperature with the appropriate alkaline phosphatase-conjugated secondary antibody (Promega) diluted in 0.2% I-Block, 0.1% Tween-20/PBS. The blots were then subjected to three 5-minute washes in 0.1% Tween-20/PBS followed by two 5-minute washes in 0.1 M diethanolamine/1 mM $MgCl_2$. 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.

Indirect Immunofluorescence. DCDML cultures grown on glass coverslips were fixed in 2% paraformaldehyde/DPBS (pH 7.5) for 30 minutes at room temperature, permeabilized in DPBS/0.1% bovine serum albumin/0.2% Triton X-100/5% normal goat serum (PBNT) for 30 minutes at room temperature, and then incubated overnight at 4°C with rabbit anti-chicken MP28 serum (6182) diluted 1:1000 in PBNT. The cells were then washed for 30 minutes with PBNT, incubated for 2 hours with rhodamine-conjugated goat anti-rabbit IgG (Pierce) diluted 1:500 in PBNT, and subjected to a final 30-minute rinse in PBNT (all at room temperature) prior to mounting on a microscope slide with MOWIOL (Calbiochem) (Le and Musil, 1998). MP28 immunoreactivity was detected with a fluorescence microscope (Leitz DMR) using a rhodamine filter set.

Explant histology. Lens central epithelial explants were fixed in 2% paraformaldehyde/DPBS (pH 7.5) for 30 minutes at room temperature and then stained with 1% thionin (Eastman Kodak Company) to facilitate visualization of the explant

during the embedding process. The fixed and stained explants were dehydrated through a graded ethanol series followed by xylene, then embedded in paraffin wax. Five micrometer-thick sections were cut, stained with Gill's #3 Hematoxylin (Fisher) and Eosin Y (LabChem, Inc.), and visualized with a Leitz DMR microscope.

Preparation of vitreous humor and vitreous body conditioned medium. Vitreous humor (VH) was prepared using a modification of the procedure of Beebe *et al.* (1980). Vitreous bodies were dissected from E10 chick eyes, pooled, and centrifuged for 10 min at 4°C at 21,000 rpm in a TLA55 rotor in a Beckman Optima Ultracentrifuge to remove cells and the fibrous elements of the vitreous body. The supernatant, referred to as vitreous humor (1 ml = ~10 vitreous bodies), was diluted with 2.3 volumes of M199 medium (= 30% VH). For vitreous body conditioned medium (VBCM), 12 vitreous bodies were removed from E10 chick eyes and transferred to the upper compartment of a 12 mm-diameter Transwell filter (polycarbonate, pore size = 0.4 μM) (Corning Costar Corp.) inserted into a 22 mm-diameter tissue culture plate well containing 1.2 ml of M199 medium. After an overnight incubation at 37°C in a 5% CO_2 incubator, the lower chamber medium (=VBCM) was collected.

Fractionation of vitreous on heparin beads. One ml of either 30% VH/M199 or VBCM was mixed end-over-end with 0.1 ml of heparin-conjugated Affigel beads (BioRad) in the presence of 0.6 M NaCl for two hours 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 minute 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 molecular mass 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.

4. Results

FGF stimulates cell proliferation in embryonic chick lens central epithelial explants. Central epithelial explants are the most commonly used culture system for the study of lens cell proliferation and differentiation in both the chick and in mammals. Explants are prepared by manually excising the fiber mass from whole lenses and then trimming the remaining epithelial monolayer (still attached to the lens capsule) to remove cells originating from the peripheral (equatorial) regions of the lens. Explants prepared from E6 embryonic chick lenses have been reported to undergo limited proliferation when cultured in the presence of 1 μg/ml insulin (Milstone and Piatigorsky, 1975). Accordingly, we found that a 24 hour treatment of such explants with 1 μg/ml insulin (or, not shown, 50 ng/ml IGF-1) more than doubled the amount of tritiated thymidine incorporated into DNA during a subsequent 12 hour labeling period (Figure 1). Notably, 25 ng/ml FGF-2 induced a similar increase in DNA synthesis under the same conditions. FGF, IGF-1, and insulin have previously been shown to be mitogenic for central epithelial explants prepared from neonatal rat (Liu *et al.*, 1996) and for cultured rabbit lenses (Reddan *et al.*, 1975; Reddan and Wilson-Dziedzic, 1983). Consistent with the findings of others (Philpott and Coulombre, 1965; Philpott and Coulombre, 1968), explants cultured in the absence of exogenously added growth factor remained viable for over 6 days as assessed either by a calcein AM/ethidium homodimer-based live/dead cell assay or by [^{35}S]-methionine incorporation into total cellular protein (data not shown). Insulin and FGF therefore appeared to be acting as bona fide stimulators of proliferation instead of as survival factors. Neither insulin nor FGF significantly increased [^{3}H]-thymidine incorporation over a five hour period if the label and growth factor were added simultaneously immediately after explant preparation (data not shown). Although other explanations are possible, the most likely reason for the failure of a previous study to detect an effect of FGF on entry of E6 chick central epithelial explants into S phase is

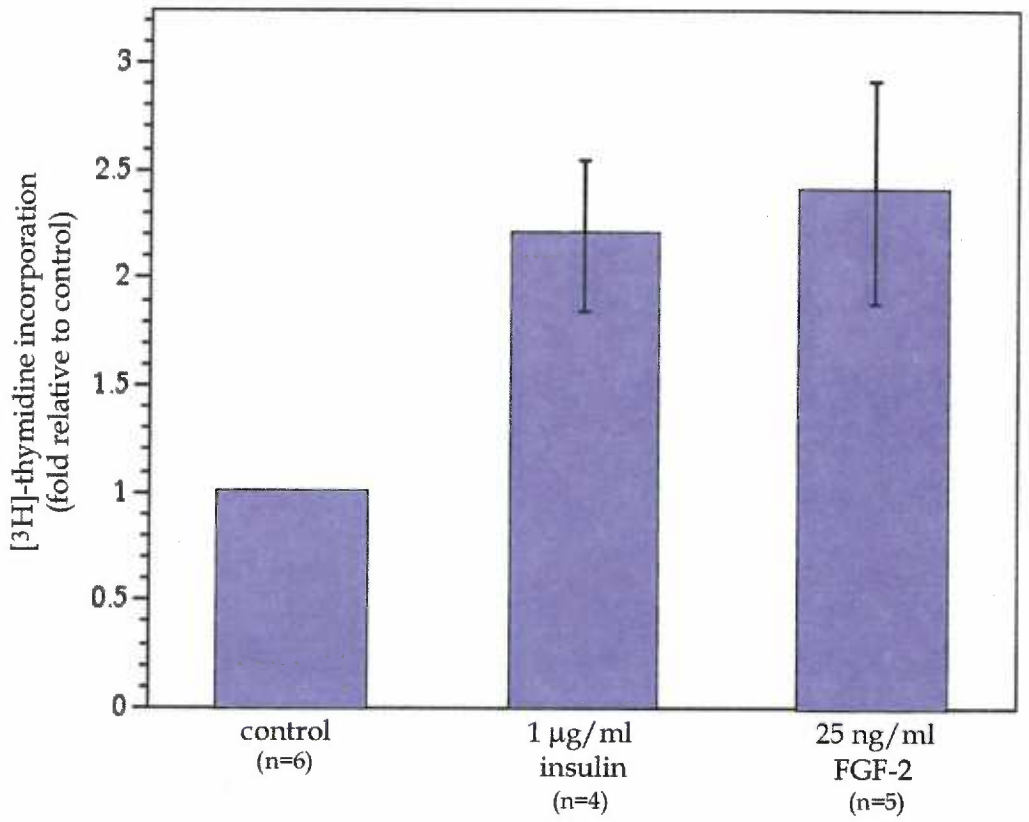

Figure 1. *FGF stimulates DNA synthesis in embryonic chick lens central epithelial explants.* Central epithelial explants were prepared from E6 chick lenses as described in Materials and Methods and cultured in M199 medium plus 0.1% bovine serum albumin in either the absence or presence of growth factor as indicated. Approximately 16 h after explantation, [^{3}H]-thymidine was added directly to the culture medium. Incorporation of radioactive thymidine into DNA during a 12 h labeling period was measured by liquid scintillation counting as described in Materials and Methods. The data are presented as the fold increase in tritiated thymidine incorporation in the presence of added growth factor relative to [^{3}H]-thymidine incorporation in untreated controls within the same experiment; each condition was tested in triplicate in each experiment. The parenthetical n-values are the number of independent experiments conducted. Untreated control explants incorporated an average of 750 counts per minute of [^{3}H]-thymidine per explant. The average background in the absence of cells was 400 counts per minute.

therefore because the treatment time was limited to 3 hours (Hyatt and Beebe, 1993).

FGF induces epithelial-to-fiber differentiation of embryonic chick lens central epithelial explants. δ-crystallin is detectable in embryonic chick lens central epithelial cells but undergoes a dramatic increase in expression during differentiation such that it constitutes up to 80% of the total protein synthesized by embryonic chick fiber cells *in vivo* (Piatigorsky *et al.*, 1972). Although upregulation of δ-crystallin is a commonly used molecular marker of epithelial-to-fiber differentiation in avian and reptilian lenses, to our knowledge the effect of FGF on this process has not been reported. Central epithelial explants were treated with or without growth factors for up to 6 days prior to metabolic labelling with [^{35}S]-methionine for 4 hours. Total cell lysates were prepared, analyzed by SDS-PAGE, and the amount of radiolabeled δ-crystallin quantitated by PhosphorImager analysis and expressed as the percent of total [^{35}S]-methionine-protein synthesized. This approach allowed comparison of δ-crystallin expression in different explants without the need to normalize to cell number. In the absence of added growth factor, δ-crystallin constituted~8-10% of the total [^{35}S]-methionine-labeled protein synthesized on days 1, 3, and 6 of culture (Figure 2A), very close to the value reported by Beebe for explants maintained under similar nondifferentiating conditions (Beebe *et al.*, 1987). A 24 hour incubation with either insulin (1 μg/ml) (Figure 2A) or 15% fetal calf serum (data not shown) increased the expression of δ-crystallin to ~14% of the total [^{35}S]-methionine-labeled protein synthesized. This enhancement is similar to that reported by Piatigorsky after incubation of explants with 1μg/ml insulin (Milstone and Piatigorsky, 1977) or 15% fetal calf serum (Milstone and Piatigorsky, 1975). Strikingly, 10-25 ng/ml FGF-2 consistently increased δ-crystallin to 25% of the total pulse-labeled protein after 24 hours of treatment. Stimulation of δ-crystallin synthesis by FGF continued to exceed that induced by insulin at days 3 and 6 of culture, with a maximum (41% at day 3) more than three-fold greater than that obtained with insulin (Figure 2A).

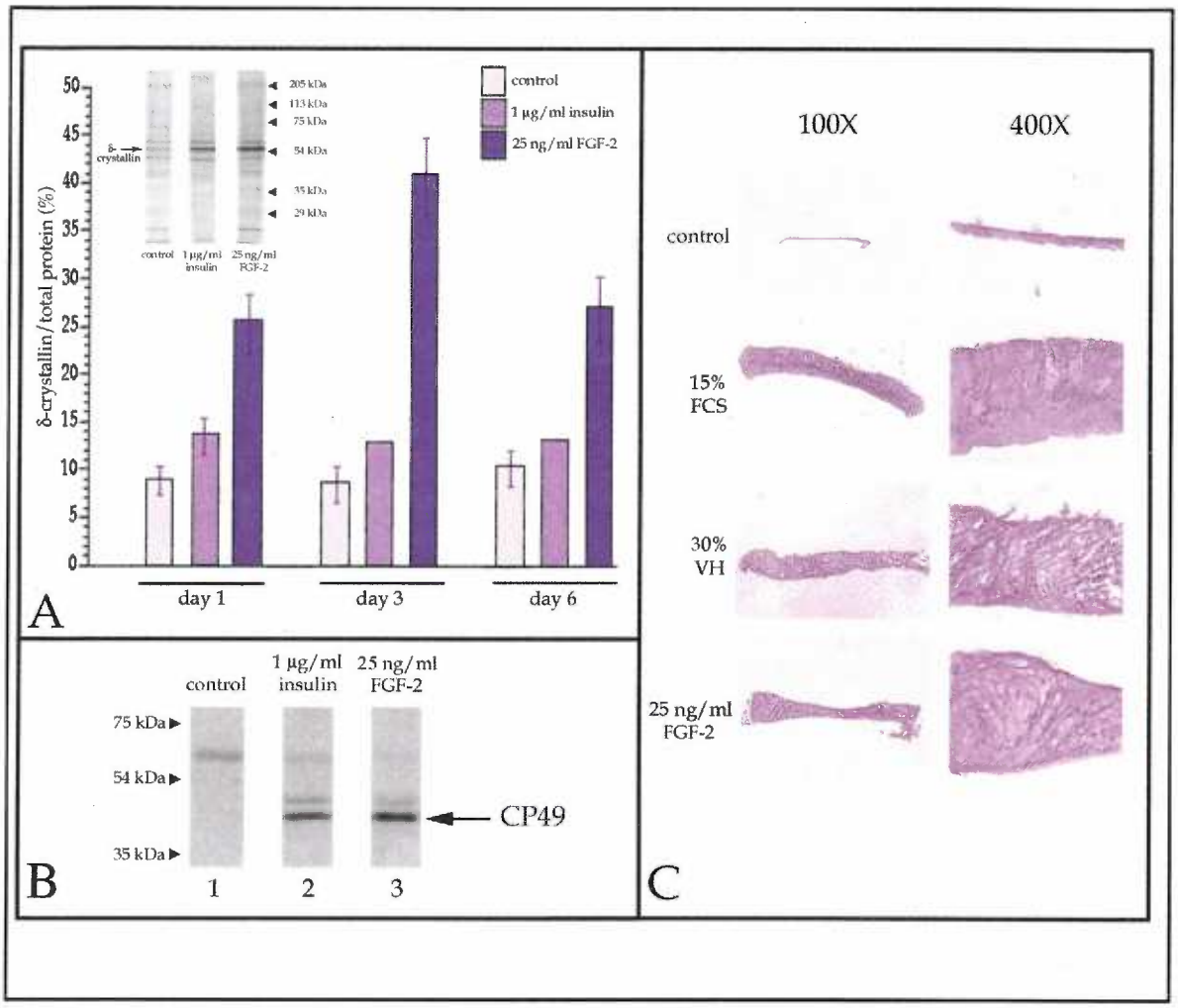

Figure 2. *FGF induces epithelial-to-fiber cell differentiation of chick lens central epithelial explants.* E6 chick lens central epithelial explants were cultured in M199 plus 0.1% bovine serum albumin in either the absence (control) or presence of the indicated additive. (A) After 1, 3, or 6 days of culture, explants were metabolically labeled with [^{35}S]-methionine for 2 h, solubilized in SDS, and the amount of radiolabeled δ-crystallin detected by SDS-PAGE expressed as the percent of total [^{35}S]-methionine-protein synthesized. A typical day 1 data set is shown in the panel inset; δ-crystallin was identified by comigration with δ-crystallin from E10 chick lens lysates. Molecular mass markers are indicated on the right. Each bar represents the average of three experiments except for the following, which are an average of two separate experiments: 1 μg/ml insulin, day 3 (δ-crystallin/total protein = 10.7%; 15.25%) and 1 μg/ml insulin, day 6 (δ-crystallin/total protein = 14%; 12.3%). (B) Total cell lysates from nine explants cultured for 6 days in the absence of exogenous growth factor (lane 1), one explant cultured for 6 days with 1 μg/ml insulin (lane 2), or one explant cultured for 6 days with FGF-2 (lane 3) were analyzed for CP49 content by immunoblotting. Data shown are typical of three independent experiments. Molecular mass markers are indicated on the left. The ~65 kDa species is nonspecifically recognized by the anti-CP49 serum; its intensity is greatest in lane 1 because of the larger number of explants analyzed. (C) Explants were cultured for 6 days in M199 plus 0.1% bovine serum albumin without additions (control), with 15% fetal calf serum (15% FCS), with 30% embryonic chick vitreous humor (30% VH), or with 25 ng/ml FGF-2 as indicated. The explants were then fixed in paraformaldehyde, dehydrated, and embedded in paraffin. Five micron sections were stained with hematoxylin and eosin and photographed at either 100X or 400X magnification.

We next investigated whether FGF could induce the *de novo* expression of a fiber-specific gene that is absent from undifferentiated epithelial cells (Figure 2B). In both birds and mammals, CP49 (also known as phakinin) is expressed only in cortical and nuclear lens fiber cells and is an essential component of beaded filaments, a lens-specific type of intermediate filament (reviewed in Quinlan *et al.*, 1996). As expected, CP49 could not be detected in undifferentiated E6 central epithelial explants maintained in unsupplemented M199 medium for 6 days, even if the whole cell lysates from nine explants were combined and analyzed by Western blotting (Figure 2B, left lane). In contrast, high levels of anti-CP49 immunoreactivity were recovered from a single explant treated for 6 days with either 1μg/ml insulin or 25 ng/ml FGF-2. Given that the increase in the number of cells per explant induced by either FGF or insulin was considerably less than 9-fold (as estimated from the number of Hoechst-stained nuclei in sections of treated and untreated explants; see Figure 2C), the rise in CP49 levels after growth factor addition must be the result of a bona fide increase in CP49 expression on a per cell basis.

As *in vivo*, differentiation of explanted chick and rodent lens epithelial cells is characterized by cell elongation. Elongation of E6 chick explants has been reported to be maximally induced by 15% fetal calf serum, resulting in a 4-fold increase in tissue thickness by day 3 of culture (Piatigorsky and Rothschild, 1972) and a 25-fold increase after 1 month (Piatigorsky, 1973). When cultured in unsupplemented M199 medium, E6 chick lens central epithelial explants remained as a flat monolayer of epithelial cells (Figure 2C). In the presence of 15% fetal calf serum, cell length increased approximately 9-fold over a 6 d period (see also Le and Musil, 1998). Similar results were obtained with medium supplemented with E6 vitreous humor, another previously characterized differentiation factor for both chick (Beebe *et al.*, 1980) and rodent explants (Lovicu *et al.*, 1995; Schulz *et al.*, 1993). Notably, 25 ng/ml FGF-2 (Figure 2C) or 100 ng/ml FGF-1 plus its cofactor heparin (1 μg/ml) (Uhlrich *et al.*, 1986) (not shown) also dramatically increased the thickness of E6 explants, typically to a slightly greater extent at the explant

periphery than in the center producing a "dumbbell" profile in cross-section. Although the distribution of nuclei in explant sections was suggestive of some cell proliferation (as expected from [^{3}H]-thymidine incorporation; Figure 1), the increase in explant thickness was due mainly to cell elongation. Both the multilayering of elongated cells and the dumbbell shape are previously reported characteristics of FGF-treated rodent explants and are largely absent from explants cultured with either insulin or an IGF (Richardson *et al.*, 1993). In the chick, Piatigorsky *et al.* (1973) have reported that insulin doubled the thickness of E6 lens central epithelial explants by 24 hours of culture but did not sustain this elongation at later timepoints. In our hands, elongation of chick explants in response to insulin or IGF-1 was inconsistent and never exceeded that obtained with either fetal calf serum or FGF at day 6 of culture (data not shown). Taken together, these results demonstrate that chick lens central epithelial explants undergo morphological as well as biochemical differentiation when treated with purified FGF at the same concentration and for the same length of time as has previously been shown to induce fiber formation in rodent explants (McAvoy and Chamberlain, 1989). Phase contrast microscopy did not reveal obvious cell elongation after exposure to FGF for 5 hours, the maximum length of time tested in previous investigations in the chick explant system (Beebe *et al.*, 1987).

