

# FGF-induced lens cell proliferation and differentiation is dependent on MAPK (ERK1/2) signalling

Frank J. Lovicu\* and John W. McAvoy

Save Sight Institute, and Department of Anatomy and Histology, Institute for Biomedical Research, University of Sydney, NSW, Australia

\*Author for correspondence (e-mail: lovicu@anatomy.usyd.edu.au)

Accepted 17 September 2001

## SUMMARY

Members of the fibroblast growth factor (FGF) family induce lens epithelial cells to undergo cell division and differentiate into fibres; a low dose of FGF can stimulate cell proliferation (but not fibre differentiation), whereas higher doses of FGF are required to induce fibre differentiation. To determine if these cellular events are regulated by the same signalling pathways, we examined the role of mitogen-activated protein kinase (MAPK) signalling in FGF-induced lens cell proliferation and differentiation. We show that FGF induced a dose-dependent activation of extracellular regulated kinase 1/2 (ERK1/2) as early as 15 minutes in culture, with a high (differentiating) dose of FGF stimulating a greater level of ERK phosphorylation than a lower (proliferating) dose. Subsequent blocking experiments using UO126 (a specific inhibitor of ERK activation) showed that activation of ERK is required for FGF-induced lens

cell proliferation and fibre differentiation. Interestingly, inhibition of ERK signalling can block the morphological changes associated with FGF-induced lens fibre differentiation; however, it cannot block the synthesis of some of the molecular differentiation markers, namely,  $\beta$ -crystallin. These findings are consistent with the *in vivo* distribution of the phosphorylated (active) forms of ERK1/2 in the lens. Taken together, our data indicate that different levels of ERK signalling may be important for the regulation of lens cell proliferation and early morphological events associated with fibre differentiation; however, multiple signalling pathways are likely to be required for the process of lens fibre differentiation and maturation.

Key words: Lens, FGF, Cell proliferation, Fibre differentiation, MAPK signalling, ERK1/2, Rat,  $\beta$ -crystallin

## INTRODUCTION

The vertebrate ocular lens is a useful model system to examine the molecular mechanisms by which growth factors regulate key developmental processes such as cell proliferation and differentiation (McAvoy et al., 1991; Hyatt and Beebe 1993; Robinson et al., 1995a; Lovicu and Overbeek, 1998; Le and Musil, 2001; Shirke et al., 2001). The distinctive architecture of the lens is established early in development, with a monolayer of epithelial cells overlying the anterior surface of the elongated fibre cells that make up the bulk of the lens. Growth of the lens throughout life involves proliferation of the epithelial cells and their subsequent differentiation into secondary fibre cells. The process of fibre differentiation in the mammalian lens is characterised by distinct molecular and morphological changes including exit from the cell cycle, cell elongation, the loss of cytoplasmic organelles and nuclei, as well as the accumulation of fibre cell-specific proteins,  $\beta$ - and  $\gamma$ -crystallins as well intermediate filaments, such as filensin and CP49 (Blankenship et al., 2001).

There is now compelling evidence from numerous studies *in vitro* and *in vivo*, that members of the fibroblast growth factor (FGF) family play an important role in the regulation of lens cell proliferation and fibre differentiation (Chamberlain and

McAvoy, 1997; McAvoy et al., 1999). The FGFs constitute a large family of at least 23 distinct polypeptide growth factors (FGF1 to FGF23) (Yamashita et al., 2000) that play pivotal roles in a variety of developmental events (Ornitz and Itoh, 2001). Support for the hypothesis that FGF signalling plays an important role in development and growth of the lens was initially proposed from findings in our laboratory (McAvoy and Chamberlain, 1989). Using rat lens epithelial explants, FGF was shown to be a potent inducer of lens fibre differentiation, as well as an inducer of lens epithelial cell proliferation and migration. Interestingly, these different cellular events could be stimulated in a dose-dependent manner: for example, a low dose of FGF induced only lens cell proliferation, whereas a high dose also induced fibre differentiation (McAvoy and Chamberlain, 1989). Further support for a role for FGF in normal lens biology stems from studies *in vivo* that demonstrated that overexpression of FGF in lenses of transgenic mice could induce inappropriate differentiation of epithelial cells, disrupting the normal polarity of the lens (Robinson et al., 1995a; Lovicu and Overbeek, 1998). Furthermore, using a similar transgenic approach, the process of lens fibre differentiation could be impaired by overexpressing either a signalling-defective, truncated FGFR (Robinson et al., 1995b; Chow et al., 1995; Stolen and

Griep, 2000) or as reported in more recent studies, a specific secreted FGF receptor (FGFR3) (Govindarajan and Overbeek, 2001).

Like most growth factors, members of the FGF family mediate cellular signalling through high-affinity cell surface receptors. The binding of FGF to its receptor leads to ligand-induced receptor dimerisation, subsequently activating its intrinsic tyrosine kinase activity, which is concomitant with tyrosine autophosphorylation (Fantl et al., 1993). Activation of the membrane-bound tyrosine kinase receptors may lead to a variety of downstream effects, including the elevation of intracellular  $Ca^{2+}$  levels, activation of target substrates, including phospholipase C $\gamma$  (PLC $\gamma$ ) (Sa and Das, 1999; Browaeys-Poly et al., 2000), as well as the activation of distinct signalling pathways such as the PI3-kinase/Akt pathway (Chen et al., 2000; Browaeys-Poly et al., 2001) and the mitogen-activated protein kinase (MAPK) pathway (Lin et al., 1998). The MAPK pathway plays an important role in modulating many cellular events, including cell cycle progression, cell differentiation and the regulation of embryonic development in a variety of biological systems (Marshall, 1995; Lewis et al., 1998; Schaeffer and Weber, 1999). The MAPK pathways are typically organised in a conserved three-kinase architecture, consisting of a MAP kinase, an activator of the MAP kinase (MAP kinase kinase; MEK) and an activator of the MAP kinase activator (MAP kinase kinase kinase; MEKK). This signalling cascade is initiated and progresses by the sequential phosphorylation and activation of its components, leading to the phosphorylation of a conserved Thr-X-Tyr motif of the MAPK. This activated MAPK is then translocated from the cytoplasm to the cell nucleus (Hulleman et al., 1999) where it activates nuclear transcription factors leading to changes in gene expression. To date, at least three distinct mammalian MAPKs have been characterised: the extracellular-regulated kinases (ERKs), Jun N-terminal kinases/stress-activated protein kinase (JNK/SAPK) and p38. The mammalian ERKs (ERK1/p44 and ERK2/p42) are generally thought to play a role in cell proliferation and differentiation, while the JNK/SAPK and p38 kinases have predominantly been implicated in responses to cellular stress, inflammation and/or apoptosis.

As mentioned, work in our laboratory has shown that FGF can induce proliferation and differentiation in lens cells, depending on its concentration; however, the signal transduction pathways by which FGF induces these different cellular responses is still not clear. Early insights into the type of signalling pathways involved in the process of lens fibre differentiation stem from transgenic studies mentioned earlier (Chow et al., 1995; Govindarajan and Overbeek, 2001), which show that impaired FGF signalling (and the associated impairment of fibre differentiation) in the mouse lens results in reduced phosphorylation of ERK. In contrast to this, more recent studies by Le and Musil (Le and Musil, 2001), have reported that although FGF can 'robustly activate' ERKs in cultured lens cells, FGF-induced proliferation and  $\delta$ -crystallin stimulation (a marker for fibre differentiation in chicks) does not require ERK activation. In light of these findings, we have set out to identify the role of the MAPK signalling pathway in the mammalian lens. More specifically, we have used the rat lens epithelial explant system to directly examine the role of ERK signalling in FGF-induced lens cell proliferation and differentiation.

## MATERIALS AND METHODS

All procedures involving animals were in accordance with the National Health and Medical Research Council (Australia) guidelines and the Association for Research in Vision and Ophthalmology Handbook for the Use of Animals in Biomedical Research (USA). All protocols were approved by the Animal Ethical Review Committee of the University of Sydney, Australia.

### Preparation of lens epithelial explants

All tissue culture was performed in Medium 199 with Earle's salts (Trace Scientific, NSW, Australia), supplemented with 0.1% bovine serum albumin (BSA), 0.5 mM L-glutamine, 100 IU/ml penicillin, 100  $\mu$ g/ml streptomycin and 2.5  $\mu$ g/ml Amphostat (all from Trace Scientific).

Untrimmed lens epithelial explants were prepared as follows: under sterile conditions, for each culture dish, two eyes were removed from neonatal (P3-P4) Wistar rats and placed in culture medium. Using a dissecting microscope, lenses were dissected from eyes and transferred to fresh culture medium. With fine forceps, the posterior lens capsule was torn and peeled from the fibre cell mass, which was discarded. The remaining lens capsule (containing the adherent epithelial monolayer) was gently pinned out flat by pressing the forceps around the edge of the explant. Culture medium was then removed and replaced with 1 ml of fresh, equilibrated (37°C, 5% CO<sub>2</sub>) medium.

For proliferation assays, explants were exposed to 5 ng/ml of FGF2 (PeproTech, Rocky Hill, NJ) and cultured for either 15 minutes, 2 hours or 2 days at 37°C in 5% CO<sub>2</sub>. At the end of the culture period, explants to be used for immunofluorescent labelling were fixed in 10% neutral-buffered formalin (NBF) for 20 minutes and rinsed in phosphate buffered saline (PBS) before immunolabelling. Explants to be used for SDS-PAGE and western blotting were rinsed in cold PBS and lens proteins extracted in lysis buffer (1 mM EDTA, 10 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1% IGEPAL, 1 mM Na<sub>3</sub>VO<sub>4</sub> and protease inhibitor cocktail (Roche, Basel, Switzerland)) for 50 minutes. Protein levels were determined using the Micro-BSA protein assay according to manufacturer's instructions (Pierce, IL).

For differentiation assays, culture dishes containing explants were supplemented with 100 ng/ml of FGF2 (PeproTech) and cultured for either 15 minutes, 2 hours, 3 days or 5 days. At the end of the culture period, explants were processed as described above.

For inhibitor studies, 2 hours before addition of growth factors, U0126 (a specific MEK1/2 inhibitor for ERK signalling (Promega, NSW, Australia)) was added to give a final concentration ranging from 0.1 to 100  $\mu$ M. The inhibitor and growth factors were only added at the start of the culture period and once added were present for the duration of the culture. Control dishes, with no inhibitor added, were supplemented with an equivalent volume of dimethylsulfoxide (DMSO; the recommended solvent for U0126), which was added at the same time the inhibitor was added to the remaining dishes.

### Immunolabelling

#### Detection of phosphorylated ERK1/2

For tissue culture studies, phosphorylated ERK1/2 was detected by SDS-PAGE and western blotting 15 minutes or 2 hours after the addition of growth factors. Immunofluorescence was also used to detect phosphorylated ERK1/2 after 2 hours, 2, 3 or 5 days.

#### SDS-PAGE and western blotting

The phosphorylation status (activation) of ERK1/2 in explant extracts was assayed using SDS-PAGE and western blotting with commercially available antibodies (New England Biolabs, NEB, MA) specific for phosphorylated and non-phosphorylated forms of ERK1/2. In brief, for each lane, up to 5  $\mu$ g of protein was electrophoresed through a 10% SDS-PAGE gel before being transferred to a PVDF membrane for western blot analysis. The membrane was blocked with 2.5% BSA before overnight incubation at 4°C with a monoclonal antibody specific

to phospho-ERK1/2 (NEB, diluted 1:1000) or a polyclonal antibody specific for phosphorylation-independent ERK1/2 (NEB, diluted 1:1000). The membrane was then incubated for 2 hours at room temperature with a secondary antibody; for colorimetric detection we used alkaline-phosphatase-conjugated rabbit anti-mouse IgG or goat anti-rabbit IgG (Sigma) and for chemiluminescence we used a HRP-conjugated rabbit anti-mouse IgG (Silenus, Hawthorn, Vic., Australia). Immunocomplexes were visualised by exposure of the membrane to NBT-BCIP substrate (Sigma) for colorimetric detection, whereas for chemiluminescence, Super Signal® (Pierce) was used. Using these same procedures, we also immunolabelled for phosphorylated p38 and JNK/SAPK using specific polyclonal antibodies (NEB).

#### Immunofluorescence

For immunofluorescent labelling of explants for phospho-ERKs, fixed explants were rinsed in PBS supplemented with BSA (PBS-BSA), followed by two rinses in PBS-BSA supplemented with 0.05% Tween 20 (PBST) and another rinse in PBS-BSA. Note that all washes, labelling and visualisation of explants was carried out in the original culture dish. Explants were incubated with 3% normal goat serum (NGS) to reduce nonspecific labelling. Excess NGS was removed and explants were incubated overnight at 4°C with a monoclonal antibody specific to phospho-ERK1/2 (NEB, diluted 1:250 with PBS-BSA). After a brief rinse in PBS-BSA, explants were incubated for 1 hour at room temperature with an anti-mouse IgG antibody conjugated to fluorescein-isothiocyanate (FITC, Silenus), rinsed again in PBS and counter-stained with 1 µg/ml bisbenzimidazole (Hoechst dye, Calbiochem, La Jolla, CA) to label nuclei. Sections were then rinsed with PBS, mounted and examined using fluorescence microscopy.

For *in vivo* immunolabelling of phospho ERK1/2, freshly dissected eyes of neonatal Wistar rats were immersed in Tissue Tek OCT compound (Miles, IN) and frozen in liquid nitrogen-cooled isopentane. Specimens were stored in liquid nitrogen until sectioned. Fresh frozen sections (10 µm) were briefly fixed in cold methanol (-20°C), air dried and incubated for 30 minutes in NGS. Sections were then incubated overnight at 4°C with a monoclonal antibody specific for phospho-ERK1/2 or polyclonal antibodies specific for either phospho-p38 or phospho-JNK/SAPK (NEB, all diluted 1:250). After a brief rinse in PBS, sections were incubated for 1 hour at room temperature with an anti-mouse Ig (for phospho-ERK1/2 labelling) or an anti-rabbit Ig (for phospho-p38 or phospho-JNK/SAPK labelling) antibody conjugated to FITC (Silenus), rinsed again in PBS and counter-labelled with Hoechst dye (Calbiochem). Sections were then rinsed with PBS, mounted and examined using fluorescence microscopy.