FGF stimulates cell proliferation in embryonic chick lens dissociated cell-derived monolayer cultures. *In vivo*, it is the epithelial cells located near the lens equator, not those in the central region, that differentiate into secondary fiber cells. Studies in the rat lens have shown that the sensitivity of these so-called peripheral epithelial cells to growth factors (including FGF) is distinct from that of epithelial cells originating from the central epithelium (Richardson *et al.*, 1993; Richardson *et al.*, 1992). To determine the effect of FGF and insulin/IGF on chick lens peripheral epithelial cells, we prepared dissociated cell-derived monolayers enriched in this population from E10 embryos. As described by Menko *et al.* (1984), such cultures undergo cell division and epithelial-to-fiber

differentiation when grown in the presence of fetal calf serum. We have reported that these cells remain viable and continue to proliferate and differentiate in a defined, growth factor-free medium (M199/BOTS), albeit to a more limited extent than in the presence of serum (Le and Musil, 1998). The ability of insulin/IGF and FGF to stimulate proliferation of chick lens peripheral epithelial cells was assessed by plating cells under serum-free conditions in either the absence or presence of added growth factor (Figure 3). Relative to untreated controls, 1 μg/ml insulin or (not shown) 15 ng/ml IGF quadrupled the amount of [^{3}H]-thymidine incorporated into DNA on the following day. Insulin had a much weaker effect at 50 ng/ml, suggesting that its activity was mediated mainly by binding to IGFR-1 receptors instead of to the higher affinity insulin receptor (Gammeltoft *et al.*, 1988). FGF-2 significantly enhanced DNA synthesis at a concentration of 1 ng/ml, and at 10 ng/ml increased [^{3}H]-thymidine incorporation 4-fold (Figure 3) resulting in a 1.7-fold increase in cell number on day 3 of culture as measured by methylene blue staining or by direct cell counting (data not shown). FGF and insulin also increased DNA synthesis if added to 1-day-old cultures, indicating that their effect was not due to either improved plating efficiency or cell spreading (see Figure 6A). In keeping with previous reports (Hyatt and Beebe, 1993; Liu *et al.*, 1994), neither recombinant human EGF (at either 1 or 100 ng/ml) nor 1-1000 ng/ml recombinant human TGF-β substantially increased proliferation under serum-free conditions despite the reported expression of their respective receptors by at least some lens cells (not shown).

FGF induces epithelial-to-fiber differentiation in embryonic chick dissociated cell-derived monolayer cultures. Epithelial-to-fiber differentiation in dissociated cell-derived monolayer cultures was assessed using the same assays as were employed with central epithelial explants. In the absence of exogenously added growth factor, δ-crystallin comprised ~14% of the total protein labeled during a 4 hour pulse with [^{35}S]methionine on day 6 of culture (Figure 4A). This value is greater than that obtained

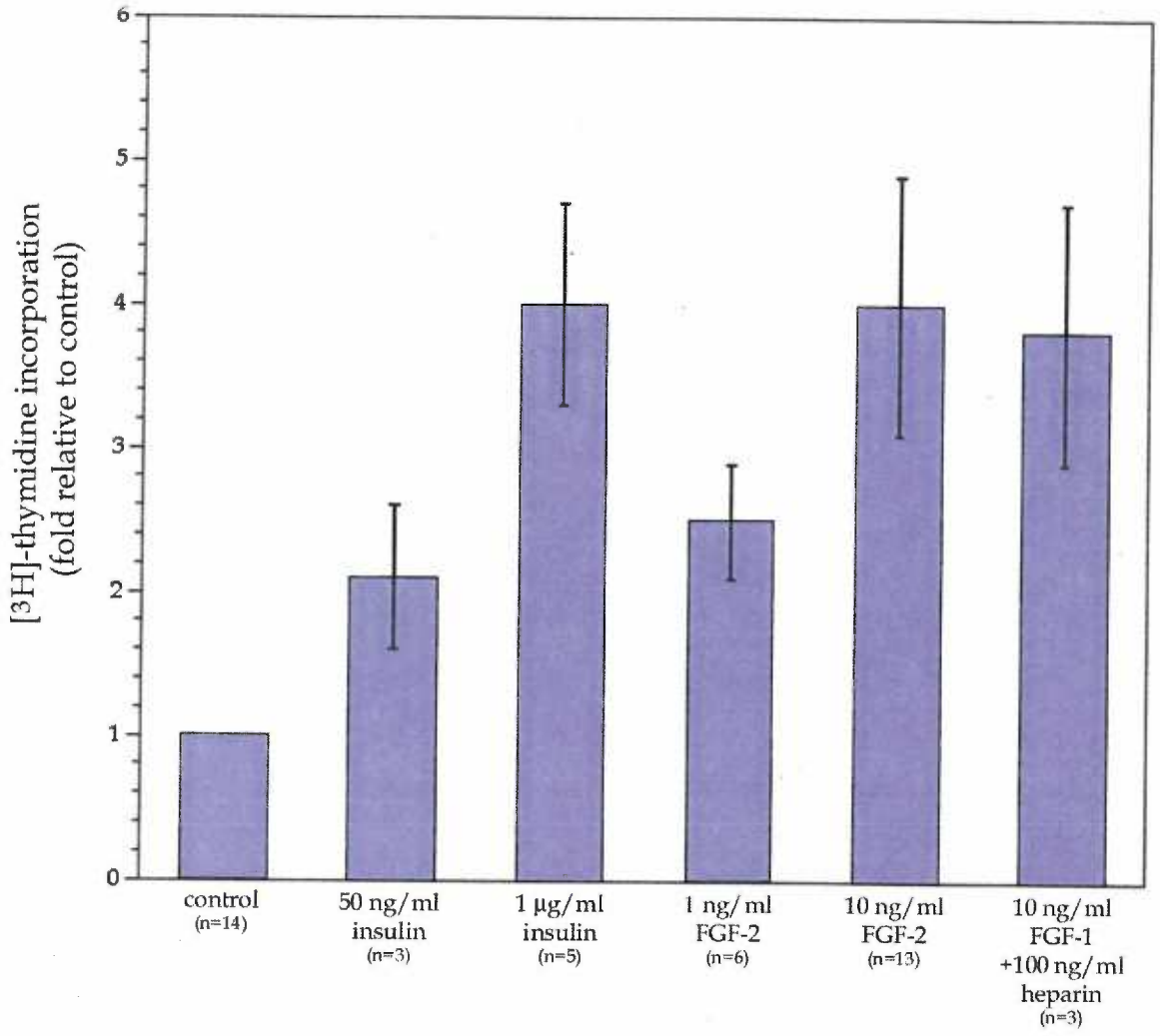

Figure 3. *FGF stimulates DNA synthesis in embryonic chick lens dissociated cell-derived monolayer (DCDML) cultures.* Dissociated cell-derived monolayer cultures were prepared from E10 chick lenses as described in Materials and Methods and plated in M199/BOTS medium in either the absence (control) or presence of the indicated growth factor. Approximately 16 h after plating, [^{3}H]-thymidine was added directly to the culture medium and the cells labeled for 12 h. The data are presented as the fold increase in tritiated thymidine incorporation in the presence of added growth factor relative to [^{3}H]-thymidine incorporation in untreated controls within the same experiment; each condition was tested in triplicate in each experiment. The n-value in parentheses indicates the number of independent experiments. Untreated control DCDML cultures incorporated an average of 16665 counts per minute of [^{3}H]-thymidine per well.

in undifferentiated explants (8-10%; Figure 2A), in keeping with the higher expression of δ-crystallin in peripheral relative to central epithelial regions of the lens (Piatigorsky *et al.*, 1972). Synthesis of δ-crystallin was increased to greater than 25% of total labeled protein if monolayers were cultured for 6 days in the presence of either 1 μg/ml insulin, 15 ng/ml IGF-1, 10 ng/ml FGF-2, or 10 ng/ml FGF-1 and its cofactor heparin. FGF-2 at 1 ng/ml only minimally stimulated δ-crystallin expression despite its ability to significantly enhance cell proliferation (see Figure 3). Moreover, no increase in [^{35}S]methionine-labeled δ-crystallin was detectable on day 6 of culture if FGF was removed on day 3. Continuous exposure to levels of FGF-2 or FGF-1 greater than those sufficient to elicit cell proliferation is also a requirement for induction of fiber-specific proteins in rodent lens cells (Klok *et al.*, 1998) (McAvoy and Chamberlain, 1989).

Low levels of CP49 were detectable by Western blot in control (no added growth factor) dissociated cell-derived monolayers, as expected given that CP49 expression is initiated in the peripheral regions of the lens (Ireland *et al.*, 1997) (Figure 4B). Expression of CP49 was upregulated by either 1 mg/ml insulin, 15 ng/ml IGF (data not shown), or 10 ng/ml FGF-2, whereas 1 ng/ml FGF had no significant effect. The increase in CP49 expression in response to 10 ng/ml FGF-2 was comparable to that elicited by cyclic AMP analogues, an experimental treatment known to uncouple upregulation of CP49 from morphological differentiation of cultured chick lens epithelial cells (Ireland *et al.*, 1997).

Epithelial-to-fiber differentiation in dissociated cell-derived monolayers cultured in the presence of FCS is associated with the formation of lentoids, clusters of enlarged cells that express high levels of the lens fiber-specific integral membrane protein MP28 (Menko *et al.*, 1984). The heterogeneity in lentoid size, the "background" of spontaneous lentoidogenesis in unsupplemented cultures, and intra- as well as inter- experimental variation precluded a meaningful quantitation of the effect of growth factors on lentoid formation under serum-free conditions. Qualitatively, however, it appeared that cells maintained in 1μg/ml insulin or (data not shown) 15 ng/ml IGF had bigger and more

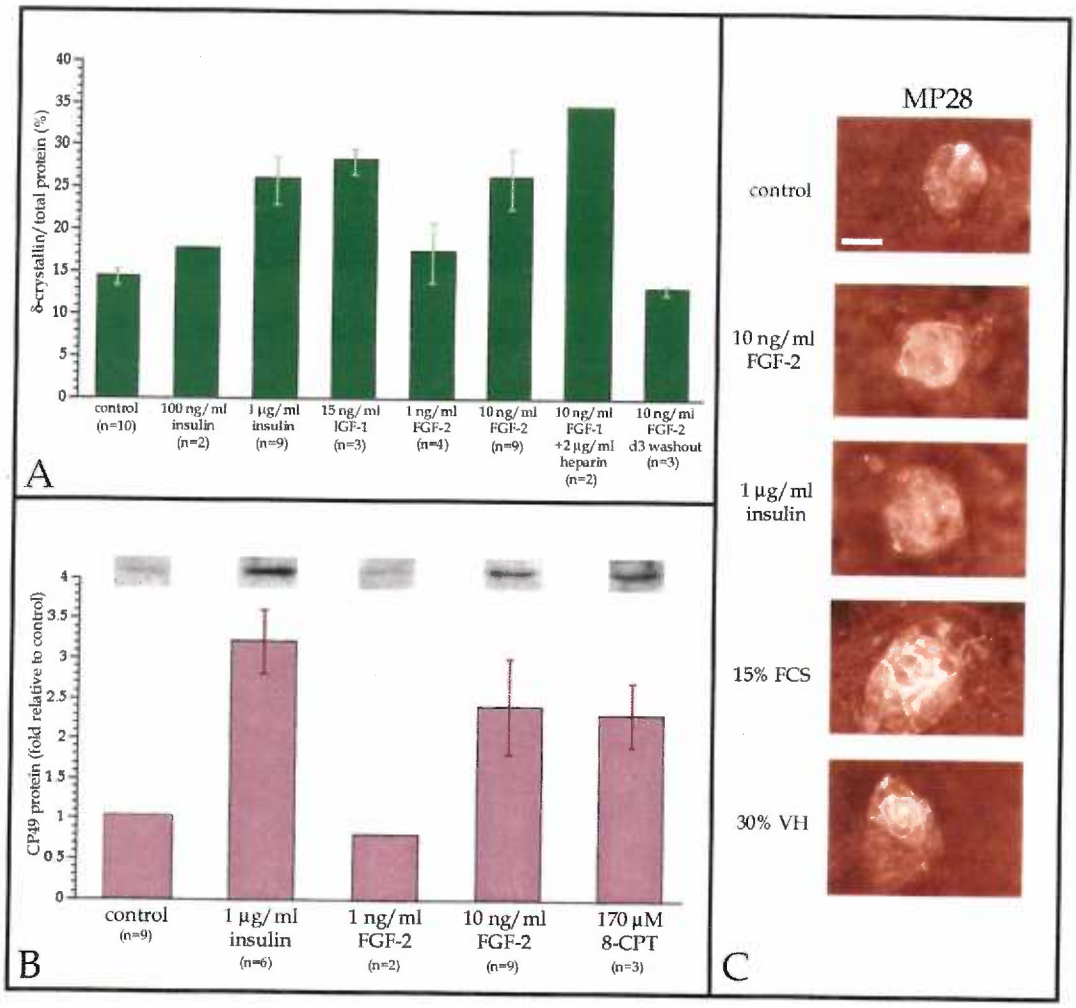

FIG. 4. FGF induces epithelial-to-fiber cell differentiation in embryonic chick lens dissociated cell-derived monolayer (DCDML) cultures. (A) DCDMLs were prepared from E10 chick lenses and cultured in M199/BOTS medium without additions for 6 days (control), with the indicated growth factor for 6 days, or with 10 ng/ml FGF-2 for 3 days followed by removal of extracellular FGF with heparin and 3 more days of culture in the absence of exogenous growth factor (d3 washout). Cells were then metabolically labeled with [35S]-methionine for 4 h, solubilized in SDS, and analyzed by SDS-PAGE. The amount of radiolabeled d-crystallin synthesized is expressed as the percent of total [35S]-methionine-labeled protein. Each bar represents the average of three or more experiments (n= number of experiments) except for the following, which are the average of two independent experiments: 100 ng/ml insulin (d crystallin/total protein = 16%; 19%) and 10 ng/ml FGF-1 + 2 mg/ml heparin (d-crystallin/total protein = 31.7%; 39.5%). (B) DCDMLs were cultured for 6 days in the absence (control) or presence of insulin, FGF-2, or the nonhydrolyzable cAMP analogue 8-CPT (8-(4 chlorphenylthio)-cyclic AMP) as indicated. Cells were solubilized in SDS, whole cell lysates (1 mg protein/lane) probed for CP49 by immunoblotting, and CP49 bands quantitated by densitometry. The data are expressed as the amount of CP49 protein in treated cells relative to the amount of CP49 in untreated controls within the same experiment; a typical blot from which these values were obtained is shown above the bar graph. Each bar represents the average of three or more experiments (n= number of experiments) except for 1 ng/ml FGF-2, which is the average of two independent experiments (CP49 protein= 0.6-fold and 0.9-fold relative to untreated control). (C) Anti-MP28 immunostaining of DCDMLs cultured for 6 days with no additions (control), FGF-2, insulin, fetal calf serum (15% FCS), or vitreous humor (30% VH) as indicated. Note that under each condition the fiber differentiation marker MP28 is concentrated in lentoids. Bar = 20 um.

numerous lentoids than either unsupplemented or FGF-treated cultures, but not as large or as many lentoids as cells grown in 15% fetal calf serum (Figure 4C). The failure of FGF to upregulate lentoids is in contrast to its effect on expression of d-crystallin and CP49 (Figure 4 A, B). A lack of correlation between the acquisition of biochemical markers of differentiation and lentoid development has previously been described in other cultured lens cells (see Discussion). Note, however, that in central epithelial explants the effects of FGF and fetal calf serum on cell elongation were comparable (Figure 2C).

Role of the ERK MAP kinase cascade in growth factor signalling in the chick lens. Taken together, the studies described in Figures 1-4 demonstrated that both FGFs and insulin/IGF-type growth factors promote cell proliferation and fiber protein expression in cultured chick lens epithelial cells. A fundamental question is whether the two classes of factors exert their effects on lens cells via the same or different signaling mechanisms downstream of receptor activation. The ERK subclass of MAP kinases is involved in the growth factor-mediated proliferation and/or differentiation of many cell types (reviewed in Lewis *et al.*, 1998). Although its role in lens development was not addressed, Chow *et al.* (1995) have shown that FGF stimulates the ERK pathway in intact rodent lens. As a first step towards determining how growth factors affect lens cell fate, we compared the ability of FGF and insulin to activate the ERK cascade in cultured lens cells.

The kinase activity of ERK1 and ERK2 is upregulated by phosphorylation of a conserved Thr-Glu-Tyr motif by the upstream kinases MEK1 and MEK2, an event that confers recognition by antibodies (anti-pERK) specific for the dually phosphorylated forms (Khokhlatchev *et al.*, 1997). Acquisition of anti-pERK immunoreactivity accurately reflects activation of the ERK cascade in chick lens cells as assessed in a series of control experiments (Figure 5A). Transient transfection of dissociated cell-derived chick lens monolayer cultures with a constitutively active form of MEK1(S217E/S221E) (Cowley *et al.*, 1994) greatly increased the immunoreactivity of whole cell lysates with