#### Lens epithelial cell proliferation assay

To examine the patterns of cell proliferation (cells in S-phase of the cell cycle) in explants treated with growth factors, incorporation of 5-bromo-2'-deoxyuridine (BrdU) was analysed using immunofluorescence. Explants to be assayed for cell proliferation were cultured for 2 days with FGF2 (5 ng/ml). Six hours before the collection of explants, BrdU (150 µg/ml) was added and at the end of the culture period, explants were fixed in NBF and processed for immunofluorescent labelling as described above. The only differences were that before washing with PBST, explants were pre-treated with 1M HCl for 20 minutes to allow epitope recognition by the anti-BrdU monoclonal antibody (Bioclone, Sydney, Australia) diluted 1:100 with PBS-BSA.

#### Lens fibre differentiation assay

To assay for the accumulation of markers for lens fibre differentiation (β-crystallin and filensin), explants were immunolabelled in the same manner as for the phospho-ERK1/2, with the exception that we used polyclonal primary antibodies for β-crystallin and filensin (both diluted 1:100 with PBS-BSA) in place of the anti-phospho-ERK1/2 monoclonal antibody.

#### Lens explant histology

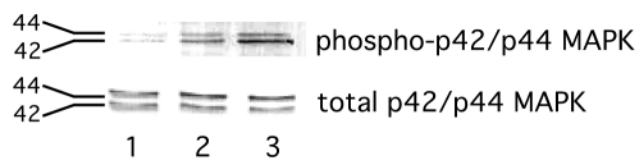
At the end of the culture period, lens explants were processed for either light or scanning electron microscopic analysis. For light microscopy, explants were fixed in NBF, rinsed in PBS and pre-embedded in 3% noble agar. Embedded explants were then dehydrated through a series of ethanol and cleared in xylene before embedding in paraffin wax. Sections (6 µm) were cut, de-waxed, hydrated and stained with Haematoxylin and Eosin. For scanning electron microscopy, explants were prepared based on earlier methods (Lovicu and McAvoy, 1989) with the exception that tissues were also fixed in 1% osmium tetroxide prior to dehydration and critical point drying.

## RESULTS

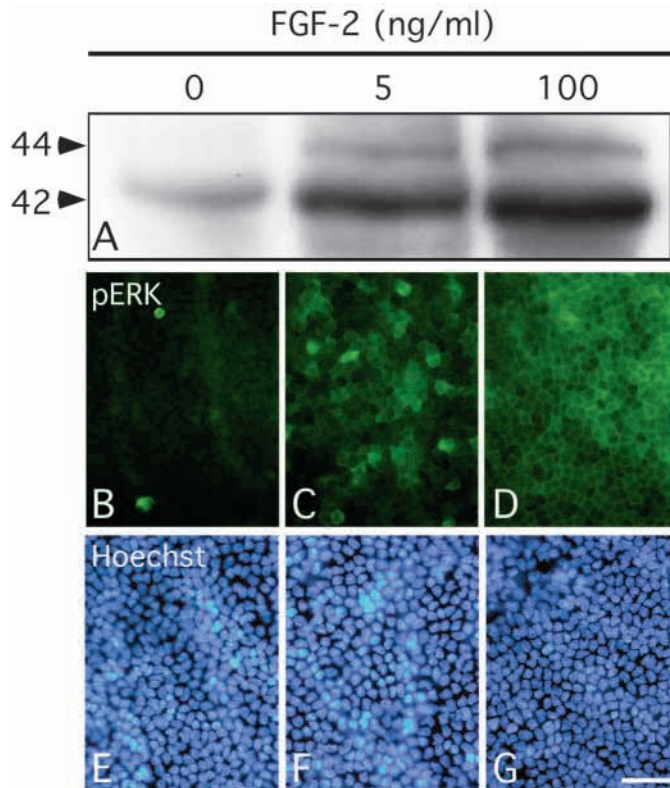
We monitored the responsiveness of lens epithelial cells exposed to different doses of FGF in the presence or absence of a specific inhibitor for ERK1/2 (MAPK) signalling. The responsiveness of these cells was analysed using routine histological evaluation (light and electron microscopy) and immunofluorescence to assay for cell proliferation (using BrdU-incorporation) and for fibre differentiation (examining the accumulation of lens fibre-specific proteins, β-crystallin and filensin).

#### FGF induces ERK activation in lens epithelial cells

An advantage of using the rat lens explant system is that epithelial cells in these explants can be stimulated to undergo a specific cellular process (either cell proliferation or fibre differentiation), depending on the dose of FGF to which they are exposed. In the present study, using lens epithelial explants, a low dose (5 ng/ml) of FGF2 was used to induce cell proliferation and a high dose (100 ng/ml) was used to induce fibre differentiation. By western blotting, low levels of the phosphorylated forms of p42 (ERK2) and p44 (ERK1) were detected in control explants (no FGF added; Fig. 1, lane 1). By contrast, as early as 15 minutes after addition of either a low or high dose of FGF2, the levels of phosphorylated (active) p42 and p44 increased markedly over levels observed in control explants (Fig. 1). Interestingly, the level of ERK activation correlated with the dose of FGF2 that the cells were exposed to. FGF2 induced a dose-dependent increase in ERK phosphorylation, with a fibre differentiating dose of FGF stimulating a greater level of ERK activation (Fig. 1, lane 3) than a proliferating dose of FGF (Fig. 1, lane 2). This labelling pattern persisted with prolonged culture (e.g. in lens epithelial cells exposed to FGF for 2 hours, Fig. 2A), and was consistent with the pattern of immunofluorescent labelling of lens epithelial cells for phosphorylated ERK1/2 (Fig. 2B-G). In control explants, few cells labelled for the phosphorylated ERK (Fig. 2B); however, after a 2 hour culture



**Fig. 1.** FGF2 induced phosphorylation of p42/p44 MAPK in a dose-dependent manner. Representative immunoblots of phospho-ERK (upper panel) and total ERK (lower panel) from lens epithelial explants exposed to no FGF2 (lane 1), 5 ng/ml FGF2 (lane 2) or 100 ng/ml FGF2 (lane 3) for 15 minutes at 37°C.

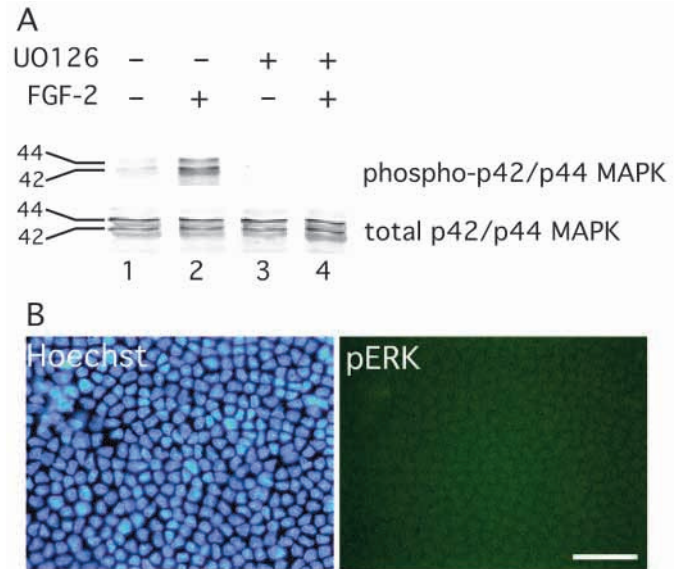


**Fig. 2.** FGF2-induced phosphorylation of p42/p44 MAPK. (A) Representative immunoblot of phospho-ERK from lens epithelial explants exposed to different concentrations of FGF2 for 2 hours at 37°C. Lens explants exposed to either no FGF2 (B,E), 5 ng/ml FGF2 (C,F) or 100 ng/ml FGF2 (D,G) for 2 hours at 37°C, immunolabelled for phospho-ERK (B-D) or counterstained with Hoechst dye (E-G). Scale bar: 40  $\mu$ m.

with a low dose of FGF2, an increased labelling in explants was observed (Fig. 2C). After a 2 hour culture with a high dose of FGF2, the majority of cells were labelled (Fig. 2D). Note that the predominantly cytoplasmic labelling shown here is characteristic of the early stages of ERK activation (see Introduction).

In contrast to the results with ERK, no significant labelling was detected in lens cell extracts of explants exposed to FGF2 for 15 to 120 minutes when using antibodies specific for the phosphorylated forms of other MAPKs, such as p38 and JNK/SAPK (data not shown).

To test the specificity of the phospho-specific antibodies for ERK as well as the activity of the MEK1 inhibitor (UO126), we exposed explants to a high dose of FGF2 (100 ng/ml) with or without a 2 hour pre-culture in the presence of inhibitor. Note that the inhibitor was present throughout the culture period and that explants not treated with UO126 were treated with an equivalent volume of DMSO (the manufacturer's recommended solvent for the inhibitor). This was the case for all subsequent experiments when testing the effects of UO126. UO126 was shown to block FGF-induced activation of ERK (Fig. 3A,B), in a dose-dependent manner (data not shown) with complete inhibition at a concentration between 25  $\mu$ M and 50  $\mu$ M. In all subsequent experiments, we used 50  $\mu$ M of UO126 to block activation of ERK. Under the same conditions, substituting

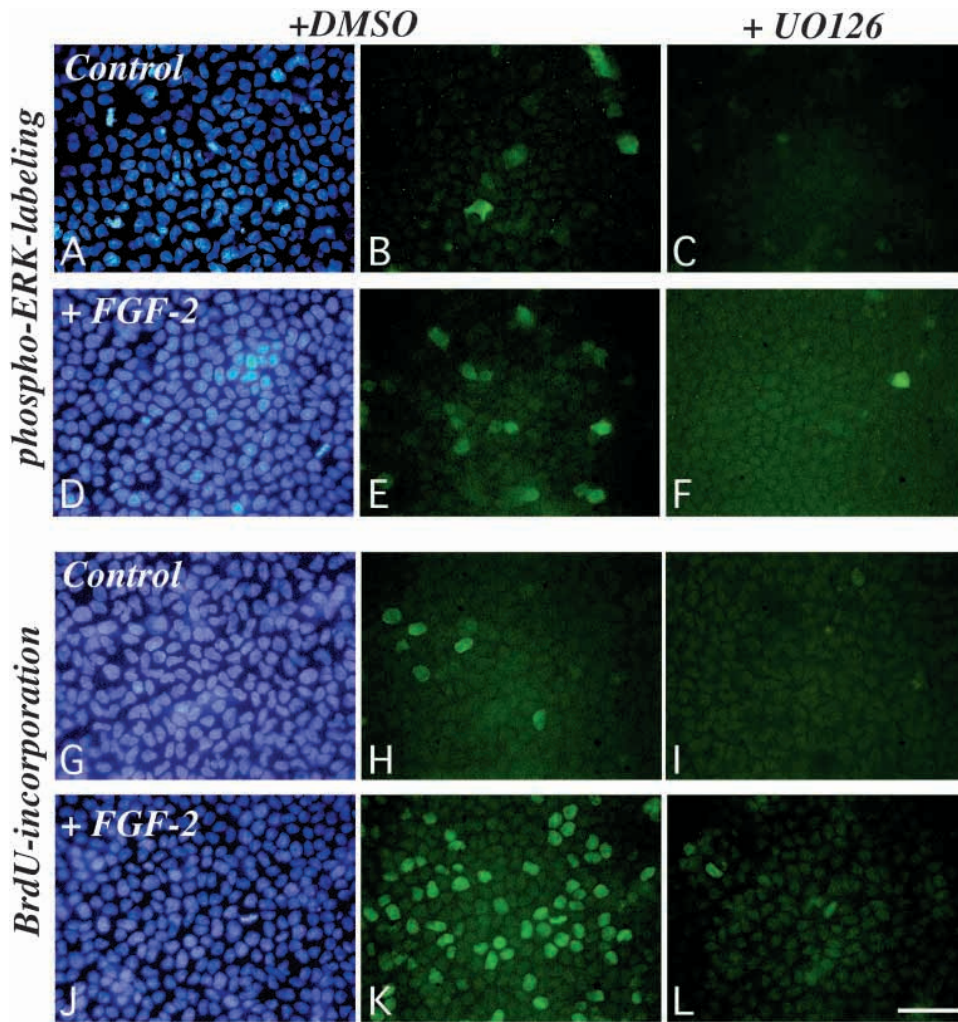


**Fig. 3.** UO126 blocks FGF-induced phosphorylation of p42/p44 MAPK. (A) Representative immunoblots of phospho-ERK (top) and total ERK (bottom) from lens epithelial explants exposed to no FGF2 (lanes 1 and 3) or 100 ng/ml FGF2 (lanes 2 and 4) for 15 minutes at 37°C, in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of UO126. (B) Lens explant exposed to 100 ng/ml of FGF2 in the presence of UO126, immunolabelled for phospho-ERK (right), counterstained with Hoechst dye (left). Control explants were treated with an equivalent volume of DMSO without UO126. Scale bar: 40  $\mu$ m.

UO126 with LY294002 (an inhibitor for the PI3 kinase pathway) did not influence the levels of ERK1/2 phosphorylated in response to FGF2 (data not shown).