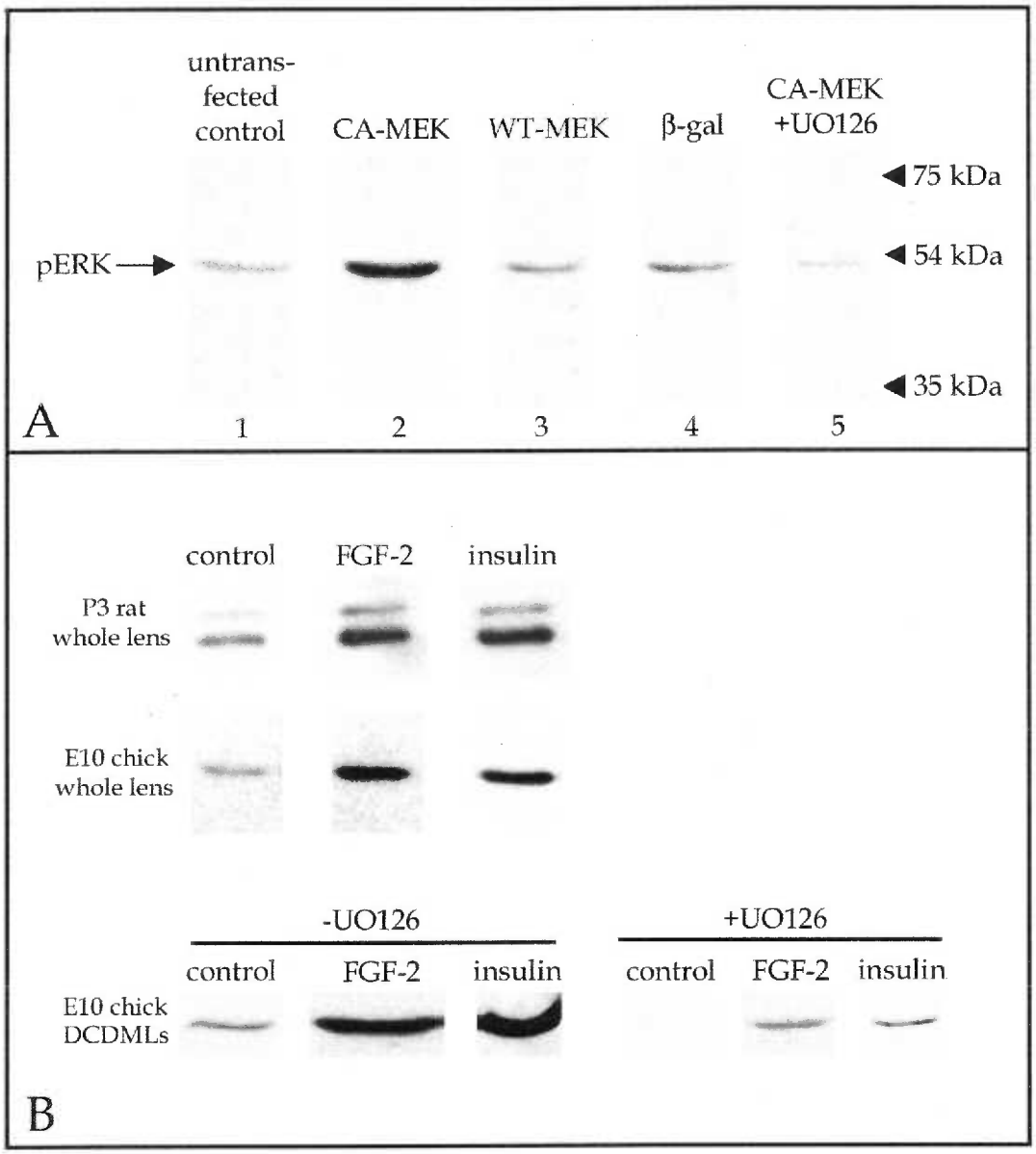

Figure 5. *Activation of the ERK MAP kinase cascade by growth factors in lens cells.* (A) Dissociated cell-derived monolayers were prepared from E10 chick lenses and transfected the day after plating with plasmids encoding a mutant form of MEK1 that constitutively activate ERKs (CA-MEK), wild-type MEK1 (WT-MEK), or an irrelevant transfection control (β-galactosidase). 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. One culture was supplemented with the MEK inhibitor UO126 (15 μM) three hours after transfection with the CA-MEK plasmid (lane 5). Molecular mass markers are indicated on the right. pERK, activated ERK. (B) Intact lenses from postnatal day 3 (P3) rats, intact lenses from embryonic day 10 (E10) chicks, or E10 chick DCDMLs cultured for 3 days in M199/BOTS were incubated for 15 min at 37°C with no additions (control), 15 ng/ml FGF-2, or 1 μg/ml insulin. The samples were then immediately solubilized in SDS and whole cell lysates assessed for activation of ERK as described in panel A. Where indicated (+UO126), E10 chick DCDML cultures were pretreated with 15 μM UO126 for 30 min prior to the 15 min incubation. Note that rodent lenses possess both the 44 kD (ERK1) and 42 kD (ERK2) MAP kinase species whereas chick lens expresses only the latter.

anti-pERK antibodies on Western blots (lane 2). In contrast, expression of wild-type MEK1 (lane 3) or a β-galactosidase control (lane 4) to the same level as the constitutively active MEK did not appreciably change anti-pERK reactivity. The ability of the constitutively active MEK1 construct to upregulate ERK phosphorylation was completely blocked by 15 mM UO126, a potent, cell permeable, nontoxic, and highly specific inhibitor of MEK1/2 and therefore of the ERK cascade (Favata *et al.*, 1998; Maher, 1999) (lane 5).

As assessed by anti-pERK Western blot analysis, both FGF-2 (1 ng/ml- 25 ng/ml) and insulin (100 ng/ml-1 μg/ml) greatly stimulated ERK activation within 15 minutes of addition to intact lenses from either P3 neonatal rats or E10 embryonic chicks (Figure 5B). The rapidity of the response indicated that the lens capsule is freely permeable to both classes of growth factors. FGF and insulin also increased anti-pERK immunoreactivity in embryonic chick lens dissociated cell-derived monolayer cultures (DCDMLs; Figure 5B) and in central epithelial explants (data not shown). As expected, activation of ERK by growth factors was completely blocked by pretreatment of cells with the MEK inhibitor UO126. Experiments in dissociated cell-derived monolayers demonstrated that this block persisted for at least eight days, yet was reversible within 15 minutes of UO126 wash-out (data not shown). As detected by indirect immunofluorescence with anti pERK antibodies, virtually 100% of the cells in the culture activated ERK in response to either FGF or insulin in a UO126-sensitive manner (not shown).

We used UO126 to test whether activation of ERKs is required for any of the effects of FGF and/or insulin on cultured lens cells. To assess the dependence of growth factor-induced proliferation on the ERK cascade, cells were plated and cultured overnight in the absence of any additions to permit progression through any cell cycles that had been initiated *in vivo*. The cells were then incubated for 12 hours with [^{3}H]-thymidine in either the absence or presence of growth factor, with or without UO126 pretreatment (Figure 6A). UO126 reduced the level of DNA synthesis in otherwise unsupplemented cultures,

most likely because of its effect on basal ERK activation (see Figure 5B) (Bost *et al.*, 1997). The attenuation of proliferation was not associated with increased cell death or with a reduction in total protein synthesis and was completely reversible upon reagent wash-out (not shown). Relative to cells treated with UO126 alone, UO126 did not significantly diminish the ability of 15 ng/ml FGF-2 or 1 μg/ml insulin to stimulate [^{3}H]-thymidine incorporation (Figure 6A). UO126 also failed to reduce FGF-simulated DNA synthesis in cells cultured for 5 days in unsupplemented M199 prior to addition of [^{3}H]-thymidine and the growth factor, indicating that mitoses initiated long after removal of cells from the *in vivo* environment were still ERK-independent (data not shown). Given that the cell cycle in embryonic chick lens epithelial cells is approximately 18 hours (Brewitt *et al.*, 1992), it is therefore unlikely that proliferation is a two-step process in which cells undergo an ERK-requiring priming step *in vivo* that renders subsequent growth factor-induced cell division in culture ERK-independent.

UO126 was next utilized to assess the role of ERK activation in the expression of fiber differentiation markers. Dissociated cell-derived monolayers were cultured in the continuous presence of growth factor and UO126 for 6 days prior to analysis of δ-crystallin and CP49 levels. UO126 did not appreciably affect basal expression of either δ-crystallin or CP49 in control (no added growth factor) cultures (not shown), nor the stimulation of CP49 synthesis by the cAMP analogue 8-CPT (Figure 6C). In contrast, UO126 blocked upregulation of CP49 in response to either FGF or insulin (Figure 6C). The two growth factors differed, however, in the pathways by which they increased δ-crystallin expression: whereas ERK activation was required for insulin or IGF-1 to significantly enhance δ-crystallin synthesis, UO126 only slightly reduced the efficacy of FGF (Figure 6B). This dissimilarity indicates that FGF and insulin/IGF-type growth factors affect lens cell fate by nonidentical pathways.

Chick vitreous humor is an *in vivo* source of an FGF-like lens differentiation factor. In order for FGF to play a physiologically important role in lens differentiation, it

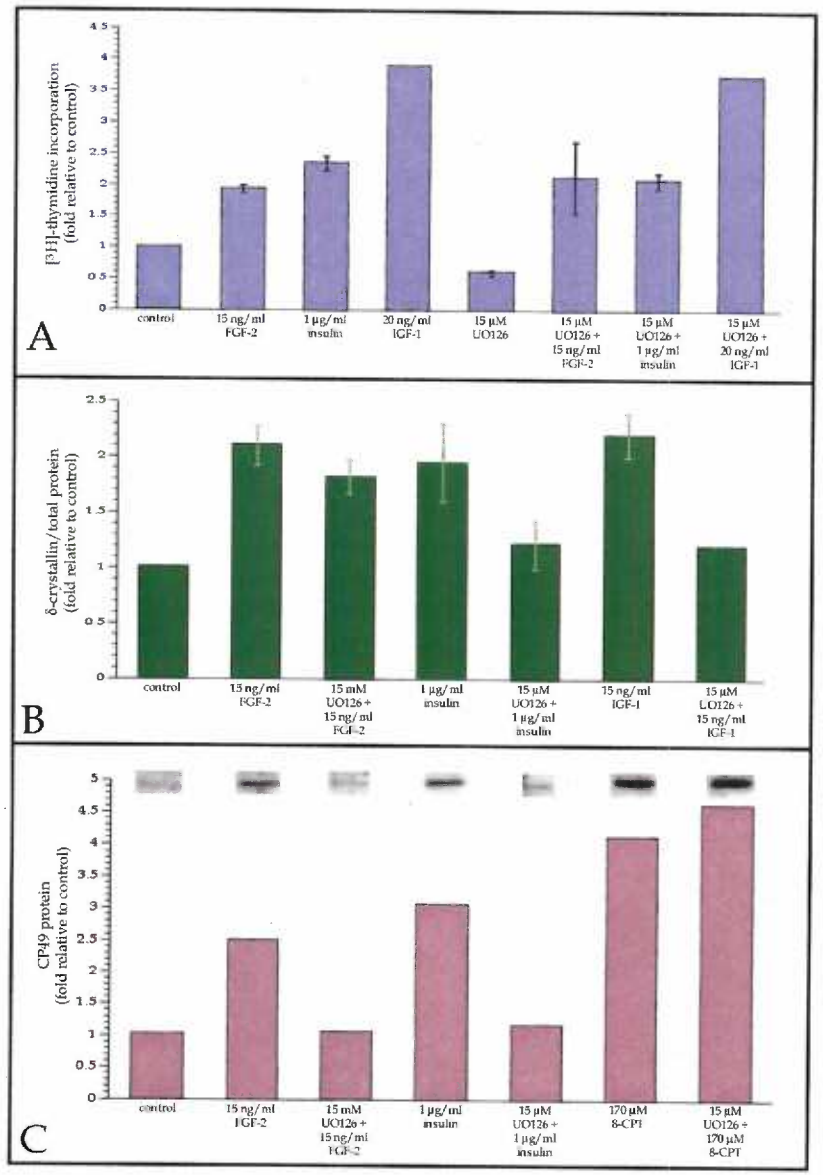

Figure 6. *FGF and insulin influence chick lens cell fate through non-identical mechanisms.* Dissociated cell-derived monolayer cultures were prepared from E10 chick lenses and plated in unsupplemented M199/BOTS medium. The day after plating, the medium was replaced with fresh M199/BOTS with or without 15 µM UO126 and the cultures incubated for 30 min. (A) After 30 min, [^{3}H]-thymidine and the indicated growth factor were added and the incubation continued for 12 h. The data are presented as the fold increase in tritiated thymidine incorporation in the presence of the indicated compound relative to [^{3}H]-thymidine incorporation in either untreated controls (for FGF-2, insulin, IGF-1, and UO126), or controls treated with UO126 alone (for UO126+ FGF-2, UO126 + insulin, and UO126 + IGF-1). Each bar represents the average of at least three experiments except for the following, which are the average of two separate experiments: 20 ng/ml IGF-1 ([^{3}H]-thymidine incorporation = 4.3-fold and 3.5-fold over untreated controls) and 20 ng/ml IGF-1 + 15 µM UO126 ([^{3}H]-thymidine incorporation = 3.4-fold and 4.1-fold over UO126-only controls). Each condition was tested in triplicate in each experiment. (B) After 30 min, the indicated growth factor was added and the incubation continued for 6 days. The cultures were then metabolically labeled with [^{35}S]-methionine for 4 h, and the amount of radiolabeled δ-crystallin quantitated and expressed as the percent of total [^{35}S]-methionine-protein synthesized as described in Fig 4A. The data are presented as the fold increase in δ-crystallin expression in cells cultured with the indicated additive(s) relative to δ-crystallin expression in untreated controls. Each bar represents the average of three or more experiments, except for 15 mM UO126 + 15 ng/ml IGF-1, which is the average of two separate experiments (δ-crystallin/total protein = 1.1-fold and 1.3-fold over control). Note that inhibition of ERK activation abolishes upregulation of δ-crystallin synthesis in response to insulin or IGF-1 but not to FGF. (C) After 30 min, the indicated growth factor was added and the incubation continued for 6 days. The cultures were solubilized in SDS and whole cell lysates (1 µg protein/lane) probed for CP49 by immunoblotting. CP49 bands were quantitated by densitometry and the data expressed as described in Fig 4B.

must be present in the ocular environment in sufficient amounts and in an appropriate location to stimulate fiber formation. In the mammalian lens, FGF (derived in part from the retina) is concentrated in the vitreous humor, from which it can be purified using heparin-Sepharose chromatography (Caruelle *et al.*, 1989; Schulz *et al.*, 1993). FGF is also present in, and can be purified from, chick vitreous humor (Mascarelli *et al.*, 1987), but was not previously considered a possible differentiation factor in this species because of the reported failure of purified FGF to stimulate elongation of E6 chick lens central epithelial cell explants (Beebe *et al.*, 1987). In light of our finding that avian lens cells respond to FGF in longer-term (> 5 h) differentiation assays, we reinvestigated the potential function of FGF in chick vitreous humor.

We first characterized the effect of vitreous humor on dissociated cell-derived embryonic chick lens cultures (Figure 7). A 12,000 X g supernatant of homogenized vitreous bodies was prepared from E10 chick embryos and (after a 3.3-fold dilution in unsupplemented M199 culture medium) assayed for differentiation-promoting activity. Over a 6-day period, the vitreous humor supplemented medium (referred to as 30% VH/M199) significantly increased the expression of both δ-crystallin and CP49 (Figure 7). The enhancement of δ-crystallin synthesis (to ~21% of total labeled protein) was very similar to that previously reported by (Beebe *et al.*, 1980) after culture of E6 embryonic chick lens central epithelial explants in 90% autologous vitreous humor (25% in 48 hours). Although 30% VH/M199 supported the formation of MP28-positive lentoids, these structures were typically not as large or numerous as in insulin-treated cells but instead resembled those in FGF treated cultures (see Figure 4C). Note, however, that FGF and vitreous humor both strongly stimulated cell elongation in the explant system (Figure 2C).

Vitreous humor is the liquid component of the gel-like vitreous body. A critical consideration is whether the differentiation-promoting activity of vitreous humor is capable of diffusing out of the vitreous body to affect lens cell fate. To examine this issue,

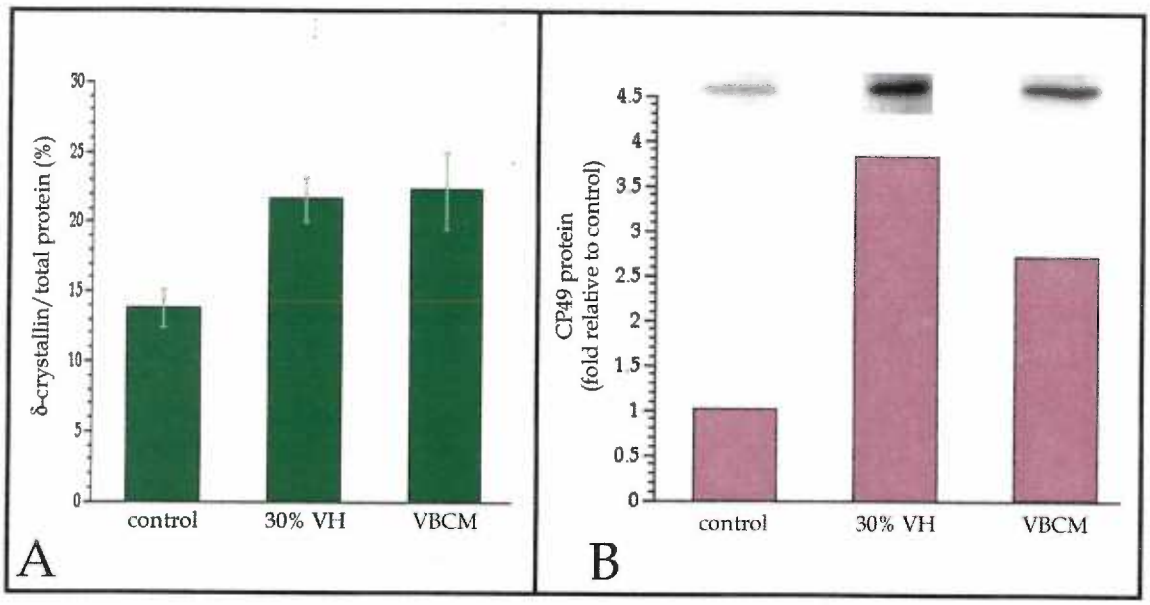

Figure 7. *Chick vitreous induces epithelial-to-fiber cell differentiation in embryonic chick lens dissociated cell-derived monolayer (DCDML) cultures.* DCDML cultures were prepared from E10 chick lenses and plated overnight in unsupplemented M199/BOTS medium. The next day, the medium was replaced with fresh M199/BOTS (control), E10 chick vitreous humor diluted with 2.3 volumes of M199/BOTS (30% VH), or M199/BOTS conditioned with intact E10 chick vitreous bodies (VBCM) as described in Materials and Methods. (A) On day 6 of culture, the cells were metabolically labeled with [^{35}S]-methionine for 4 h and the amount of radiolabeled δ-crystallin synthesized expressed as the percent of total [^{35}S]-methionine-labeled cellular protein. Each bar represents the average of three experiments. (B) On day 6 of culture, the cells were solubilized in SDS and whole cell lysates (1 μg protein/lane) probed for CP49 by immunoblotting. CP49 bands were quantitated by densitometry and the data expressed as in Fig 4B.

intact vitreous bodies were removed from E10 chick eyes and placed in the upper compartment of a Transwell chamber containing unsupplemented M199. After an overnight incubation, the medium was removed from the lower compartment and tested for its effect on the synthesis of δ-crystallin and CP49 in dissociated cell-derived monolayer cultures. As shown in Figure 7, vitreous body-conditioned medium (VBCM) increased the expression of both fiber markers to an extent comparable to 30% VH/M199.

The effect of vitreous humor on the morphology of cultured chick lens cells (induction of cell elongation in explants; failure to stimulate lentoid formation in dissociated cell-derived monolayers) more closely resembled that of purified FGF than of either insulin or IGF. Supporting evidence that the differentiation-promoting activity of chick vitreous humor was more FGF-like than insulin/IGF-like came from two additional experiments (Figure 8 and Figure 9). Based on the results shown in Figure 6B, the ability of vitreous humor to increase δ-crystallin expression would be expected to be effectively blocked by the MEK inhibitor UO126 if mediated by an IGF/insulin-type factor, but only minimally reduced if an FGF were responsible. Anti-pERK immunoblotting revealed that 30% VH/M199 robustly activated ERK2 in dissociated cell-derived chick lens monolayer cultures in a UO126-inhibitable manner (Figure 8A). Despite its potency in preventing ERK activation, UO126 did not reduce the ability of vitreous humor to upregulate δ-crystallin expression (Figure 8B). A similar result was obtained in an experiment with vitreous body conditioned medium (data not shown).