### FGF-induced lens cell proliferation

To determine whether FGF-induced ERK activation plays a role in FGF-induced lens cell proliferation, we cultured lens explants with or without UO126 for 2 hours before the addition of FGF. A low dose of FGF was then added to half the dishes, with the other half left untreated (control explants). All explants were cultured for 2 days. Immunofluorescent labelling with anti-phospho-ERK1/2 antibodies demonstrated that in control explants, even after 2 days in culture, a few cells continued to label for phospho-ERK1/2 (Fig. 4B) indicating low level activation of endogenous ERKs. In the presence of FGF, increased levels of ERK activation were observed, with an increased number of cells immunolabelling for phospho-ERK1/2 (Fig. 4E), indicating that FGF could still induce ERK activation after 2 days in culture. The potency and stability of the MEK1 inhibitor was also confirmed after 2 days culture, with little to no labelling for phospho-ERK1/2 observed in control or FGF-treated explants cultured in the presence of UO126 (Fig. 4C,F). To assay for cell proliferation, we immunolabelled for BrdU incorporation which was administered to the cells 6 hours before collection of explants for analysis. Control explants demonstrated low levels of BrdU-reactive cells (Fig. 4H); however, in the presence of a low dose of FGF2, a marked increase in the number of cells labelled for BrdU-incorporation was observed. Consistent with the reduced labelling for



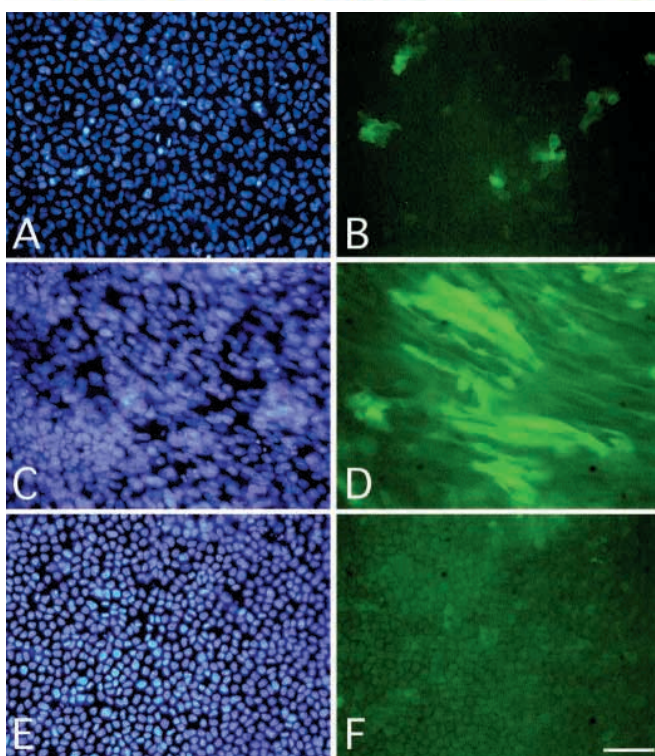
**Fig. 4.** UO126 blocks FGF-induced phosphorylation of p42/p44 MAPK and BrdU-incorporation. Representative micrographs of cells in lens explants exposed to no FGF (A-C,G-I) or 5 ng/ml FGF-2 (D-F,J-L) in the presence of DMSO (A,B,D,E,G,H,J,K) or DMSO with UO126 (C,F,I,L), immunolabelled for phospho-ERK (B,C,E,F) or BrdU-incorporation (H,I,K,L), counterstained with Hoechst dye (A,D,G,J). After 2 days culture, a low dose of FGF could still induce an increase in ERK phosphorylation (E), together with a marked increase in BrdU-incorporation (K). In the presence of UO126, however, phosphorylation of ERK and the number of cells that incorporated BrdU was markedly reduced both in control explants (C,I), and in explants treated with a low dose of FGF2 (F,L). Scale bar: 40  $\mu$ m.

phospho-ERK1/2 in the presence of UO126, FGF-induced lens cell proliferation was shown to be ERK dependent, with explants cultured in the presence of UO126 demonstrating little to no labelling for BrdU incorporation. In control explants, no BrdU-labelled cells were observed in the presence of UO126 (Fig. 4I) indicating that the endogenous mitogen active in these explants is ERK dependent. In

FGF-treated explants exposed to UO126, we noted a marked reduction in the number of BrdU-labelled cells (Fig. 4L) with only a small number of reactive cells for BrdU-incorporation occasionally observed. It was noted that these few cells were predominantly found at the explant periphery (data not shown).

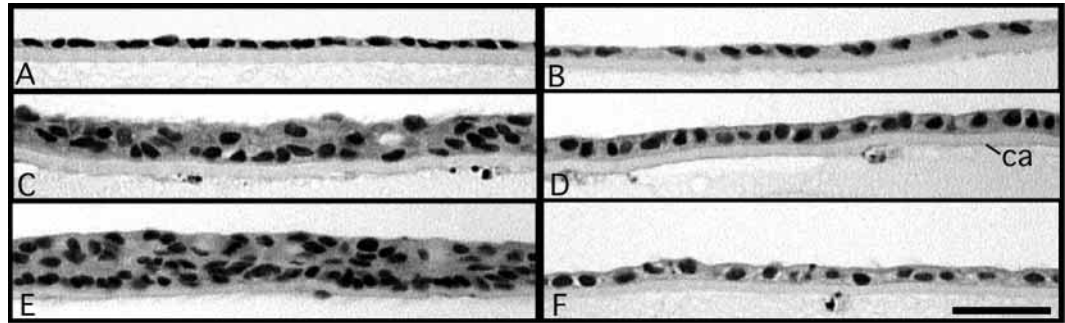
#### FGF-induced lens fibre differentiation

Adopting a similar approach used to examine the role of ERK signalling in FGF-induced lens cell proliferation, we examined the role of ERK signalling in FGF-induced lens fibre



**Fig. 5.** FGF-induced fibre cell elongation and multilayering accompanied phosphorylation of ERK and could be blocked by UO126. Representative micrographs of cells in lens explants not exposed to FGF2 (A,B) or exposed to 100 ng/ml FGF2 (C-F) in the presence of DMSO (A-D) or DMSO with UO126 (E,F), immunolabelled for phospho-ERK (B,D,F) or counterstained with Hoechst dye (A,C,E). After 5 days culture with FGF, cells have elongated and multilayered as shown by the overlapping large ovoid nuclei (C). These cells also displayed a marked increase in phospho-ERK labelling (D) compared with epithelial cells of control explants (B). This FGF-induced activation of ERK was blocked in the presence of UO126 (F), which not only resulted in inhibition of cell elongation (compare D with F) but also multilayering of the cells (compare C with E). Scale bar: 50  $\mu$ m.

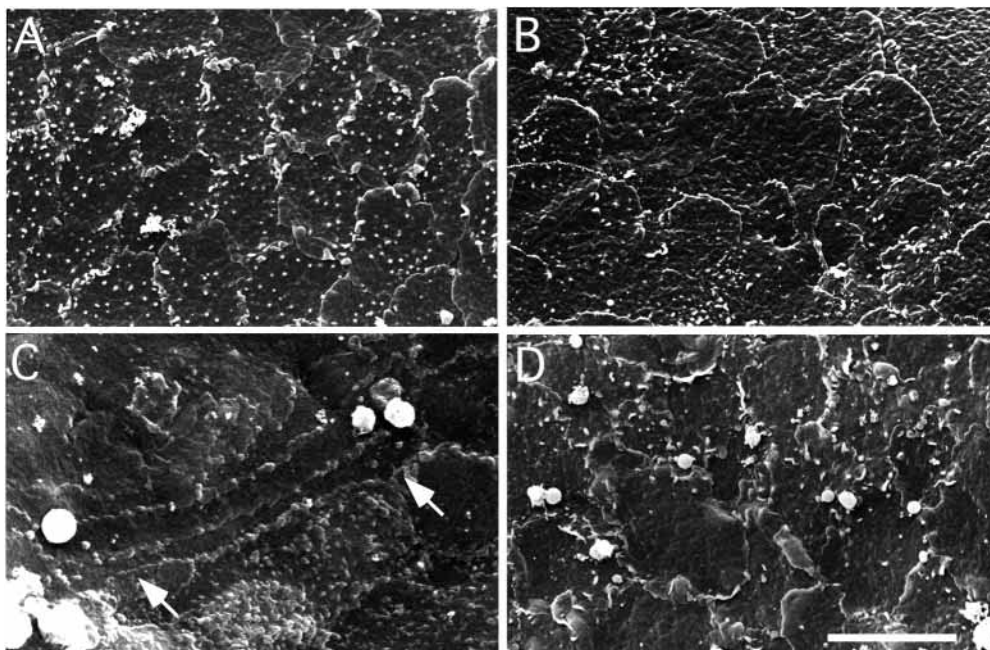
**Fig. 6.** UO126 blocks FGF-induced cell multilayering in lens explants. Representative sections of explants cultured for either 3 (C,D) or 5 days (A,B,E,F) with no FGF2 (A,B) or 100 ng/ml of FGF2 (C-F) in the presence of DMSO (A,C,E) or DMSO with UO126 (B,D,F). FGF2 induced a progressive thickening of lens explants over the culture period, as cells elongated and multilayered (C,E). In the presence of UO126, the multilayering could be blocked with cells remaining as a monolayer on the lens capsule (D,F), similar to control explants (A). Abbreviations: ca, lens capsule. Scale bar: 20  $\mu$ m.



differentiation by culturing explants over 5 days in the presence of UO126, only now with an increased dose of FGF2 (100 ng/ml) to induce lens fibre differentiation. We found that after 5 days in culture, a fibre-differentiating dose of FGF induced a marked activation of ERK in lens cells (Fig. 5). Unlike cells in control explants which remained in a monolayer (Fig. 5A,B), cells in explants cultured for 5 days in the presence of FGF elongated and multilayered, and this was accompanied by increased immunolabelling for phospho-ERK1/2 (Fig. 5C,D), indicating that this pathway plays a role in this process. Consistent with earlier findings, in the presence of UO126, no labelling for phospho-ERK1/2 was evident in these explants after 5 days culture (Fig. 5E,F), confirming the activity and stability of this inhibitor even during extended periods of culture. It was also noted that in the presence of UO126, cells cultured with FGF appeared to remain as a monolayer (similar to control explants) and did not show any signs of elongation. This was confirmed in histological sections of these explants (Fig. 6). Explants cultured in the presence of FGF over 5 days underwent a marked increase in thickness, owing to multilayering associated with fibre differentiation (Fig. 6C,E). Control explants remained as a monolayer of epithelial cells over the 5

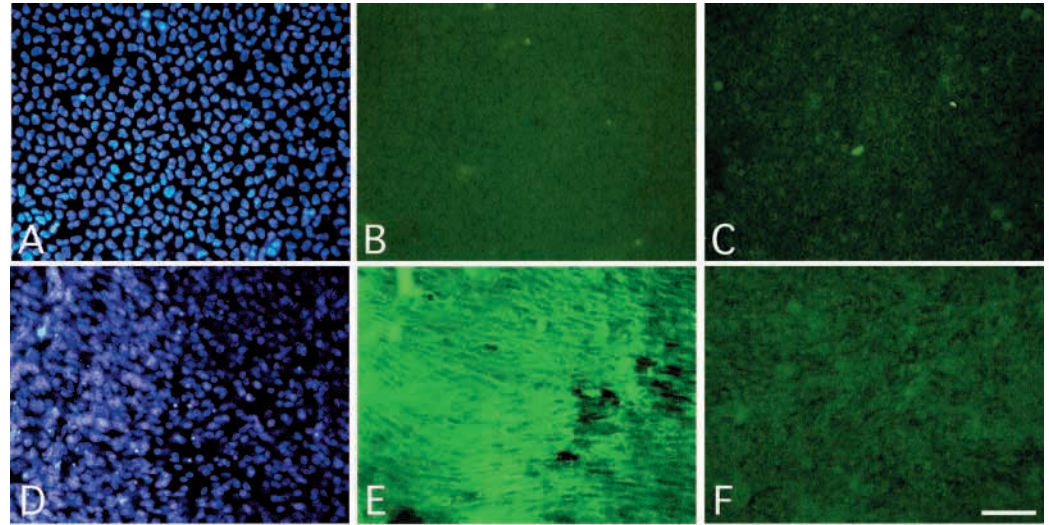
day culture period (Fig. 6A). In the presence of UO126, explants remained as a monolayer of epithelial cells whether FGF was added (Fig. 6D,F) or not (Fig. 6B). Scanning electron microscopy analysis demonstrated that only explants cultured with FGF in the absence of UO126, underwent cell elongation characteristic of the fibre differentiation process (Fig. 7C, arrows). Cells in explants cultured in the presence of a differentiating dose of FGF and UO126, retained an epithelial-like morphology (Fig. 7D), similar to that of cells in control explants (Fig. 7A,B).

To confirm that we were indeed inhibiting FGF-induced lens fibre differentiation by blocking the activation of ERK signalling, we assayed for changes in the expression of other fibre differentiation markers, namely, the accumulation of filensin and  $\beta$ -crystallin. Filensin is an intermediate filament, a component of the beaded filament, unique to fibre cells of the lens (Blankenship et al., 2001). Expression of this cytoskeletal structure accompanies the elongation of fibre cells. This is clearly shown after 5 days culture with a fibre-differentiating dose of FGF, which induces a marked increase in filensin reactivity in lens explants (Fig. 8D,E), when compared with control explants (Fig. 8A,B). Consistent with our earlier findings that showed that the morphological changes associated with FGF-induced fibre differentiation were dependent on ERK signalling, little to no labelling for filensin was apparent when explants were cultured in the



**Fig. 7.** UO126 blocks FGF-induced cell elongation in lens explants. Representative scanning electron micrographs of cells in explants cultured for 5 days in the absence of FGF2 (A,B) or with 100 ng/ml of FGF2 (C,D) in the presence of DMSO (A,C) or DMSO with UO126 (B,D). FGF2 induced the elongation of lens epithelial cells (C, arrows). In the presence of UO126, this elongation could be blocked (D), with cells demonstrating a similar morphology to cells in control explants (A,B). Scale bar: 5  $\mu$ m.