As a final test of the FGF-like nature of the δ-crystallin-promoting activity of chick vitreous humor, we examined its ability to interact with heparin-conjugated beads (Figure 9). A defining characteristic of all members of the FGF family is their high affinity for heparin, heparin sulfate, and heparin sulfate proteoglycans (Ornitz, 2000). Control experiments verified that the ability of M199 medium containing 15 ng/ml recombinant FGF to stimulate expression of δ-crystallin was abolished if the medium was preabsorbed with heparin-Affigel in 0.1 M NaCl. In contrast, this treatment had no effect

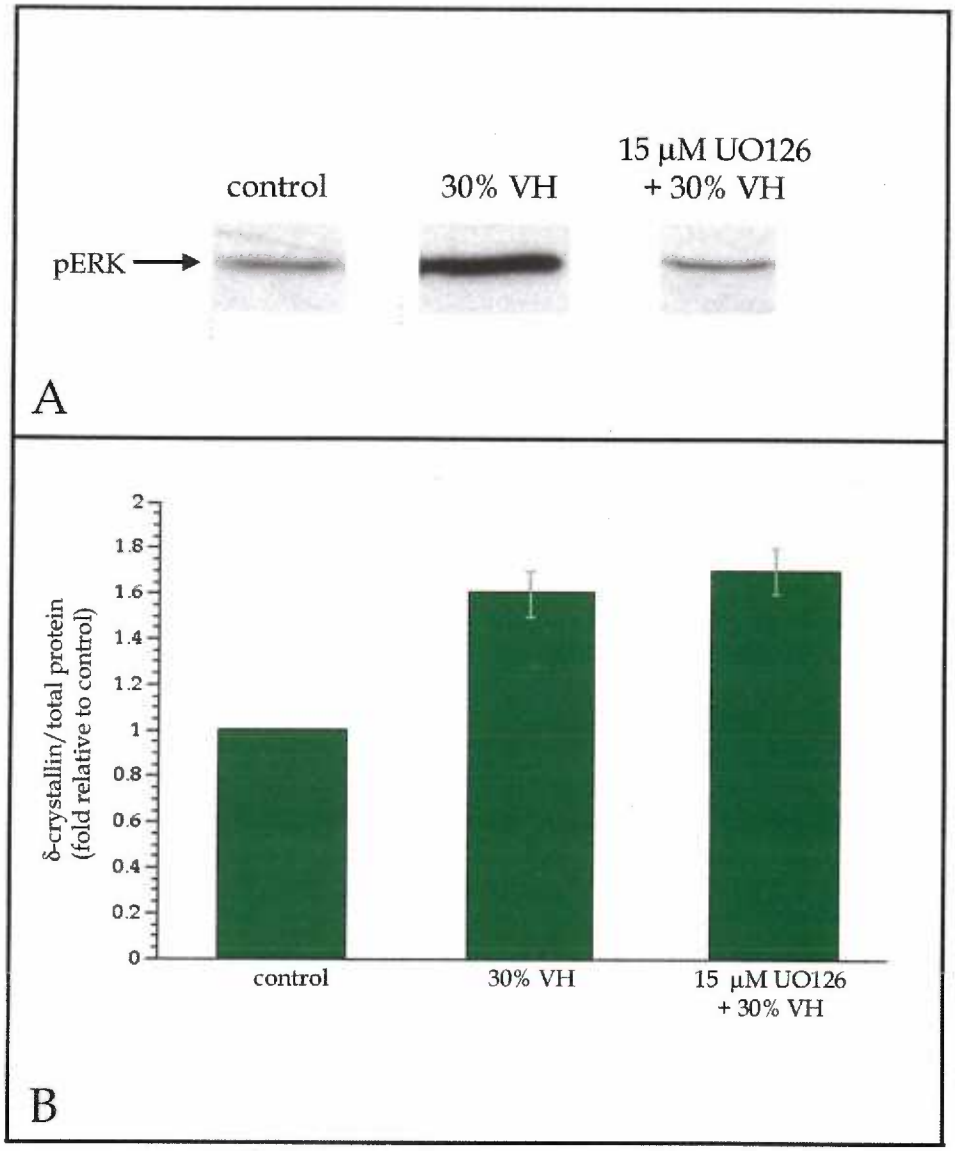

Figure 8. *Upregulation of δ-crystallin synthesis by chick vitreous is ERK-independent.* (A) Dissociated cell-derived monolayers (DCDMLs) prepared from E10 lenses were cultured for three days in M199/BOTS and then incubated for 15 min in fresh M199/BOTS (control), 15 min in M199/BOTS containing 30% vitreous humor (30% VH), or 30 min with 15 μM UO126 followed by 15 min in M199/BOTS containing 15 μM UO126 and 30% vitreous humor (15 μM UO126 + 30% VH). The cells were lysed and assessed for activation of ERK as described in Fig 5. (B) DCDMLs were plated in M199/BOTS medium. The day after plating, the cells were incubated for 30 min with or without 15 μM UO126, after which the medium was replaced with fresh M199/BOTS (control), M199/BOTS containing 30% vitreous humor (30% VH), or M199/BOTS containing 15 mM UO126 and 30% vitreous humor (15 μM UO126 + 30% VH). The cells were cultured for 6 days and then metabolically labeled with [^{35}S]-methionine for 4 h. The amount of radiolabeled δ-crystallin synthesized was quantitated as described in Fig 6B. Each bar represents the average of three or more independent experiments.

on the competence of either 1 μg/ml insulin or 15 ng/ml IGF-1 to upregulate δ-crystallin synthesis (data not shown). The δ-crystallin-promoting activity of 30% VH/M199 or vitreous body conditioned medium was quantitatively removed when incubated with immobilized heparin in salt concentrations as high as 0.6 M NaCl, but did not bind to unconjugated Affigel beads. The activity was largely recovered from the heparin beads by incubation in 2.5 M NaCl (Figure 9). This elution behavior is typical of an FGF (Mascarelli *et al.*, 1987; Schulz *et al.*, 1993; Seed *et al.*, 1988) and distinguishes the δ-crystallin promoting activity of chick vitreous humor from either PDGF or vEGF, both of which bind to heparin with a lower affinity than FGFs (Sakurada *et al.*, 1996; Vlodavsky *et al.*, 1987). Identification of the operative FGF species in vitreous humor was precluded by the unavailability of blocking antibodies capable of selectively neutralizing the activity of each of the 18 known members of the FGF family.

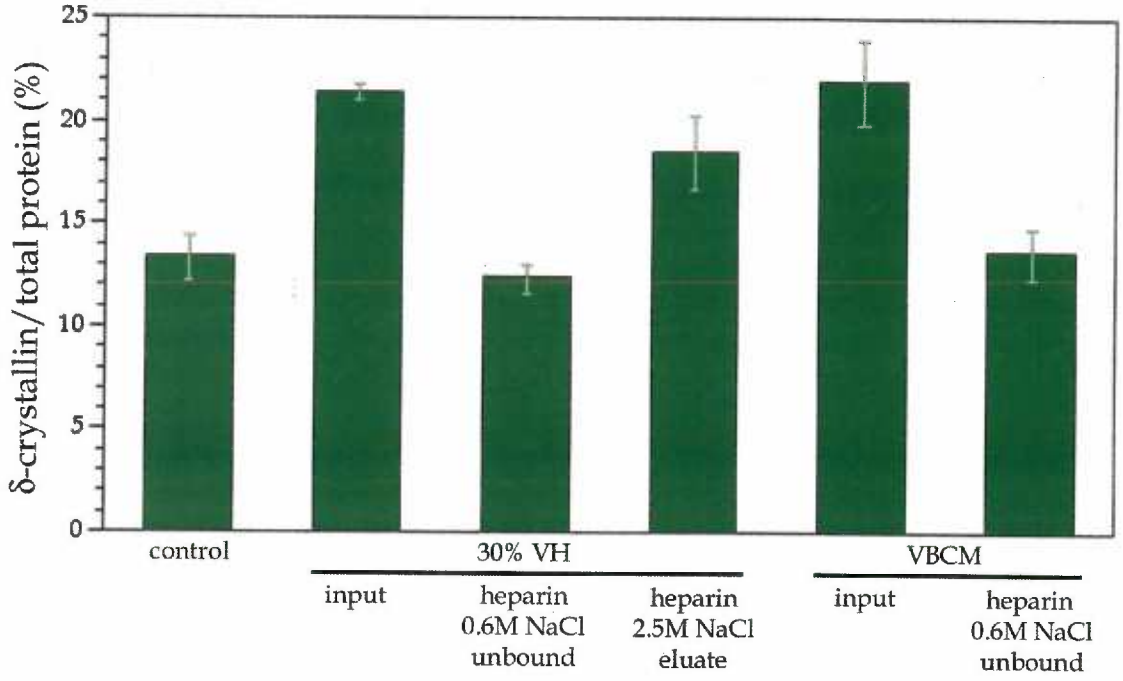

Figure 9. *The δ-crystallin-promoting activity of chick vitreous has FGF-like properties.* E10 chick vitreous humor diluted with 2.3 volumes of M199/BOTS (30% VH), or M199/BOTS conditioned with intact E10 chick vitreous bodies (VBCM), was incubated with heparin-conjugated Affigel beads in the presence of 0.6 M NaCl as described in Materials and Methods. The beads were pelleted, and after removal of the supernatant (= heparin 0.6 M NaCl unbound), FGF-like activity was eluted with 2.5 M NaCl (= heparin 2.5 M NaCl eluate). The fractions were brought to 0.15 M NaCl by repeated rounds of concentration and dilution with M199 medium. Dissociated cell-derived monolayers (DCDMLs) were cultured for six days in M199/BOTS (control), unfractionated 30% VH (30% VH input), unfractionated VBCM (VBCM input), or the indicated heparin-Affigel fraction for 6 days. The amount of radiolabeled δ-crystallin synthesized was quantitated as described in Fig 6B. Each bar represents the average of three or more independent experiments.

5. Discussion

Although the key processes in lens development were first recapitulated in culture over 30 years ago (Philpott and Coulombre, 1965), the molecular mechanisms underlying these events have not been elucidated. A fundamental issue is the identity of the stimuli that regulate lens epithelial cell proliferation and differentiation into fiber cells *in vivo*. In mammals, both *ex vivo* and *in vivo* data strongly indicate (although do not unequivocally prove) that one or more members of the FGF family are involved in secondary fiber formation (see Introduction). In contrast, FGF-2 was reported to have no effect on explanted central epithelium from embryonic chick lenses despite the presence of both FGF and FGF receptors in the avian lens (Beebe *et al.*, 1987). Instead, insulin/IGF-type factors were proposed to act as differentiation agents, whereas neither insulin, IGF-1, nor IGF-2 induce epithelial-to-fiber differentiation in cultured rodent lenses (Beebe *et al.*, 1987; Hyatt and Beebe, 1993; Lang, 1999; Richardson *et al.*, 1993). These findings are consistent with any of three mutually exclusive possibilities. First, the epithelial-to-fiber differentiation program in birds and mammals may be controlled by fundamentally different systems (IGF/IGF receptors and FGF/FGF receptors, respectively) despite the overall similarity in the process of lens development among vertebrate species. Second, fiber differentiation in chick and mammalian lenses may be mediated by the same factor(s), but this factor is neither an FGF nor an insulin/IGF. In this model, FGF (but not insulin/IGF) is sufficient, but not necessary, for mammalian fiber differentiation; conversely, insulin/IGF (but not FGF) is sufficient, but not necessary, for the same process in the chick lens. The third possibility is that the reported differences between chick and mammalian lenses in growth factor response is more apparent than real, reflecting differences in experimental design or interpretation. In the current study, we rule out the first two scenarios by demonstrating that chick lens cells are capable of proliferating and upregulating the expression of fiber cell markers in response to FGF, provided they are

incubated with the growth factor for periods exceeding the 5 hours maximum used in previous investigations. Such longer-term treatments have been routinely used in studies with mammal-derived lens cultures and are physiologically relevant given that the lens is continuously exposed to growth factors in the ocular environment.

Effect of FGF on epithelial-to-fiber differentiation in chick lens cells. We tested the effect of FGF-2 and FGF-1 on two previously characterized model systems of chick lens development: central epithelial explants and dissociated cell-derived monolayer cultures. In explants, FGF upregulated each of the markers of epithelial-to-fiber differentiation assayed to an extent equal to (expression of CP49) or greater than (stimulation of δ-crystallin synthesis; cell elongation) insulin or IGF-1 (Figure 2). In monolayer cultures, the effects of FGF on δ-crystallin and CP49 levels were also comparable to those of insulin/IGF (Figure 4 A and B). Unlike insulin/IGF, however, FGF failed to increase the number or size of lentoids, the clusters of MP28-expressing, enlarged cells commonly associated with epithelial-to-fiber differentiation in dissociated cell-derived cultures (Figure 4C). Given that the mechanisms underlying cell enlargement during fiber differentiation remain obscure, it is not possible to molecularly dissect the basis for this phenomenon. An imperfect correlation between expression of fiber-specific proteins and cell elongation has, however, previously been described in other lens-derived culture systems. For example, exposure of neonatal rat explants to FGF-2 for only 2 hours followed by insulin induces the full spectrum of differentiation-associated crystallin expression, but fails to stimulate epithelial cell elongation in the majority of cells (Klok *et al.*, 1998). Conversely, the nontransformed N/N1135A rabbit lens epithelial cell line forms raised lentoids composed of enlarged cells that lack even the earliest of fiber differentiation markers (Kidd *et al.*, 1994). Lentoidogenesis is therefore neither a necessary nor sufficient indication of fiber differentiation. Significantly, vitreous humor, the most likely in vivo source of differentiation-promoting activity, imparts the same

morphological phenotype as FGF in both explants (extensive cell elongation; Figure 2C) and dissociated cell-derived monolayer cultures (no increase in lentoids relative to untreated controls; Figure 4C).

The ERK cascade in FGF- and insulin/IGF- stimulated lens cell proliferation and differentiation. Our studies show that FGF, insulin, and IGF-1 each stimulate both division and differentiation of cultured chick lens epithelial cells. A similar dual function has been demonstrated for FGF in rodent lens explants (McAvoy and Chamberlain, 1989) and in adrenal chromaffin cells (Claude *et al.*, 1988); IGF-1 promotes both proliferation and differentiation of rat myoblasts (Florini *et al.*, 1996) and fetal brown adipocytes (Porras *et al.*, 1998). Studies in nonlenticular systems have elucidated two mechanisms by which a single growth factor can mediate these two very different processes within the same cell type. In some cases, proliferation and differentiation are controlled by separate signaling cascades downstream of receptor activation (Coolican *et al.*, 1997; Porras *et al.*, 1998). In other systems, the same pathway is the predominant mediator of both division and differentiation, with the outcome determined by the duration and/or intensity of signaling through this pathway (reviewed by Marshall, 1995). As a first step towards determining which (if either) mechanism is operative in lens cells, we tested whether the ERK pathway was required for any of the observed effects of growth factors on dissociated cell-derived embryonic chick lens monolayer cultures. Despite their participation in a wide variety of growth factor-induced phenomena in most cell types, the role of the ERK family of MAP kinases in lens development has, to our knowledge, not previously been investigated in any species. Using an antibody that specifically recognizes the active form of ERK1/2, we have shown that FGF, insulin, and IGF all robustly activate ERKs in whole rodent and chick lenses as well as in chick lens epithelial cell cultures (Figure 5). In many, although not all (Dufourny *et al.*, 1997; Maher, 1999) cell types, FGF- and/or insulin/IGF- induced cell proliferation is ERK-dependent. It was

therefore notable that blocking ERK activity with UO126 did not diminish the ability of either FGF or insulin to stimulate [^{3}H]-thymidine incorporation in dissociated cell-derived chick lens monolayer cultures (Figure 6A). ERK activation was, however, essential for upregulation of expression of the fiber differentiation marker CP49 by either FGF or insulin (Figure 6C), indicating that the signal transduction cascades that govern differentiation in these cells are at least partially distinct from those that effect DNA synthesis. FGF and insulin/IGF differed, however, in that blocking the ERK cascade abolished the ability of insulin/IGF to upregulate δ-crystallin expression whereas induction by FGF was much less affected (Figure 6B). The latter finding indicates that FGF and insulin/IGF influence chick lens cell fate through non-identical mechanisms. *In vivo*, multiple independent pathways leading to the same endpoint may serve as back-up systems to ensure continued development of the lens should one pathway become compromised.

In vivo role of FGF and insulin/IGF-type factors in chick lens development. Our finding that cultured chick lens epithelial cells respond to purified recombinant FGF-2 and FGF-1 as well as to insulin and IGF-1 raised the question of whether one or more of these factors is likely to contribute to lens development *in vivo*. IGF-1 and both FGFs have been detected in vitreous humor (Arnold *et al.*, 1993; Caruelle *et al.*, 1989; Mascarelli *et al.*, 1987), which serves as a reservoir of various ocular growth factors and which lens inversion experiments have implicated as the most likely source of epithelial-to-fiber differentiating activity in the eye (Coulombre and Coulombre, 1963; Yamamoto, 1976). Consistent with such a function, crude vitreous humor prepared from either chick or mammalian eyes has been reported to stimulate differentiation of (respectively) chick (Beebe *et al.*, 1980) and rodent (Schulz *et al.*, 1993; Lovicu *et al.*, 1995) lens central epithelial explants. We have reproduced the effect of chick-derived vitreous humor on autologous explants (Figure 2C) and demonstrate for the first time that vitreous humor

also increases the expression of fiber cell markers in dissociated cell-derived monolayer cultures (Figure 7). Most importantly, we also show that the differentiation-stimulating activity of vitreous humor can be recovered from medium in which intact vitreous globes were incubated overnight. The active substance is therefore freely diffusible, as would be required if it were to play an important role in lens development in vivo. The epithelial-to-fiber differentiating activity of vitreous humor bound heparin with high affinity (Figure 9) and increased δ-crystallin expression in an ERK-independent manner (Figure 8). Both properties are consistent with an FGF but are distinct from those of either insulin or an IGF. Taken together, these results support a role for FGFs, but not insulin/IGF-type factors, in lens development *in vivo*. Our findings cannot, however, rule out the possibility that a heparin-binding factor other than, or in addition to, FGF is responsible for the differentiating activity of chick vitreous humor. Attempts to resolve this issue using the reportedly FGFR-selective inhibitor SU5402 (Mohammadi *et al.*, 1997) were unsuccessful because the compound also blocked processes mediated by other receptor tyrosine kinases including the IGF receptor when added to chick lens cells (data not shown). Likewise, studies in mammals strongly indicate, but do not definitively prove, a physiologically important role for FGFs in lens development (Lang, 1999). When expressed under the control of a lens-specific crystallin promoter, dominant interfering mutant forms of FGFR-1 with the capacity to inhibit signalling of all known FGF family members disrupted lens development and function in transgenic mice (Chow *et al.*, 1995; Robinson *et al.*, 1995; Stolen and Griep, 2000). Although diminished, fiber differentiation was not completely abolished. It is currently unknown whether this partial effect is a consequence of inadequate levels of expression of the transgene (especially in cells undergoing the initial stages of epithelial-to-fiber differentiation; (Stolen and Griep, 2000; Robinson *et al.*, 1995) or instead indicates that some non-FGF factor is a major determinant of lens differentiation in transgenic and/or wild-type mice. Although targeted disruption of the FGF-2 and FGF-1 genes apparently does not grossly perturb lens

development (Miller *et al.*, 2000), it remains to be determined whether any of the 16 other known FGFs is the true *in vivo* lens differentiation factor or is able to compensate for the absence of FGF-2 or FGF-1 in knockout mice. With regard to proliferation, neither our studies nor those in mammals address whether FGF or insulin/IGF serve as a physiologically important lens cell mitogen. Other growth factors, including PDGF, have been proposed to play such a role but this possibility has not been supported by the phenotypes of knock-out mice (Potts *et al.*, 1994; Potts *et al.*, 1998).