**Fig. 8.** UO126 blocks FGF-induced upregulation of filensin expression, which accompanies fibre differentiation. Representative micrographs of cells in lens explants cultured for 5 days in the absence of FGF2 (A-C) or with 100 ng/ml FGF2 (D-F) in the presence of DMSO (A,B,D,E) or DMSO with UO126 (C,F), immunolabelled for filensin (B,C,E,F) or counterstained with Hoechst dye (A,D). FGF2 induced a marked increase in filensin expression (E). In the presence of UO126, no reactivity was observed in response to FGF2 (F), with levels comparable with control explants (C). Scale bar: 50  $\mu$ m.



presence of both FGF and UO126 (Fig. 8F). No differences in filensin labelling was observed in control explants either in the presence (Fig. 8C) or absence (Fig. 8B) of UO126. In contrast to the ERK-dependent expression of filensin in response to FGF in lens explants, we found that FGF-induced  $\beta$ -crystallin accumulation associated with fibre differentiation was independent of ERK activation (Fig. 9). FGF-induced fibre differentiation in lens explants is accompanied by the accumulation of  $\beta$ -crystallin (Fig. 9D,E) as cells elongate and multilayer. Control explants demonstrate little to no reactivity for  $\beta$ -crystallin (Fig. 9B). In the presence of a fibre-differentiating dose of FGF and UO126, consistent with earlier findings, lens cells remain in a monolayer and retain epithelial-like packing. However, unlike other fibre cell markers we examined, these cells retain the ability to synthesise and accumulate  $\beta$ -crystallin (Fig. 9F). No differences in  $\beta$ -crystallin labelling was observed in control explants either in the presence (Fig. 9C) or absence (Fig. 9B) of UO126.

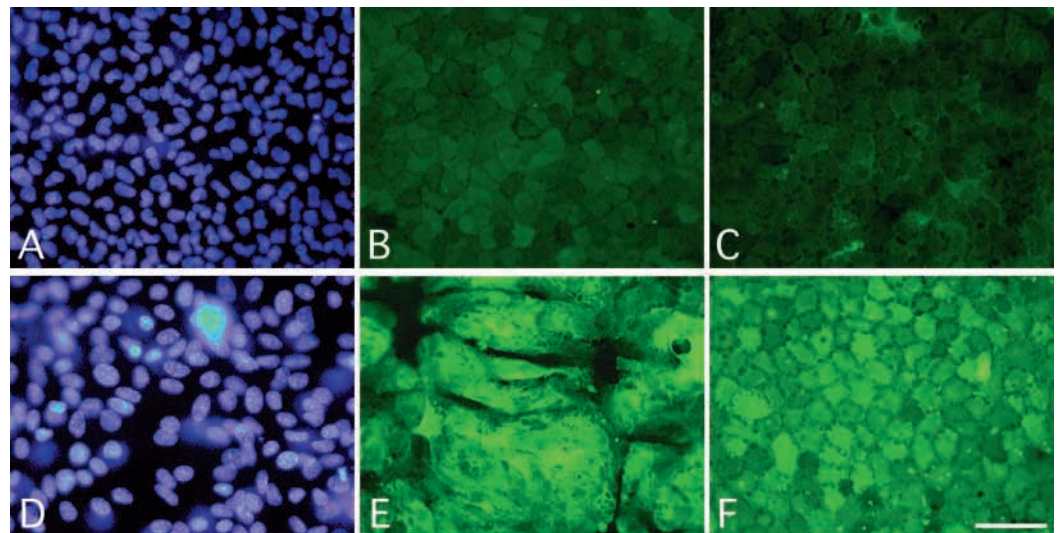
#### In vivo expression of phospho-ERK1/2

To determine the labelling pattern of the phosphorylated ERKs *in vivo*, we immunolabelled frozen sections of whole neonatal lenses with anti-phospho-ERK1/2 antibodies. We detected strong punctate nuclear reactivity throughout the epithelial cells, extending into the transitional zone where cells are undergoing the earliest changes associated with fibre differentiation (Fig. 10A,B).

Fibre cells slightly deeper in the lens cortex demonstrated a marked reduction in labelling with no reactivity apparent in these maturing cells (Fig. 10A,B, arrow). Note that we could not detect any labelling for the phosphorylated forms of p38 (Fig. 10C) or JNK/SAPK (data not shown) in the lens.

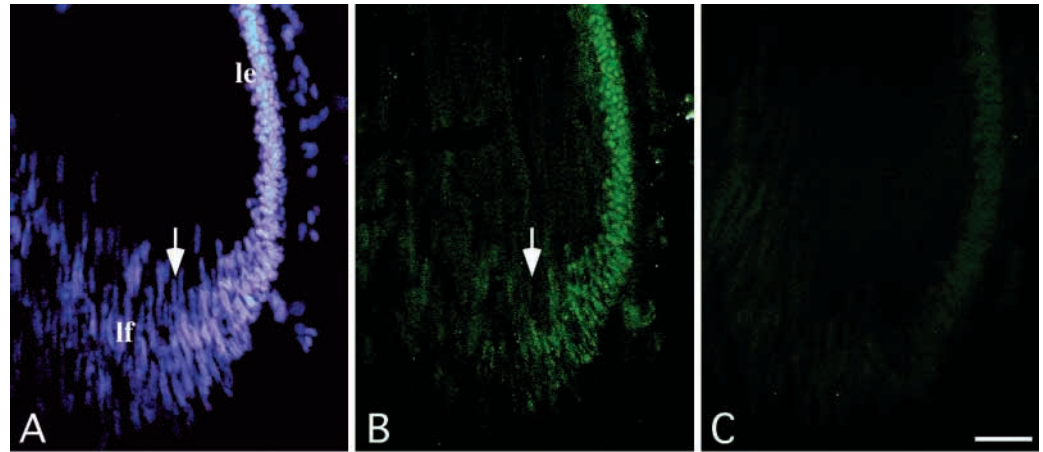
#### DISCUSSION

Over the past decade, there has been increasing support for a role for growth factors in regulating the cellular events involved in lens differentiation and growth, namely cell proliferation and fibre differentiation (Chamberlain and McAvoy, 1997). Members of the FGF family have been shown to induce both



**Fig. 9.** UO126 does not block FGF-induced  $\beta$ -crystallin expression, which accompanies fibre differentiation. Representative micrographs of cells in lens explants cultured for 5 days in the absence of FGF2 (A-C) or with 100 ng/ml FGF2 (D-F) in the presence of DMSO (A,B,D,E) or DMSO with UO126 (C,F), immunolabelled for  $\beta$ -crystallin (B,C,E,F) or counterstained with Hoechst dye (A,D). FGF2 induces a marked increase in  $\beta$ -crystallin expression (E) with little to no reactivity detected in control explants (B). In the presence of UO126, although the cells retain an epithelial morphology in response to FGF2, they express  $\beta$ -crystallin (F). Scale bar: 40  $\mu$ m.