Regardless of the identity of the *in vivo* lens proliferation and differentiation factor(s), our results demonstrate that the effect of exogenously added FGF on cultured chick lens cells is very similar to that previously described for mammalian *ex vivo* systems. The ability of lens epithelial cells to transduce an FGF signal into DNA synthesis and secondary fiber formation is therefore conserved among vertebrate species. Although not directly addressed in the current work, recent studies by others have indicated that differences between chick and mammalian lens cells in their response to insulin/IGF may also be less absolute than originally proposed. Klok *et al.* (1998) and Richardson *et al.* (1993) have demonstrated that IGF-1 induces a subpopulation of cells in P3 rat lens central epithelial explants to express even late-onset markers of terminal fiber differentiation such as gamma crystallin. Ibaraki *et al.* (1995) have reported that 10 ng/ml IGF-1 is as potent as 10 ng/ml FGF-2 in stimulating morphological differentiation of human lens epithelial cells cultured in the presence of 20% fetal calf serum. These studies indicate that insulin/IGF is capable of evoking at least some aspects of fiber differentiation in mammalian cells. It is also noteworthy that Piatigorsky and colleagues have demonstrated that insulin increases epithelial-to-fiber differentiation in explants dissected from embryonic day 6-15 chicks, but only proliferation in those from E19 or older animals (Milstone and Piatigorsky, 1977; Piatigorsky and Rothschild, 1972). Given that rodent explants are routinely prepared from postnatal lenses, it is conceivable that the dissimilarity in the response of E6 chick explants and P3 rat explants to insulin/IGF

reflects age-dependent instead of species-specific differences.

6. Acknowledgments

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C. Chapter 3

Novel Role of FGF and ERK Kinase in Gap Junction-Mediated Intercellular Communication in the Lens

Anh-Chi N. Le

and Linda S. Musil

Vollum Institute for Advanced Biomedical Research and

Department of Cell and Developmental Biology

Oregon Health Sciences University

3181 SW Sam Jackson Park Road

Portland, OR 97201

1. Abstract

The vertebrate lens maintains metabolic homeostasis via an extensive network of intercellular gap junctional channels. Gap junction coupling is higher at the lens equator than at either pole, a property thought to be essential for lens transparency. Because the equatorial and polar regions express the same complement of gap junction proteins (connexins) at similar levels, the higher coupling at the equator appears to be due to greater flux through equatorial gap junctional channels. We show here that FGF (either FGF-2 or FGF-1 plus heparin) increases gap junction-mediated intercellular dye transfer in primary cultures of embryonic chick lens cells without detectably increasing connexin expression or gap junction assembly. Insulin and IGF-1, although as potent as FGF in inducing lens cell differentiation, had no effect on gap junctions. Upregulation of intercellular communication by FGF in cultured lens cells was reversible, mediated by sustained activation of ERK MAP kinases, and was mimicked by expression of a constitutively active MEK-1 mutant. We also identify the 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 or core fibers. Taken together, these findings support a model in which regional differences in FGF signalling through the ERK pathway are responsible for the asymmetry of gap junctional intercellular coupling in the lens. The lens is the first system in which ERK has been demonstrated to positively regulate gap junction permeability.

2. 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 a core of elongated, crystallin-rich fiber cells that differentiate from the epithelial cells at the lens equator (reviewed by McAvoy *et al.*, 1999; Piatigorsky, 1981; Wride, 1996). 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.

The unique optical properties of the lens are due in part to the absence of either blood vessels or nerves and to the extraordinarily tight cell-to-cell packing of the fibers that make up the bulk of the organ. The question then arises as to how this solid, ever-expanding mass of cells remains in metabolic and ionic homeostasis (and thus transparent) through a lifespan that can exceed 100 years. 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 (reviewed by Goodenough, 1992). Gap junctions are clusters of transmembrane channels that connect the plasma membranes of two adjoining cells (reviewed by Goodenough *et al.*, 1996). Substances under ~1 kDa 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. Three members of the connexin family have been identified in the lens: connexin43 (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 (Jiang *et al.*, 1994; Musil *et al.*, 1990a; Paul *et al.*, 1991; Rup *et al.*, 1993; White *et al.*, 1992). Between one-third and one-half of the cell surface of mature fiber cells is occupied by gap junctions, 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 is not uniform throughout the lens but is instead higher at the equator than at either the anterior or posterior poles of the organ (see Figure 10 for a diagram of lens anatomy). Based largely on vibrating probe current measurements (Parmelee *et al.*, 1985; Robinson and Patterson, 1982) and impedance studies (Baldo and Mathias, 1992; Mathias *et al.*, 1985), 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 follow, establishing a non-vascular microcirculatory system that brings nutrients deep into the lens and flushes out waste products. The asymmetric distribution of 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 (reviewed in Goodenough, 1992; Mathias *et al.*, 1997).

Despite its physiological significance, it is not known how the regional differences in gap junctional coupling in the lens are generated. As assessed by immunofluorescent microscopy, assembly of each of the known fiber connexins into gap junctions at the equator is comparable to that at the poles (Berthoud *et al.*, 1994; Dahm *et al.*, 1999;

Gruijters *et al.*, 1987). A study in human lens utilizing freeze-fracture electron microscopy, which detects gap junctions by their unique structural features instead of by immunoreactivity, also failed to reveal noticeable quantitative differences between equatorial and polar fibers in gap junction channel content (Vrensen and Van Marle, 1992). There is therefore no compelling evidence that the estimated 14-fold to 335-fold increase in intercellular electrical conductance at the lens equator (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. In the lens as in other organs, gap junction-mediated intercellular communication 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 (reviewed in Bruzzone *et al.*, 1996). Although in most cases the mechanisms underlying these effects 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 ERK-type MAP kinases has been reported to 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 years 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 (Hyatt and Beebe, 1993; McAvoy and Chamberlain,

1989; Schulz *et al.*, 1993). In mammals, both *ex vivo* and *in vivo* studies (including lens-specific expression of dominant-negative mutant forms of FGFR-1 in transgenic mice) strongly implicate one or more members of the FGF family in the differentiation process (Chow *et al.*, 1995; McAvoy *et al.*, 1999; Robinson *et al.*, 1995; Schulz *et al.*, 1993). 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 intact chick vitreous bodies to effect fiber formation (Le and Musil, 2000). Taken together, these studies strongly support (although do not definitively prove) a physiologically important role for FGF in both mammalian and avian lens development.

The evolutionarily conserved response of lens cells to FGF and the high concentrations of this growth factor in vitreous humor (Caruelle *et al.*, 1989; Mascarelli *et al.*, 1987; Schulz *et al.*, 1993) led us to consider whether this growth factor might be involved in the upregulation of gap junction function at the lens equator. 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 than does not involve an increase in either connexin synthesis or in gap junction assembly. Insulin and IGF-1, although comparable to FGF in their ability to induce epithelial-to-fiber differentiation, had no effect on gap junctions. Both purified recombinant FGF and the FGF-like activity of vitreous humor induced sustained activation of ERK kinases in lens cells, an event that is necessary as well as sufficient to increase 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 fibers. These studies support a model in which the distribution of FGF-induced ERK signalling in the lens plays a central role in establishing the gradient of gap junctional coupling believed to be

essential for lens transparency. Our results also identify upregulation of gap junction-mediated intercellular communication as a new function for sustained ERK activation and change the current paradigm that the ERK pathway only negatively regulates gap junction channel activity.

3. Materials and Methods

Materials. Recombinant human FGF-1 and bovine FGF-2, kind gifts from Dr. Felix Eckenstein (Oregon Health Sciences University), were prepared and assessed for biological activity as previously described (Shipley, 1986; Stock *et al.*, 1992). 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 analog of human IGF-1 with reduced affinity for IGF binding proteins, was from *GroPep* (Adelaide, Australia). The nonhydrolyzable cAMP analog 8-CPT-cAMP (8-(4-chlorphenylthio)-cyclic AMP), forskolin, and RO-20-1724 were purchased from CalBiochem. 18β-glycyrrhetinic acid 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 generously provided by Dr. James Trzaskos (DuPont Pharmaceuticals). The anti-p44/42 MAP kinase polyclonal rabbit antibody (recognizes both activated and inactive forms of ERK) and the anti-phospho-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 kindly provided by Drs. Jean Jiang (University of Texas Health Science Center) and Daniel Goodenough (Harvard Medical School) (Jiang *et al.*, 1995). 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 X 10^5 cells/well onto laminin-coated 96-well tissue culture plates as previously described in Le and Musil (Le and Musil, 1998). Cells were cultured for up to 7 days 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 two days with fresh medium. For FGF reversal experiments (Figure 2C), cells cultured for 48 hours 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. One day after plating, DCDML cultures were transfected in M199 medium without BOTS or antibiotics using Lipofectamine PLUS (GibcoBRL) 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; Pharmacia) 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 hours, the transfection medium was supplemented with BOTS, penicillin G, and streptomycin and the cells cultured for an additional 48 hours prior to 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 in 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 one minute, the culture was quickly rinsed three times with HBC and then incubated for an additional 8 minutes (Lucifer Yellow) or 2 minutes (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). 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). Unless otherwise indicated, cells were assayed on day 3 after plating.

Paracellular permeability assays. 1.8 X 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 µm) (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) was added to the upper chamber of the Transwell unit. At 2 hours, a 0.1-ml aliquot were 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 minutes, 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 anti-phospho-p44/42 MAP kinase E10 monoclonal mouse antibody followed by alkaline phosphatase-conjugated goat anti-mouse IgG as described by Le and Musil (2000). 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 reprobed with the anti-p44/42 MAP kinase polyclonal antibody 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, three-day-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 previously described (Le and Musil, 1998) and boiled for 3 minutes. 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 hour 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 minutes each), incubated for 2 hours 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 (BCIP; Sigma) and nitro blue tetrazolium (NBT; Sigma).

Microdissection and analysis of embryonic chick lenses. Lenses were excised from E13 chick embryos and incubated in unsupplemented M199 medium for 16 hours 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 hours either with or without FGF. Thereafter, the lenses were carefully rolled on a piece of Kimwipe to remove non-lenticular 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 regions (central epithelium, equatorial region, and polar and core fibers). Immediately after dissection, the fractions were boiled for 3 minutes in lysis buffer (see above). Ten micrograms of protein from the equatorial region and 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 2X 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 days in either the absence or presence of FGF were fixed and stained for Cx43, Cx45.6, or Cx56 as previously described in Le and Musil (1998).

Preparation of vitreous humor and vitreous body conditioned medium. As described in Le and Musil (2000), vitreous bodies were dissected from E10 chick eyes and either centrifuged for 10 min at 4°C at 12,000 X g to remove cells and fibrous elements (= vitreous humor), or transferred to the upper compartment 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 chamber medium (= vitreous body conditioned medium) was collected.

Fractionation of vitreous humor and FGF on heparin beads. One ml of either vitreous humor diluted with 2.3 volumes 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 (BioRad) 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 minute 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 molecular weight 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.

4. 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; Menko *et al.*, 1987; TenBroek *et al.*, 1994). This system also most faithfully reiterates fiber-type gap junction formation (FitzGerald and Goodenough, 1986; Jiang *et al.*, 1993; Menko *et al.*, 1987). Dissociated cell-derived monolayer cultures prepared from E10 chick lenses as described by Menko et al. (1984) (termed DCDMLs) are enriched in cells originating from the equator of the lens, the region that undergoes both fiber differentiation and upregulation of gap junctional intercellular coupling *in vivo*. When maintained in fetal calf serum, DCDML cultures become confluent and then differentiate into masses of elongated, gap junction- and crystallin- rich cells that are ultrastructurally indistinguishable from fiber cells *in vivo* (Menko *et al.*, 1987). We have previously shown that these cells 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, 2000). 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 Figure 1A, the membrane impermeant, low-molecular weight fluorescent dye Lucifer Yellow was introduced into DCDMLs 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

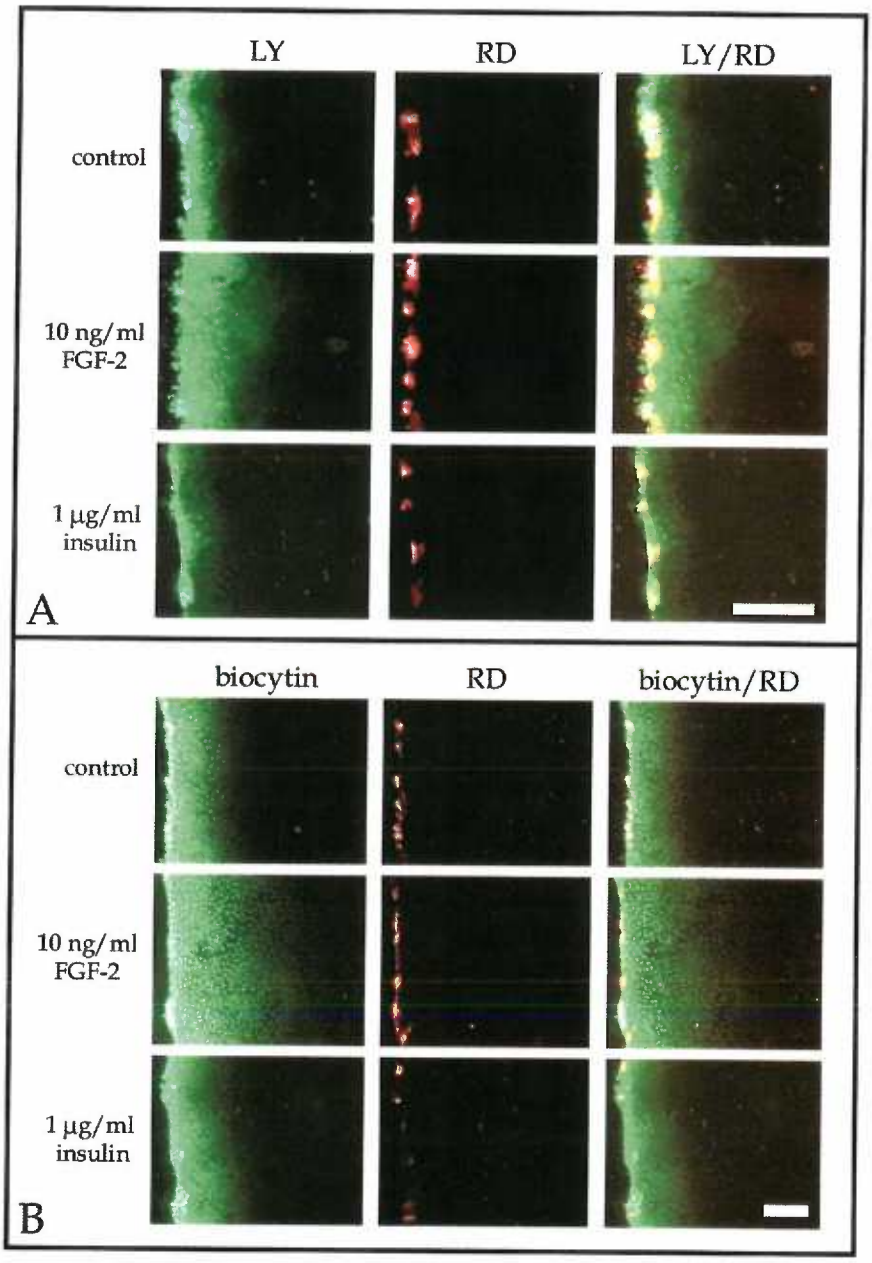


Figure 1. *FGF, but not insulin, increases gap junctional intercellular communication in chick lens dissociated cell-derived monolayer cultures (DCDMLs).* One-day-old DCDML cultures prepared from E10 chick lenses were incubated for an additional 48 h in either the absence (control) or presence of the 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 2 min (A) or 8 min (B), the cells were fixed and the dyes visualized by fluorescence microscopy. The M_r =10 kDa 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 at right (LY/RD and biocytin/RD) Bar: 100 µm for both A and B.

unwounded cells, cultures maintained in the absence of added growth factor were moderately well coupled by gap junctions. Intercellular transfer of Lucifer Yellow was clearly elevated in cells cultured for 2 days in 15 ng/ml FGF-2, even as assessed by this relatively insensitive assay. In contrast, 1 μg/ml insulin, although comparable to 15 ng/ml FGF-2 in its ability to upregulate expression of fiber differentiation markers (Le and Musil, 2000), did not detectably affect 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) (Figure 1B). The increase in intercellular dye transfer in FGF-treated cells was prevented by the gap junction blocker 18β-glycyrrhetinic acid (18β-GA) (Davidson *et al.*, 1986; Le and Musil, 1998), indicating a bona fide increase in gap junction-mediated intercellular communication (Figure 2A).

The effect of exogenously added growth factors on junctional coupling in DCDML cultures was characterized in a series of experiments summarized in Figure 2A. Upregulation of gap junction-mediated intercellular dye transfer required concentrations of FGF-2 greater that 1 ng/ml and was maximal at 10-50 ng/ml (higher doses of FGF-2 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. FGFs enhanced dye transfer to a much greater extent than 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 (Atkinson *et al.*, 1995; Saez *et al.*, 1986; van Rijen *et al.*, 2000). Despite their ability to stimulate cell proliferation and/or fiber differentiation in DCDML cultures, insulin (at up to 1 μg/ml) and the IGF-1 analogue R^3IGF-1 (at 15-50 ng/ml) failed to increase junctional transfer of either Lucifer Yellow or biocytin. Moreover, insulin neither enhanced nor interfered with the effect of FGF on gap junction activity (not shown). None of the other purified growth factors tested (TGFα, TGFβ, and

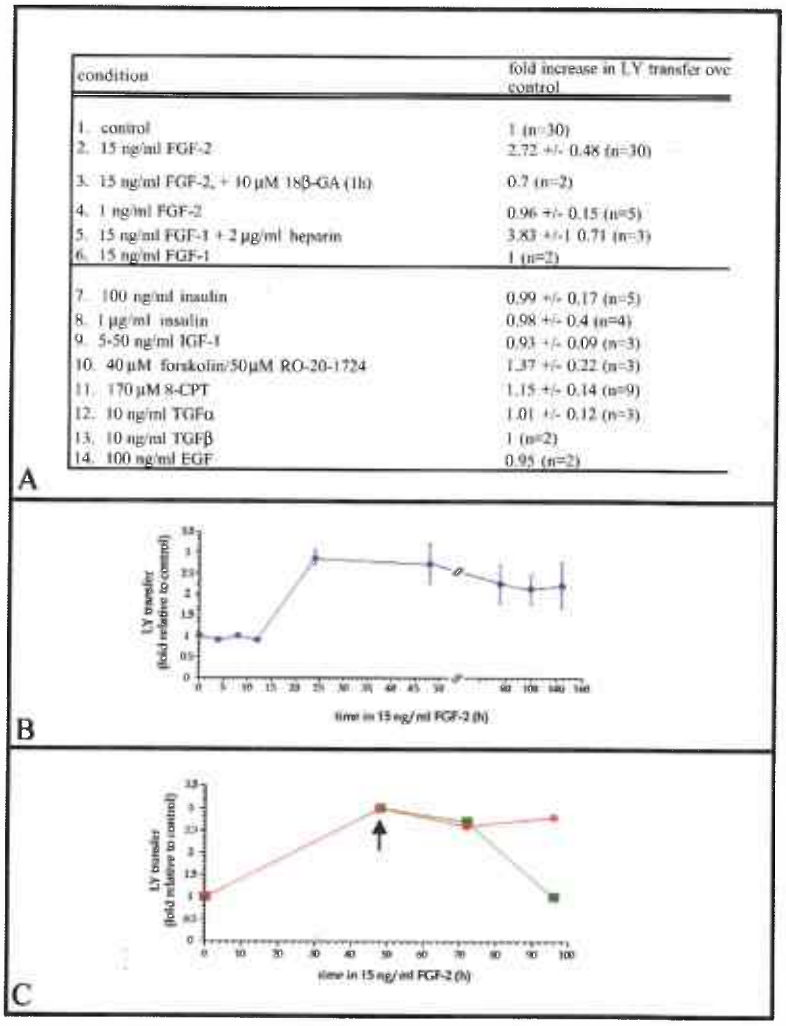
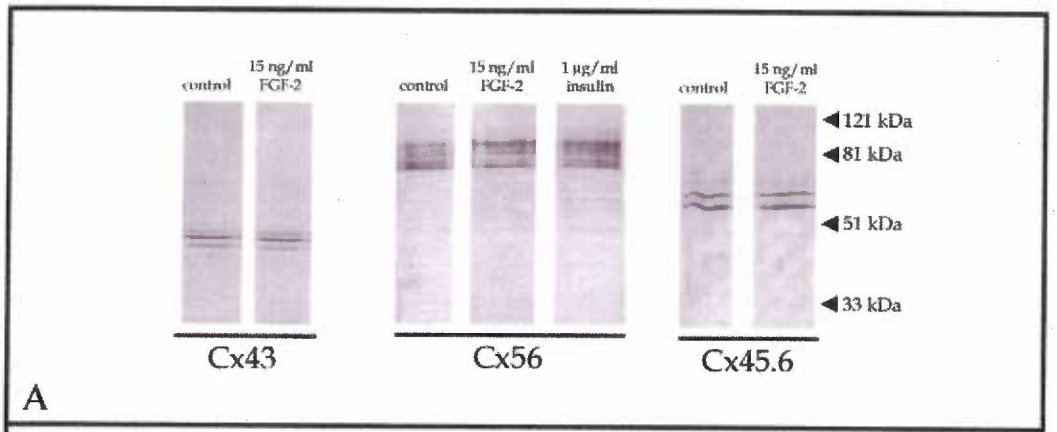


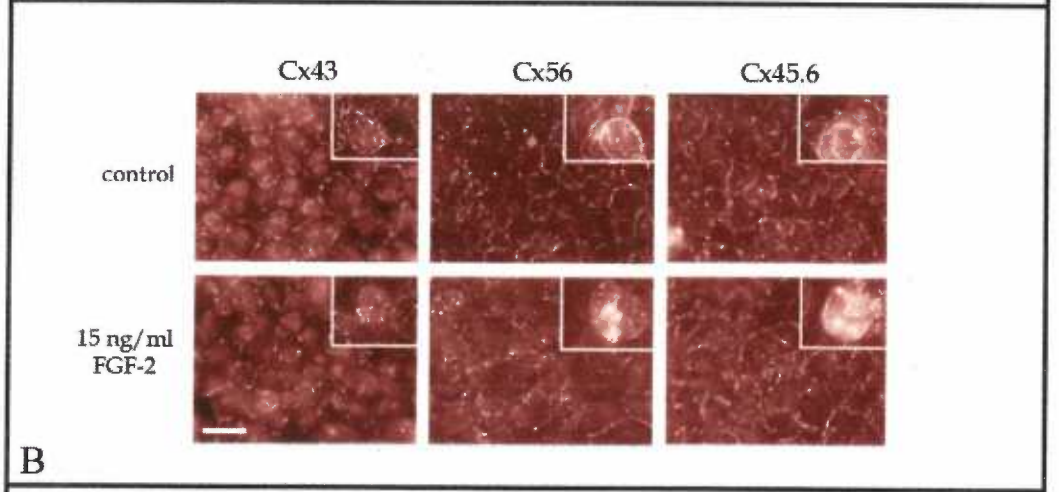
Figure 2. *Summary of effects of growth factors on gap junctional intercellular communication in chick lens DCDML cultures.* (A) One-day-old DCDML cultures were incubated for an additional 48 h in either the absence (control) or presence of the indicated additive and then assayed for gap junction-mediated intercellular communication as described in Figure 1A. Cell-cell coupling was quantitated by measuring the distance that Lucifer Yellow was transferred into the monolayer perpendicular to the line of rhodamine dextran-positive cells at the wound edge. At least three measurements were made per monolayer, and the averaged results expressed relative to Lucifer Yellow transfer in control cells within the same experiment. None of the compounds tested significantly affected the diameter of cells within the monolayer, and thus the distance of Lucifer Yellow transfer is directly proportional to the number of coupled cells. The effect of the gap junction blocker 18β-glycyrrhetinic acid (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 µM 18β-GA. Each data set is the average of 3 or more experiments (n= number of experiments), except for the following which are the average of 2 independent experiments: 3) LY transfer = 0.6-fold and 0.7-fold relative to control, 13) LY transfer = 0.9-fold and 1.1-fold relative to control, and 14) LY transfer = 0.9-fold and 1.0-fold relative to control. (B) DCDMLs were incubated with 15 ng/ml FGF-2 for 4, 8, 12, 24, 48, 72, 96, or 144 h, after which intercellular transfer of Lucifer Yellow was quantitated as in (A). (C) One-day-old DCDMLs were cultured for an additonal 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 h in fresh M199/BOTS medium with (red line) or without (green line) 15 ng/ml FGF and assessed for intercellular transfer of Lucifer Yellow as in (A). The plotted results are a representative data set from one of three similar experiments.

EGF) affected intercellular transfer of Lucifer Yellow under the conditions tested.

Time course experiments revealed that upregulation of cell-cell coupling required greater than 12 hours of continuous exposure to FGF and reached a maximum by 24 hours of treatment (Figure 2B). At all shorter timepoints assayed (30 minutes, 4 hours, or 8 hours), FGF neither enhanced nor inhibited junctional transfer of Lucifer Yellow. The level of cell-cell coupling achieved after 24 hours of FGF exposure persisted for at least 6 days (the longest period tested), provided the cells were fed every two days 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 hours but returned to basal levels by 48 hours after FGF washout (Figure 2C). The growth factor therefore did not permanently change the junctional phenotype of lens cells. Insulin did not appreciably affect intercellular coupling at any of the timepoints tested (30 minutes, 1 hour, 3 days, 4 days, or 6 days) (not shown).

FGF-induced upregulation of gap junctional coupling is not associated with 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, FGF-4 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 days 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,





	Permeation of FITC-dextran (fold relative to control)	TER ($\Omega \bullet cm^2$)	
	d4	d4	d6
control	1.00	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

C

Figure 3. *FGF does not increase connexin protein expression or gap junction assembly, nor selectively affect cell-cell adhesion in chick lens cells.* (A) One-day-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. 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 immunostained for Cx43, Cx56, and Cx45.6 as indicated. Connexin immunoreactivity was concentrated at cell-cell interfaces throughout the epithelial monolayer and in differentiating, multilayered lentoids (insets). Bar: 20 µm. (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 (transepithelial electrical resistance; 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 data set is the average of 3 experiments.

Cx45.6, or Cx56 levels was observed (Figure 3A). Each connexin migrated on SDS-PAGE as multiple species, presumably as a result of post-translational phosphorylation events. Phosphorylation of Cx43 to the lowest mobility form (Cx43-P_2) is closely correlated with its assembly into functional gap junctions in multiple cell types (Laird *et al.*, 1995; Musil and Goodenough, 1991; 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). FGF treatment did not, however, specifically affect the band pattern of any of these proteins. 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 anti-connexin 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 (Figure 3B). 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 (Le and Musil, 1998; Menko *et al.*, 1984).

A caveat to the experiment shown in Figure 3B is that light microscopy may not be able to distinguish assembled gap junctional intercellular channels from nonjunctional (and therefore nonfunctional) connexins on the plasma membrane. In some cell types, the efficiency with which cell surface connexins are assembled into gap junctions 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 (Figure 3C). 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 (M_r= 40 kDa) 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. This response did not, however, 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 the 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 Figure 3 do not support increased connexin expression or assembly as the mechanism by which FGF upregulates intercellular communication in cultured lens cells. FGF therefore most likely acts at the level of gating of the gap junctional channel (see Discussion). Elevated gap junction-mediated intercellular coupling without a concomitant increase in gap junction number is also a feature of the equatorial region of the lens *in vivo*.

Role of ERK kinase 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, 2000). Activation of ERKs by growth factors or other stimuli has been causally linked to a transient inhibition of Cx43-mediated intercellular

communication in some cell types (Hossain *et al.*, 1998; Warn-Cramer *et al.*, 1998; Zhou *et al.*, 1999) but not, to our knowledge, to a post-translational 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 MAPK cascade on gap junction function. To distinguish between these two possibilities, we utilized UO126, a potent, cell permeable, nontoxic, and highly specific inhibitor of the kinases (MEK 1/2) immediately upstream of ERK in the MAPK cascade (Favata *et al.*, 1998; Le and Musil, 2000). 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 (Figure 4A). This effect persisted for at least a week, provided the cells were refed with fresh UO126-containing medium every two days. 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 insensitive to inhibition of the ERK pathway (Figure 4B). 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 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,

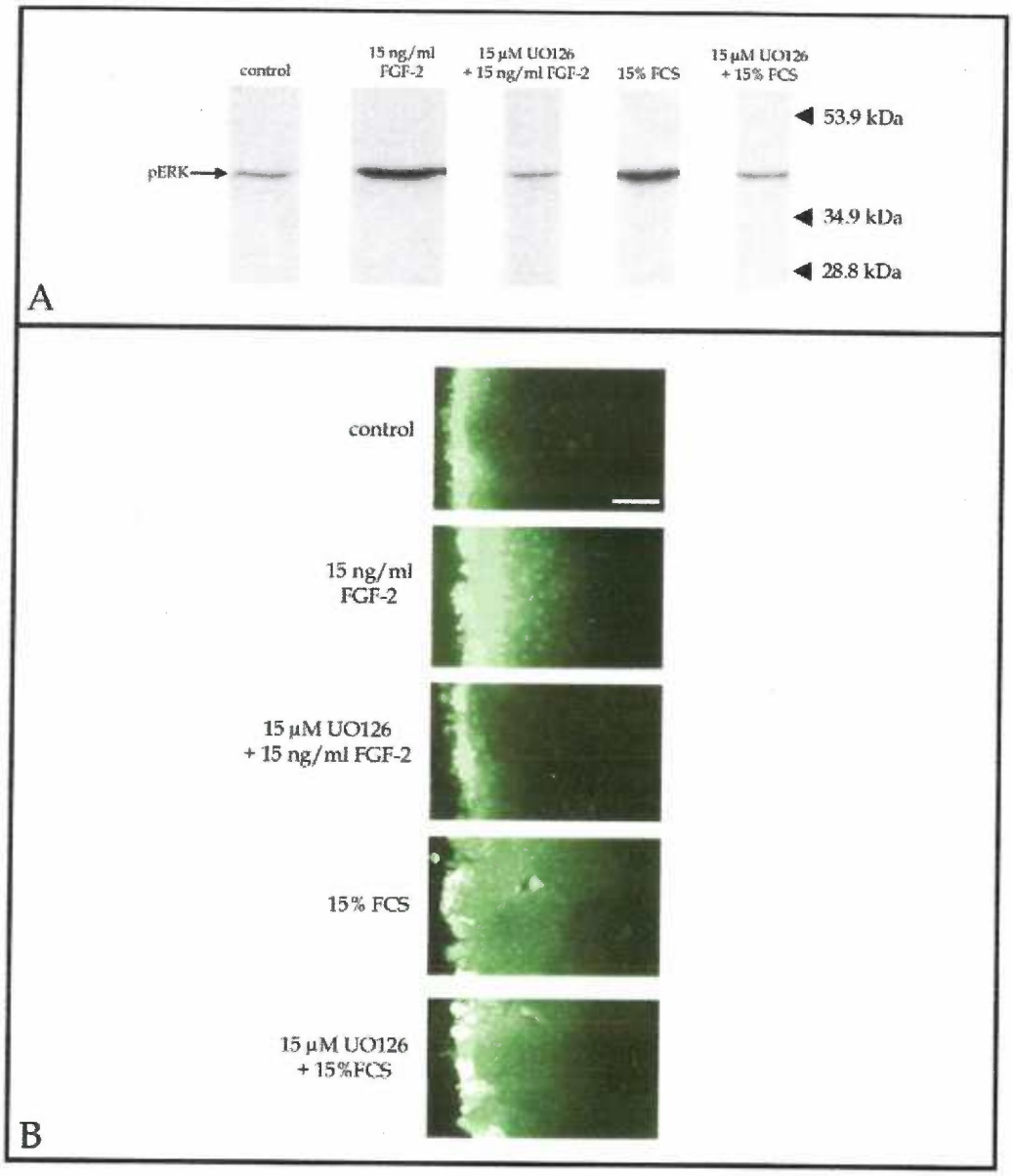


Figure 4. *ERK activation is required for FGF to increase gap junctional intercellular communication in chick lens cells.* (A) Three-day-old DCDMLs, cultured in M199/BOTS, were incubated for 15 min at 37¡C with no additions (control), 15 ng/ml FGF-2, or 15% fetal calf serum (FCS). Where indicated (+UO126), cells were pretreated with 15 µM UO126 for 30 min prior to addition or either FGF or fetal calf serum. 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 kDa (ERK2) MAP kinase species. (B) One-day-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 as indicated. The cells were then assayed for gap junction-mediated intercellular communication as described in Figure 1A. Only Lucifer Yellow immunofluorescence is presented; rhodamine dextran was confined to a single row of cells immediately bordering the wound (see Figure 1). Data typical of 3 independent experiments. UO126 alone did not affect the basal level of intercellular dye transfer (not shown). Bar: 50 µm.

2000). Immunocytochemistry with anti-phosphoERK antibodies revealed that the CA-MEK construct activated endogenous ERK in 60-70% of the cells in the culture (data not shown). Expression of constitutively active MEK1 induced a large increase in the amount of phosphorylated ERK detectable by immunoblotting (Figure 5, top) and significantly increased the intercellular transfer of Lucifer Yellow (Figure 5, bottom) in cells cultured in the absence of FGF or other added growth factors. Both effects were completely blocked by the MEK inhibitor UO126. Neither wild-type MEK1 (Figure 5; WT-MEK) nor the irrelevant transfection control β-galactosidase (not shown) stimulated ERK activation or gap junctional coupling. 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 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, 2000), yet have no effect on gap junctions. One of the most important determinants of the biological outcome of MAP kinase signalling is the length of time that a stimulus activates ERKs (Marshall, 1995). As assessed by quantitative anti-phosphoERK 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 minutes of addition to DCDML cultures (Figure 6). Only the treatments capable of upregulating junctional communication (15 ng/ml FGF-2 or FGF-1 plus heparin; see Figure 2A), however, sustained ERK activation for greater than 8 hours.

To determine whether the observed correlation between FGF-mediated sustained

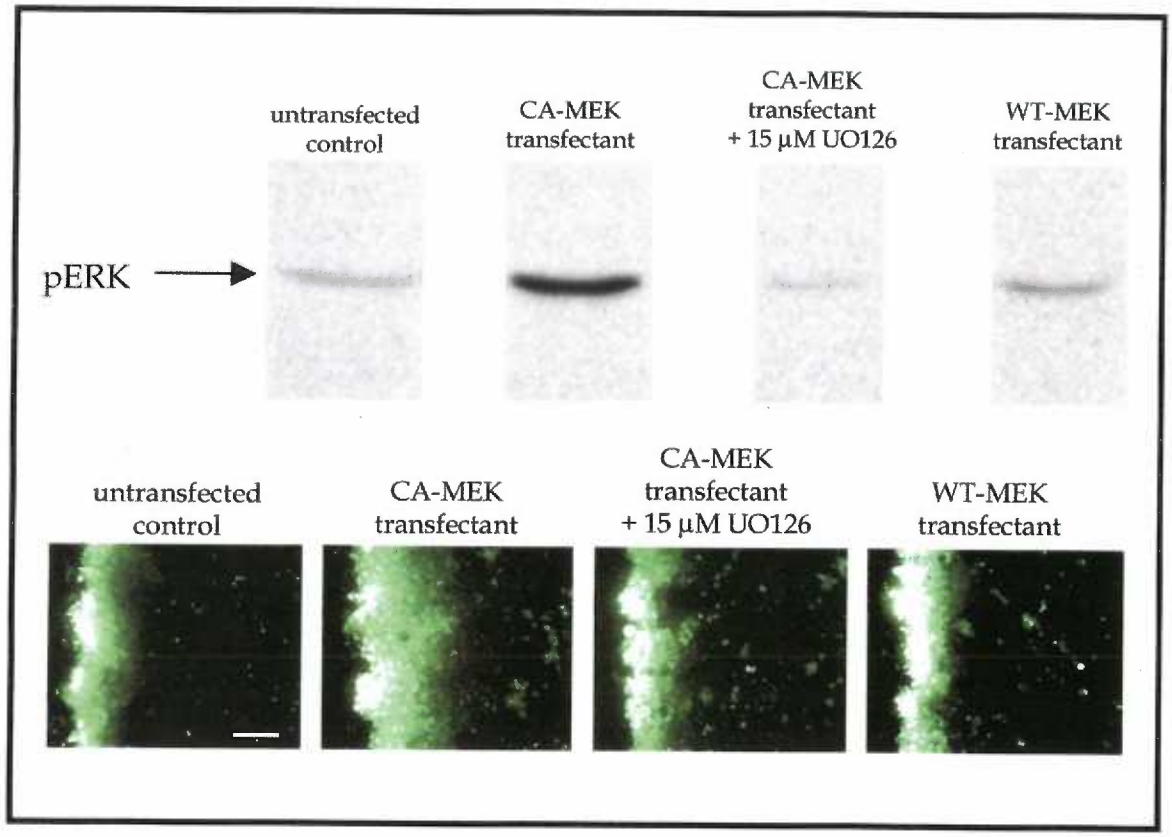


Figure 5. *ERK activation is sufficient to upregulate gap junctional intercellular communication in chick lens cells.* DCDMLs were transfected the day after plating with plasmids encoding either a mutant form of MEK1 that constitutively activate ERKs (CA-MEK), or wild-type MEK1 (WT-MEK). Where indicated, the MEK inhibitor UO126 (15 μM) was added 3 h after transfection. Top row: 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, 2000). Molecular mass markers are indicated on the right. Bottom row: Gap junctional intercellular communication was assessed 48 h after transfection as described in Figure 1A. Only Lucifer Yellow immunofluorescence is presented; rhodamine dextran was confined to a single row of cells immediately bordering the wound (see Figure 1). Representative of 5 experiments. Bar: 50 μm.

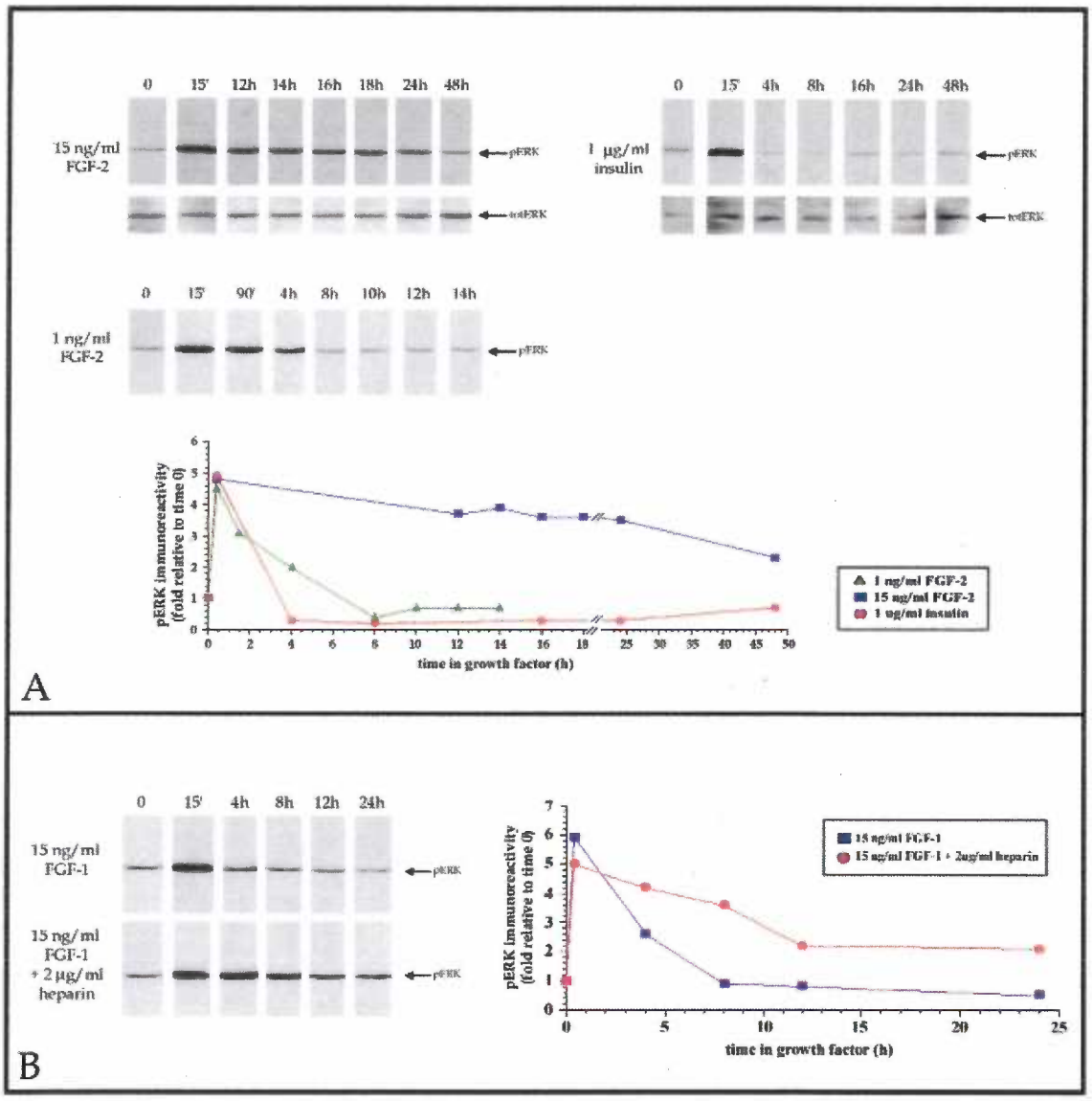


Figure 6. *FGF-mediated upregulation of gap junctional intercellular communication in chick lens cells is correlated with sustained activation of ERK.* The indicated growth factor was added directly to the culture medium of 3-day-old DCDMLs plated and maintained in M199/BOTS. After the indicated period (15 min - 48 h) at 37¡C, the cultures 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. After quantitation of the pERK bands by densitometry, the blots were stripped and reprobed with the anti-p44/42 MAP kinase polyclonal antibody to verify that the expression level of total ERK protein (both activated and inactive ERK; totERK) remained constant throughout the experiment. 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 3 independent experiments.

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 (Figure 7). 15 ng/ml FGF-2 was added to lens cells and, after 4, 8, or 12 hours, UO126 was included and the incubation continued for a total of 24 hours in FGF. PhosphoERK blots verified that addition of UO126 reduced the level of activated ERK to that of untreated controls within 90 minutes (Figure 7A, lane 3), and that this inhibition persisted for over 8 hours (lane 5). Scrape-load dye transfer analysis revealed that ERK had to be active for at least 12 hours in order for FGF to increase gap junctional coupling (Figure 7B). Given that expression of constitutively active MEK also induces long-term activation of ERKs and increased junctional coupling (Figure 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. In order 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 (Caruelle *et al.*, 1989; Mascarelli *et al.*, 1987; Schulz *et al.*, 1993). We have recently shown that crude vitreous humor diluted 1:2.3 in M199/BOTS medium (termed 30% vitreous humor; 30% VH) increases expression of fiber differentiation markers including δ-crystallin in embryonic chick lens DCDML cultures. Moreover, M199/BOTS conditioned overnight with intact 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, 2000). Both 30% vitreous humor and vitreous body conditioned medium (VBCM) increased intercellular transfer of Lucifer Yellow to an extent comparable to 15 ng/ml purified recombinant FGF (Figure 8). Moreover, they also

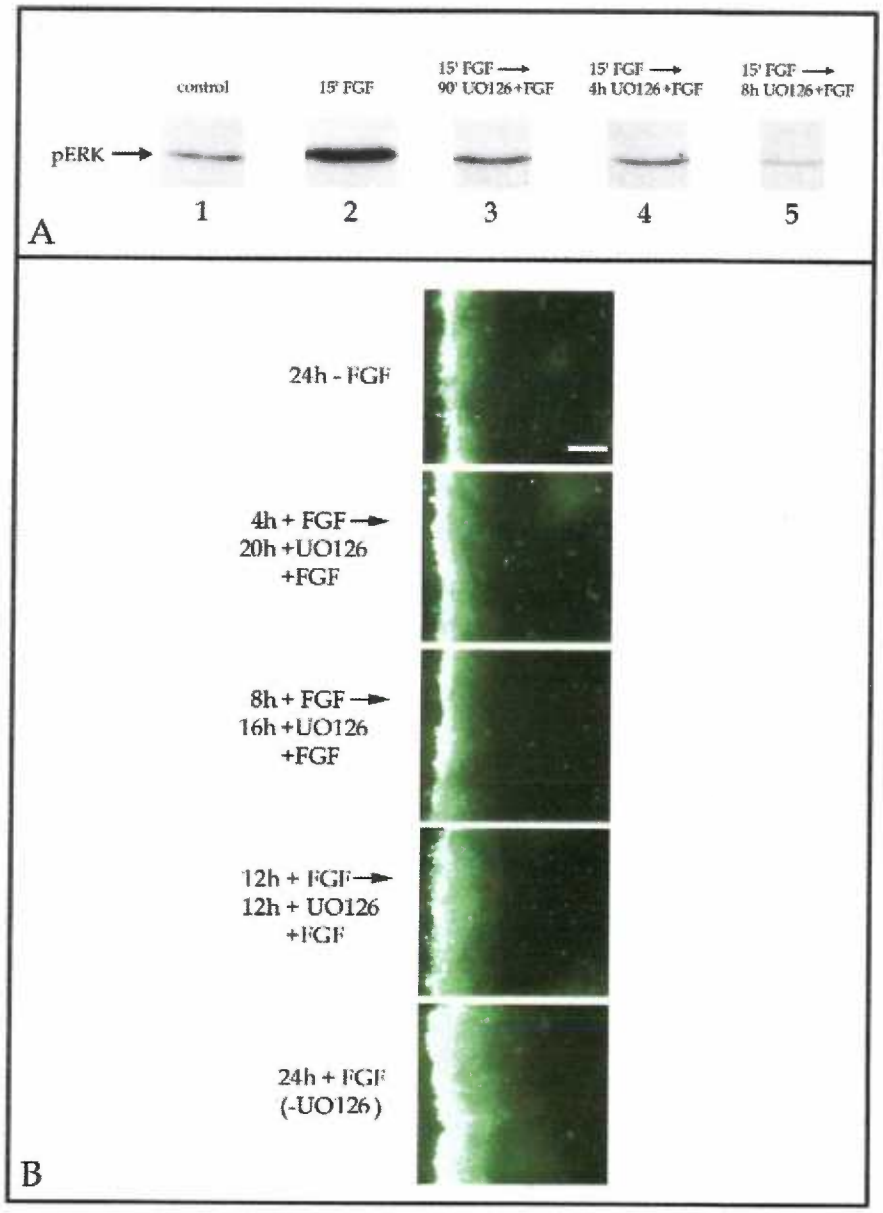


Figure 7. *FGF-mediated upregulation of gap junctional intercellular communication in chick lens cells requires sustained activation of ERK.* (A) Three-day-old DCDMLs cultured in M199/BOTS were incubated for 15 min at 37¡C in the absence (lane 1; control) or presence of 15 ng/ml FGF-2. In lanes 3-5, 15μM UO126 was then added to inhibit further activation of ERK. At the indicated times, the cells were solubilized in SDS and whole cell lysates 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, 2000). (B) Three days 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 prior to addition of 15 μM 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 Figure 1A. Only Lucifer Yellow immunofluorescence is presented; rhodamine dextran was confined to a single row of cells immediately bordering the wound. Note that at least 12 h of ERK activation was required for FGF to stimulate intercellular transfer of Lucifer Yellow. Representative data set from 4 independent experiments. Bar: 50 μm.

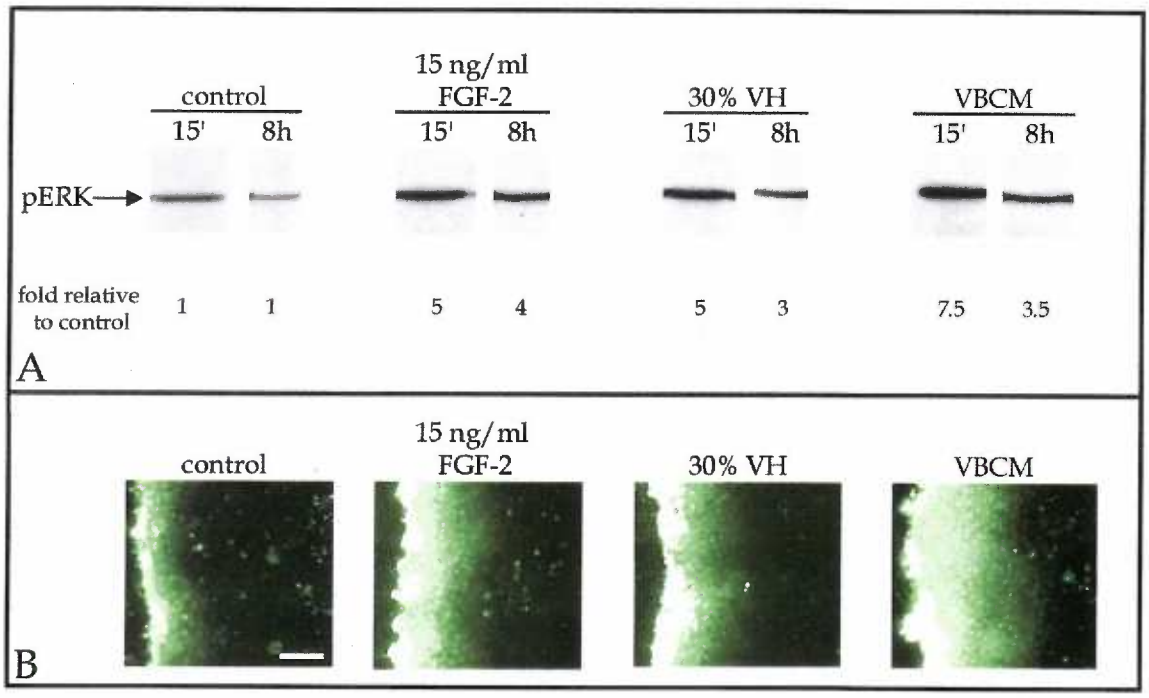


Figure 8. *Vitreous humor induces sustained ERK activation and increases gap junctional intercellular communication in chick lens cells.* (A) Three-day-old DCDMLs cultured in M199/BOTS were incubated at 37¡C in fresh M199/BOTS medium without additions (control), in fresh M199/BOTS with 15 ng/ml FGF-2, in chick vitreous humor diluted with 2.3 volumes of M199/BOTS (30% VH), or in M199/BOTS conditioned with intact E10 chick vitreous bodies (VBCM) 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 relative to the amount of pERK in controls incubated in M199/BOTS without additions for the same period. The amount of pERK in control cells is greater at 15 min than at 8 h in because medium removal and replacement transiently (< 30 min) activated ERKs. (B) Gap junctional intercellular communication in DCDMLs cultured for 2 d in the absence (control) or presence of 15 ng/ml FGF-2, 30% vitreous humor, or vitreous body conditioned medium was assessed as described in Figure 1A. Only Lucifer Yellow immunofluorescence is presented; rhodamine dextran was confined to a single row of cells immediately bordering the wound. Representative results from 3 independent experiments. Bar: 50 µm.

induced sustained activation of ERKs as assessed by anti-phosphoERK immunoblotting. The effect of vitreous on junctional communication, like that of recombinant FGF, was sensitive to the gap junction blocker 18β-glycyrrhetinic acid, 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).

A defining characteristic of all members of the FGF family is their high affinity for heparin (Ornitz, 2000). Control experiments verified that the ability of M199 medium containing 15 ng/ml recombinant FGF to enhance intercellular transfer of Lucifer Yellow was abolished if the medium was preabsorbed with heparin-Affigel beads in 0.1 M NaCl, but was eluted from the beads with 2.5 M NaCl (Figure 9, top row). The communication-promoting activity of 30% vitreous humor (or, not shown, vitreous body conditioned medium) was quantitatively removed when incubated with immobilized heparin in 0.1 M NaCl, and all but a small fraction also bound to heparin in 0.6 M salt. The bound activity was recovered from the heparin beads by incubation in 2.5 M NaCl (Figure 9, bottom row) and increased intercellular dye transfer in a UO126-inhibitable manner (not shown). This elution behavior is typical of an FGF (Mascarelli *et al.*, 1987; Schulz *et al.*, 1993; Seed *et al.*, 1988) and is distinct from that of other growth factors known or suspected to be present in the eye. The δ-crystallin-promoting activity of vitreous also binds to heparin with high affinity (Le and Musil, 2000), consistent with a role for FGF 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 ERK activation. 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

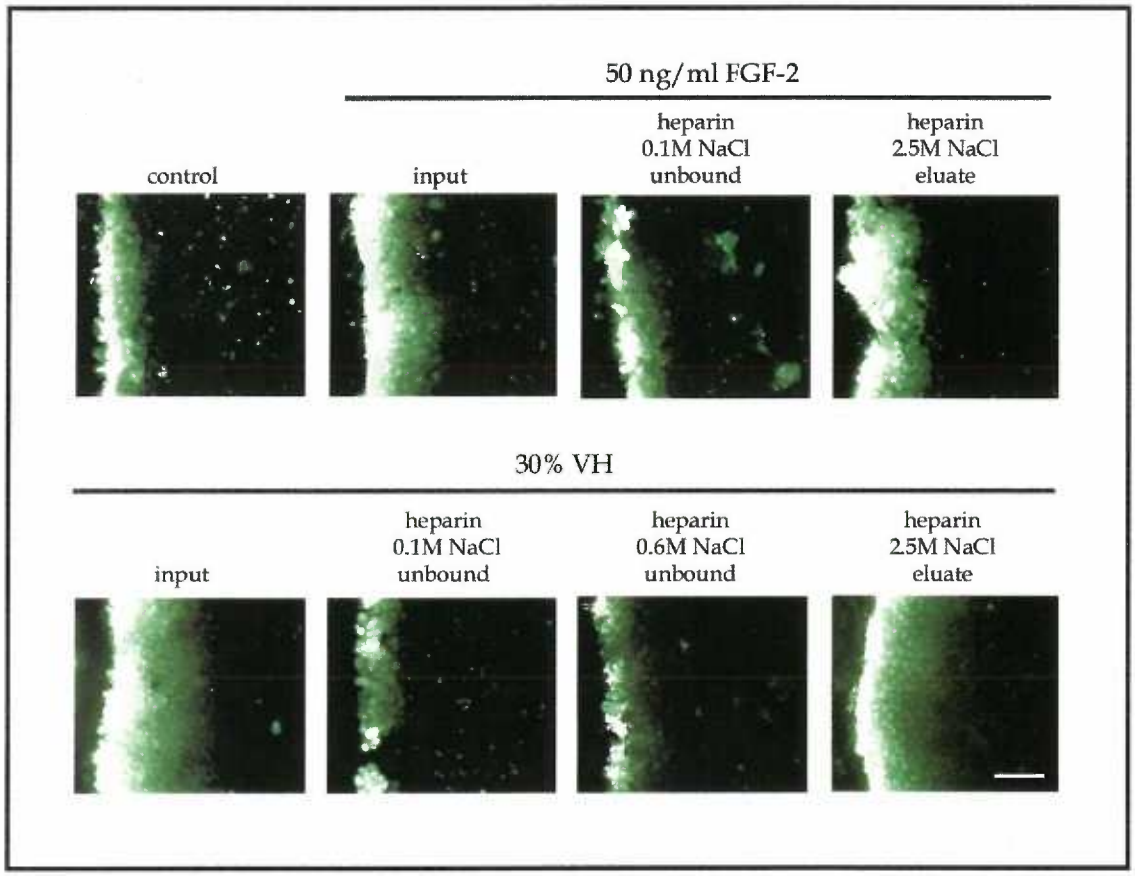


Figure 9. *The gap junctional intercellular communication-promoting activity of vitreous humor has properties of an FGF.* 50 ng/ml FGF-2 in M199/BOTS or E10 chick vitreous humor diluted with 2.3 volumes of M199/BOTS (30% VH) was incubated with heparin-conjugated Affigel beads in the presence of 0.1 M 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 brought to 0.15 M NaCl by repeated rounds of concentration and dilution with M199 medium. Dissociated cell-derived monolayers (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 Figure 1A; 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 3 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). Bar: 50 μm.

recombinant FGF-2 for 2 hours. 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 remainder of the lens constituting the polar and core fibers (see diagram, Figure 10). Each fraction was assayed by immunoblotting for activated (pERK) as well as for total (totERK) ERK. As shown in Figure 10, FGF efficiently induced ERK activation in the central epithelium and in the equatorial regions, which in other experiments (not shown) was confirmed to be sustained for over 12 hours. 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-sensitive ERK supports a model in which regional differences in FGF signalling are responsible for the asymmetry of gap junctional intercellular coupling in the lens (see Discussion).

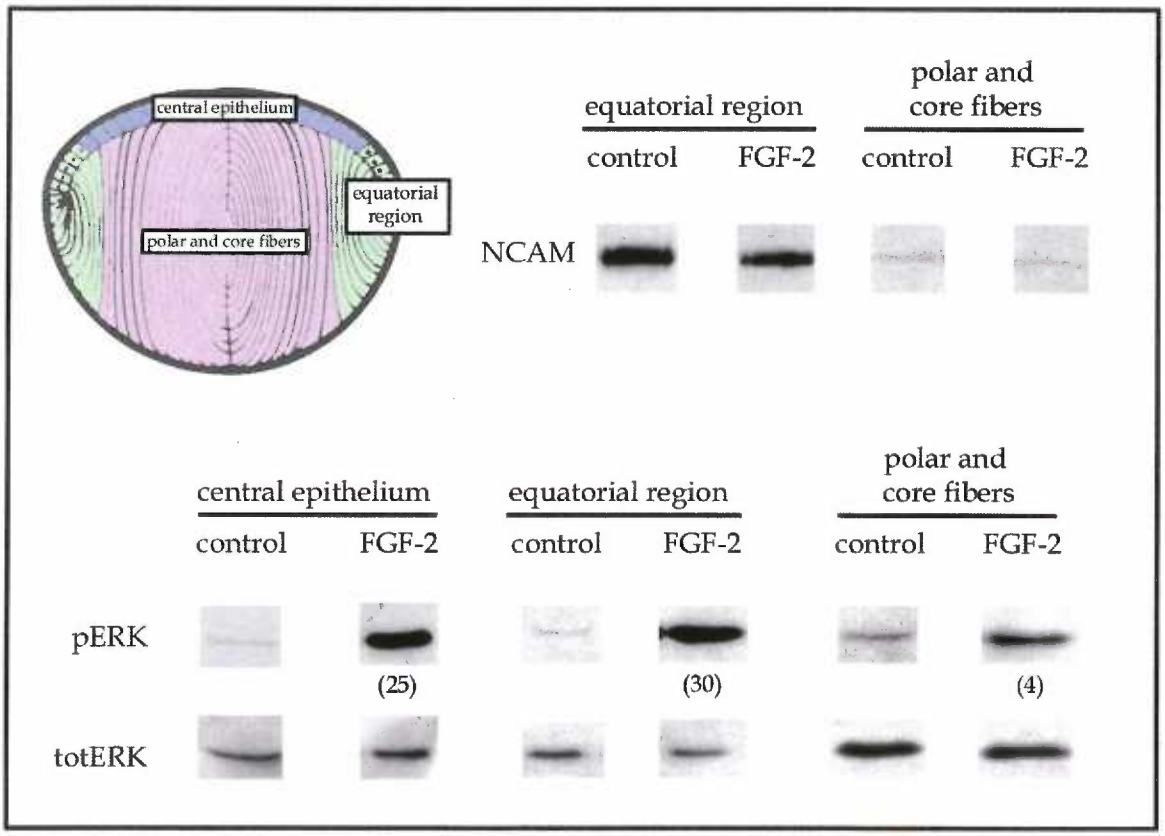


Figure 10. *FGF-induced activation of ERK is higher in the equatorial region than in polar and core fibers.* Intact lenses from embryonic day 13 (E13) chicks were incubated overnight in M199 medium and then treated for 2 h at 37¡C with no additions (control) or with 25 ng/ml FGF-2. The lenses were then snap-frozen in liquid nitrogen and dissected into three regions: the central epithelium, the equatorial region, and polar and core fibers (see diagram at left). The samples were solubilized in SDS and whole cell lysates assessed for NCAM expression and activation of ERK 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 a resident of peripheral, but not polar or core, fiber cells in the embryonic chick lens (Watanabe *et al.*, 1989). The pERK blots were subsequently stripped and reprobed for total (active and inactive) ERK with the anti-p44/42 MAP kinase polyclonal antibody (totERK). 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.

5. 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 and coworkers, this asymmetry is believed to be essential for the circulation of substances through the lens and therefore for lenticular homeostasis (Mathias *et al.*, 1997). In this study, we have demonstrated that FGF upregulates gap junctional communication between cultured chick lens cells and present evidence that FGF signalling may play an important role in establishing the gradient of intercellular coupling in the lens *in vivo*.

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, 2000). In the current investigation, we found that these same concentrations of FGF also increase gap junction-mediated intercellular dye transfer in the same system. Upregulation of intercellular coupling required sustained activation of ERK kinases, 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 much higher in the equatorial region than in polar or core fibers (Figure 10). Taken together, these results support (although do not prove) a model in which regional differences in

FGF signalling through the ERK pathway are responsible for the asymmetry of gap junctional intercellular coupling in the lens (Figure 11). We propose that the low level of FGF in the aqueous humor (Schulz *et al.*, 1993; Tripathi *et al.*, 1992) is inadequate to support either epithelial-to-fiber differentiation or enhanced intercellular coupling in the anterior 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. Posterior pole and core fiber cells also face the vitreous body. Unlike equatorial cells, this population does not, however, efficiently activate ERKs in response to exogenous FGF (Figure 10). During the ongoing process of fiber formation, cells at the lens equator gradually become displaced towards the lens core as they are buried by newer generations of fiber cells. If exposure of cells to FGF at the lens equator 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. Our results in cultured lens cells demonstrate, however, that FGF-induced upregulation of gap junction function is reversed within two days of FGF withdrawal. We propose that the reduction in FGF signalling 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 region cells to extralenticular FGF. The first, and most straight-forward, would be a loss of expression of high affinity FGF receptors in post-equatorial 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 fibers than in more equatorially located cells (de Iongh *et al.*, 1997; de Iongh *et al.*, 1996). Alternatively or in addition, the diffusion of vitreous humor-derived FGF through the interfiber space to receptors on lateral cell surfaces may be more restricted at the poles than at the equator. It is also

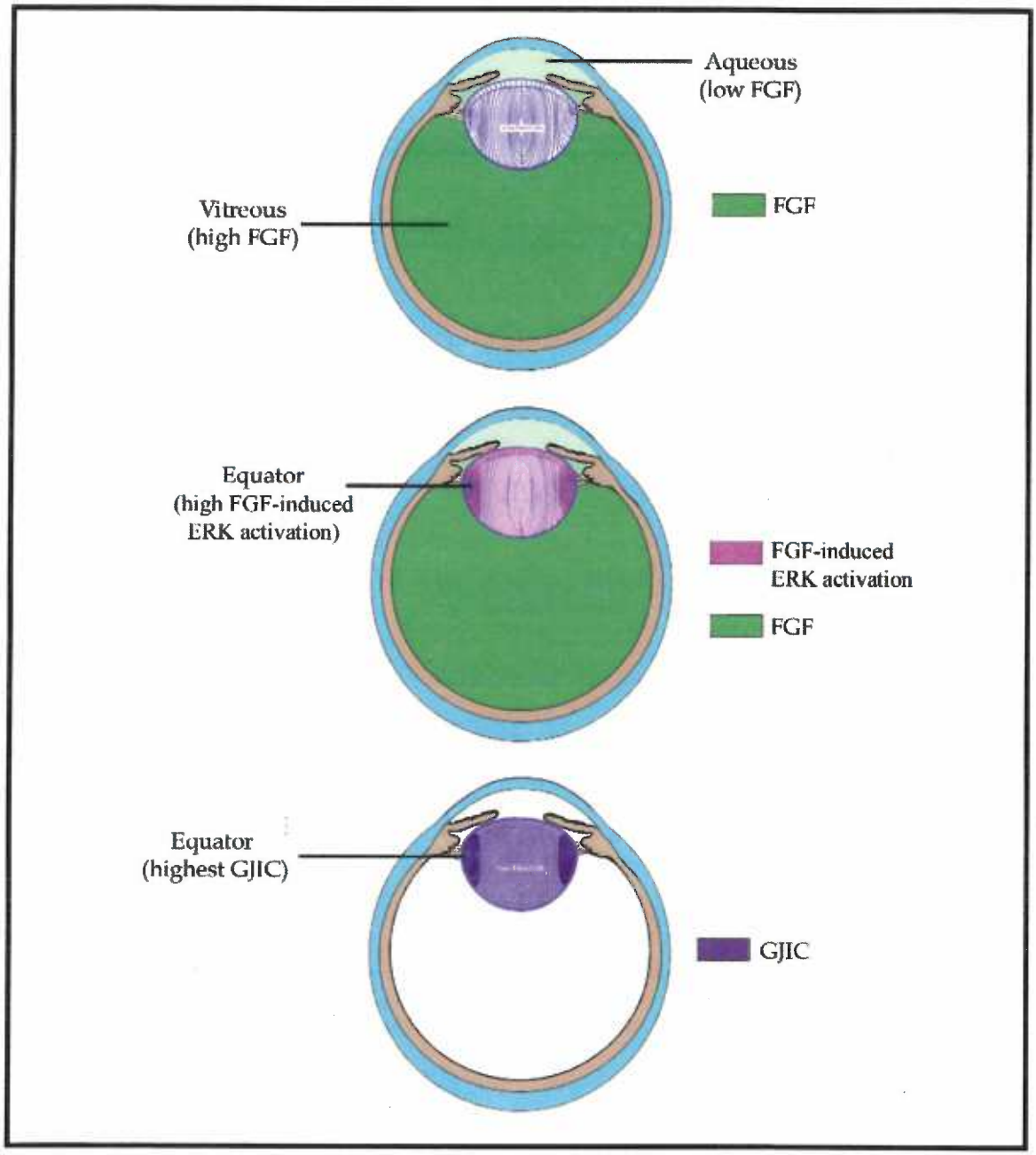


Figure 11. *Model of FGF induction of regional differences in gap junction-mediated intercellular communication (GJIC) in the lens.* Top, the concentration of FGF is known to be higher in the vitreous than in the aqueous humor. Middle, the central epithelium and equatorial regions, but not the polar and core fibers, are capable of sustained activation of ERK when isolated lenses are incubated with FGF (Figure 10). We propose that the level of FGF in the aqueous humor is too low to promote either gap junctional communication or fiber differentiation in the central epithelium. Both the equatorial and polar/core fiber region cells have access to the high levels of FGF that diffuse from the vitreous body, but only the former cell population responds to this FGF by sustained activation of ERKs. Sustained ERK activation leads to an increase in gap junctional intercellular communication in equatorial cells. The reduction in FGF signalling in polar and core fiber cells results in a reduction in intercellular coupling, thereby producing the observed equator to-pole gradient of lenticular communication (bottom). See discussion for details.

conceivable that polar fibers are deficient in a protein or activity required for transduction of the FGF signal to ERKs.

Mechanism of FGF-induced upregulation of gap junctional communication. The macroscopic gap junctional conductance between two cells (g_j) is given by:

$$g_j = f\, n \times P_o \times \gamma_j$$

in which n= the total number gap junctional channels, f = the fraction of those channels that are active at a given time, P_o = the single channel open probability, and γ_j is the single channel conductance. As summarized in the Introduction, the increase in g_j at the lens equator is not associated with a proportional increase 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 had no detectable effect on either the expression level or gap junction assembly of Cx43, Cx45.6, or Cx56 under conditions in which intercellular dye transfer was elevated (Figure 3). Upregulation of gap junction function at the lens equator *in vivo* as well in FGF-treated lens cells in culture is therefore unlikely to involve 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, 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 junction channels, than increasing the fraction of active channels would be an efficient mechanism by which FGF could increase junctional coupling without affecting total channel number. Although beyond the scope of this study, we plan to use the DCDML system and a combination of electrophysiological and morphological techniques to address the mechanisms by which FGF increases gap junction function. Primary cultures of embryonic chick lens epithelial cells have previously been used to analyze gap junction activity at the single channel level (Miller *et al.*, 1992).

If FGF influences channel gating instead of connexin expression, then why should its effect on intercellular dye transfer take more than 12 hours to manifest itself? The lengthy lag phase makes it unlikely that FGF upregulates gap junction-mediated intercellular communication by modulating the activity of pre-existing cellular components. Instead, FGF may change the expression of potential regulators of gap junction function such as protein kinases or phosphatases. It has recently been suggested that binding of the scaffolding protein ZO-1 to Cx43 and possibly certain other connexin species may serve to bring signalling complexes to the vicinity of gap junctional channels (Giepmans and Moolenaar, 1998; Toyofuku *et al.*, 1998). FGF could conceivably affect the expression or activity of such accessory proteins which in turn could influence gap junctional gating.

Novel role of ERK in gap junction-mediated intercellular communication in the lens. The results depicted in Figures 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 either no effect or to transiently (<1 h) reduce cell-cell coupling (Hill *et al.*, 1994; Hossain *et al.*, 1998; Warn-Cramer *et al.*, 1998). In mammalian cells,

the latter phenomenon 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 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, this appears unlikely (Musil *et al.*, 1990a). 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 continued transfer through Cx45.6 and 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, 2000), yet have no effect on gap junction function. Second, a detectable increase in the expression of fiber markers in response to FGF requires more than three days, whereas

junctional coupling is maximally stimulated within 24 hours 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.

6. Acknowledgments

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IV. Discussion

As summarized in the abstract (pp.1-2), the studies presented in this thesis have addressed several fundamental issues in lens development and function. Although many questions were answered, the findings obtained have also raised new topics for future investigations. Some of the key issues that have emerged during the course of this project are discussed separately below.

A. How can FGF enhance both the proliferation and differentiation of lens epithelial cells? FGFs can bind to more than one type of FGF receptor with different affinities. This raises the possibility that the physiological response to FGFs may be mediated by binding to alternate receptor species that activate distinct signaling pathways leading to different biological outcomes. For example, rat lung epithelial cells express FGF-R2 and -R4. FGF-2, which binds to both receptors with different affinities, increases cellular proliferation and differentiation. FGF-2-induced proliferation requires 10 ng/ml of FGF-2 and is mediated by a MAP-kinase dependent mechanism. In contrast, expression of a differentiation-specific gene required 100 ng/ml of FGF-2, was ERK-independent, and was partially inhibited by wortmannin, a PI3-kinase inhibitor. It was suggested that these two very different events may be mediated by separate FGF receptors acting through different signaling pathways (Matsui *et al.*, 1999). In the lens, FGF increases cell division in proliferative zone epithelial cells and fiber differentiation in the equatorial region. FGF-R2 (IIIc) is most strongly expressed in lens epithelial cells whereas other FGF receptor species (FGF-R1, FGF-R3) are most highly expressed in the differentiating equatorial cells (de Iongh *et al.*, 1997; de Iongh *et al.*, 1996; Orr-Urtreger *et al.*, 1993; Peters *et al.*, 1993). Thus, expression of different receptor subtypes may play a role in dictating the biological outcome of FGF signaling in the two regions.

In a variety of cell types, it has been demonstrated that translocation of FGF-1 and FGF-2 into the nucleus is necessary for their mitogenicity (Baldin *et al.*, 1990; Bouche *et al.*, 1987; Imamura *et al.*, 1990; Wiedlocha *et al.*, 1994; Wiedlocha *et al.*, 1996; Zhan *et al.*, 1992). It was recently shown that a single point mutation in FGF-2 prevents it from interacting with a protein kinase (CK2) in the nucleolus (Bailly *et al.*, 2000). When expressed in PC12 cells, this FGF-2 mutant had reduced mitogenic activity but undiminished differentiation activity. Thus, the subcellular localization of the FGFs may determine the biological outcome of FGF signaling.

B. What is the true in vivo role of FGF signaling in chick lens development? The role of FGF in chick lenses *in vivo* could be addressed by ectopically expressing a truncated FGF receptor that acts as a dominant negative mutant. The mutated receptor could be delivered to chick lens cells by infecting shell-less or in-shell cultured chick embryos with a replication-competent RCAS virus encoding the truncated receptor (Flanagan-Steet *et al.*, 2000) before the lens vesicle pinches off at embryonic day two (Hamburger and Hamilton, 1951). If FGF plays an important role *in vivo*, then perturbing FGF signaling with the mutated receptor should result in lens growth and differentiation defects.

C. How does FGF induce sustained activation of ERK in lens cells? The data presented in Chapter Three provide the first demonstration, to our knowledge, that lens cells are capable of both transient and sustained ERK activation leading to different biological outcomes. Transient ERK activation is required for the insulin-induced upregulation of δ-crystallin expression, whereas sustained ERK activation is required for the FGF-induced increase of GJIC. The mechanism by which FGF induces sustained ERK activation in lens cells is not known. Stork and colleagues have shown that the sustained phase of ERK activation by NGF in PC12 cells is mediated by the small G-

protein, Rap-1 (York *et al.*, 1998). We could not detect expression of Rap-1 in chick lens, nor did transfecting a plasmid encoding a constitutively active form of Rap-1 increase ERK activation in primary chick lens cell cultures. Future experiments will therefore focus on other potential mechanisms for sustained activation of ERK. A report by Sellers (1999) indicated that sustained activation of ERKs in Chinese hamster ovary (CHO) cells induced by somatostatin was dependent on protein kinase C (PKC). The role of PKC in FGF-induced sustained ERK activation in lens cells could be addressed utilizing specific PKC inhibitors (RO-31-0432, Calphostin C, chelerythrine, Go6976). It has also been suggested that the number of receptors activated at the cell surface is a determinant of the duration of ERK activation (Marshall, 1995; Traverse *et al.*, 1994). It would therefore be of interest to investigate whether overexpressing IGF-1-type receptors in primary chick lens cells increases the length of ERK activation in response to IGF-I and converts IGF-1 into a GJIC-promoting factor. Finally, it is possible that FGF may induce sustained ERK activation by inactivating a phosphatase which dephosphorylates ERKs. Protein phosphatase type-2A (PP2A) is a general ser/thr phosphatase that upon growth factor stimulation becomes tyrosine phosphorylated and (unlike the ERK-specific phosphatases MKP-1 and MKP-2) is inactivated (Chen *et al.*, 1992). Decreased ERK activation in renal mesangial cells was correlated with an increase in PP2A activity (Parameswaran *et al.*, 2000). In chick lens cells, inactivation of PP2A in response to FGF could render it incapable of dephosphorylating ERKs and thereby prolong the ERK signalling. Future experiments will examine the role of phosphatase activity in the FGF-induced increase of GJIC in primary chick lens cells.

D. What is the mechanism by which FGF upregulates GJIC in the lens? In contrast to the ERK-independent effects of FGF on chick lens cell proliferation and differentiation (δ-crystallin expression), upregulation of GJIC in response to FGF was shown to be dependent on sustained ERK activation (Chapter Three). This is the first

demonstration, to our knowledge, that ERK signalling can *increase* GJIC. This is a significant finding because it contradicts the current dogma based on results with PDGF, EGF, and insulin in other cell types, in which ERK activation is associated with a transient downregulation of GJIC (Homma *et al.*, 1998; Hossain *et al.*, 1998; Kanemitsu and Lau, 1993). In the few studies in which GJIC is reported to be increased in the presence of growth factors, the role of the ERK pathway was not addressed. In these cases, increased GJIC was correlated with a rise in connexin protein expression (Doble and Kardami, 1995; Pepper and Meda, 1992; Rivedal *et al.*, 1994). In contrast, FGF-induced GJIC in lens cells is not accompanied by a detectable change in either Cx43, Cx45.6, or Cx56 protein expression or the number of immunohistochemically detectable gap junctional plaques. Thus, the increase in GJIC induced *in vitro* by FGF, like that in the bow region of the lens *in vivo*, is likely to be due to an increase in either the time and/or frequency of opening of gap junction channels. This possibility can be investigated by using electrophysiological techniques to compare various gap junction parameters (unitary conductance, mean open probability, permselectivity, and/or active fraction of gap junctional channels) in chick lens epithelial cells cultured in either the absence or presence of FGF.

Another possibility is that FGF may be increasing GJIC by inducing a new connexin species. This possibility could be explored by generating a chick eye cDNA library from rat or chick lenses that presumably were exposed *in vivo* to FGF from the vitreous humor, designing a probe against a consensus region of know connexins, and performing low-stringency hybridization. One could also use a PCR-based approach using degenerate oligonucleotide primers corresponding to conserved regions of the connexin sequence. However, others have used similar methods and have only been able to identify the three previously described connexins in vertebrate lenses. It is therefore unlikely that *in vivo* exposure to FGF from the vitreous humor induces the synthesis of a novel connexin.

E. Is FGF involved in establishing the asymmetry of GJIC required for lens function in vivo? In order to test the *in vivo* model proposed in Chapter Three, transgenic mice could be generated that express wildtype FGF receptors under the control of the γF-crystallin promoter, which directs protein expression to the central nuclear fiber cells (Goring *et al.*, 1987). Overexpression of functional FGF receptors should increase GJIC in the polar and core fibers and by doing so, disrupt the asymmetry of gap junctions in the lens that is thought to be necessary for lens transparency. Cataract formation would be predicted to ensue.

F. Concluding remarks. Gap junctions play an essential role in homeostatic mechanisms necessary for lens clarity. Elucidating the mechanisms that regulate gap junctions in the lens is therefore important for understanding both normal lens function and cataractogenesis. We are confident that the data presented in this thesis will provide a framework for future investigations into the role of growth factors and gap junctions in lens biology.

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