**Fig. 10.** Representative frozen sections of neonatal lenses counterstained with Hoechst dye (A) or immunolabelled for phospho-ERK (B) or phospho-p38 (C). Strong punctate nuclear reactivity for phospho-ERK was detected throughout the epithelial cells, extending into the transitional zone where cells are elongating into fibres (A,B). Fibre cells deeper in the cortex demonstrated a marked reduction in labelling with no reactivity apparent in these maturing cells (A,B, arrows). No labelling for phospho-p38 was detected in the lens (C). Abbreviations: le, lens epithelium; lf, lens fibres. Scale bar: 40  $\mu$ m.



lens cell proliferation and differentiation; however, to date, the signal transduction pathways by which FGFs exert their effects on the lens are poorly understood. As a means of addressing this issue, we used a rat lens epithelial explant system to examine the signalling pathways activated in response to FGF, and the role that these pathways play in lens cell proliferation and fibre differentiation.

Earlier studies from our laboratory using lens explants have shown that FGF can induce lens cell proliferation and differentiation in a dose-dependent manner: a low dose of FGF induces lens cell proliferation but not fibre differentiation, whereas a high dose of FGF can also induce fibre differentiation (McAvoy and Chamberlain, 1989). This observation that a single growth factor can induce different responses in a single cell type, led us to investigate how each of these cellular events may be regulated. As a first step in identifying the intracellular signalling pathways involved in this process, we have shown that FGF is a potent inducer of ERK activation in lens epithelial cells. Consistent with a recent study by Le and Musil (Le and Musil, 2001), we report that with a 15 minute exposure to FGF, lens epithelial cells demonstrate a marked increase in ERK activation. In addition, in our study we found that a fibre-differentiating dose of FGF can stimulate a greater increase in ERK phosphorylation than a lower proliferating dose of FGF. This dose-dependent differential activation of ERK was more pronounced after a 2 hour exposure to FGF using both western blotting and immunofluorescent detection of the phosphorylated forms of ERK. Activation of ERK was also monitored over longer periods of culture by immunofluorescent labelling to examine its relationship with FGF-induced proliferation and differentiation. Explants exposed to a proliferating dose of FGF continued to demonstrate an increased number of lens epithelial cells with increased levels of ERK phosphorylation, compared with control explants (not exposed to FGF), after 2 days. In explants exposed to a fibre differentiating dose of FGF for 5 days, elongating cells displayed strong immunoreactivity for phospho-ERK. At this stage of the study, it appeared that the events involved in both FGF-induced lens cell proliferation and differentiation may be initiated by a common MAPK (ERK) signalling pathway.

To more directly address the issue of whether FGF-induced activation of ERK signalling was required for the induction of

lens cell proliferation and differentiation, we employed a highly potent and specific inhibitor of MEK1/2 (UO1216) (Favata et al., 1998) in our tissue culture studies. By immunolabelling for phospho-ERK, we demonstrated that with a single 2 hour pre-incubation of explants with UO126, we could not only block the basal levels of phosphorylated ERK in control explants but effectively block FGF-induced ERK activation in a dose-dependent fashion. Using immunofluorescence, we found that 50  $\mu$ M UO126 was the lowest dose we found to block reproducibly and effectively most, if not all, ERK activation induced by a fibre-differentiating dose of FGF. Providing the inhibitor was present throughout the culture period, we found that FGF-induced ERK activation could be continually blocked for up to 5 days (see Fig. 5). In light of this, we assayed for FGF-induced lens cell proliferation and differentiation in the presence or absence of UO126.

We used immunolabelling of BrdU-incorporation as a marker for cell proliferation induced in rat lens explants cultured for up to 2 days with a low dose of FGF. As with earlier studies in our laboratory, we demonstrated a marked increase in the levels of cell proliferation in explants incubated with FGF when compared with control explants (see Fig. 4). This increased cell proliferation was shown to be ERK dependent. Consistent with earlier studies that have shown that inhibition of ERK signalling leads to inhibition of DNA synthesis (Pagés et al., 1993; Brondello et al., 1995; Hulleman et al., 1999), we found that in the presence of UO126, virtually all FGF-induced cell proliferation could be blocked, as assessed by the marked reduction in the number of cells incorporating BrdU, with only a few cells at the explant periphery still BrdU positive. These findings are in contrast to a recent study showing that 15  $\mu$ M UO126 did not diminish the ability of FGF to stimulate cell proliferation in a monolayer culture of dissociated chick lens epithelial cells (Le and Musil, 2001). The difference between the findings of this study and the present study may be attributed to several factors, including the source and type of cell culture system used (dissociated chick lens cells primarily derived from the peripheral regions of the epithelium) as well as the lower concentration of UO126 employed. In our system (intact rat lens epithelial explants) we found that 12 to 25  $\mu$ M UO126 was not sufficient to block FGF-induced ERK phosphorylation completely. Interestingly, consistent with the fact that basal



levels of phosphorylated ERK were blocked in our control explants in the presence of UO126, we also observed the inhibition of basal levels of cell proliferation in these explants. This indicates that the endogenous mitogen active in the lens explant system is dependent on ERK activation, supporting our finding that ERK signalling is important for lens cell proliferation.

To assess if UO126 was having a similar inhibitory effect on FGF-induced fibre differentiation, we examined the morphology of cells in explants treated with or without this inhibitor, as well as assayed for the presence of lens fibre-specific molecular markers, filensin and  $\beta$ -crystallin. Our first indication that UO126 was having a profound effect on FGF-induced differentiation was when we examined explants labelled with Hoechst dye, which stained cell nuclei. The nuclei of epithelial cells in control explants are notably round, typical of the cuboidal epithelial cell morphology (Fig. 5). As these epithelial cells differentiate in response to FGF, the cells multilayer as they begin to elongate into fibre cells. The elongation of the cells is accompanied by the nuclei also becoming larger, more elongate and ovoid in shape (see Fig. 5). We found that in the presence of UO126, the cells in explants cultured with a high dose of FGF did not multilayer but remained as a monolayer. Furthermore, the size and shape of the cell nuclei did not change and appeared similar to that typically seen in control explants (see Fig. 5). To further examine this, we carried out a histological analysis of explants cultured with a high dose of FGF, in the presence or absence of UO126. In the presence of UO126, cells cultured with a fibre-differentiating dose of FGF remained as a monolayer and did not elongate, as assessed by scanning electron microscopy. By contrast, explants exposed to FGF in the absence of the inhibitor, progressively thickened as a result of cell multilayering and elongation. Thus, although other studies have reported an association between ERK signalling and FGF-mediated lens fibre differentiation (Chow et al., 1995; Govindarajan and Overbeek, 2001; Le and Musil, 2001), this is the first report that the morphological changes associated with FGF-induced lens fibre differentiation are directly dependent on ERK activation.

Further characterisation of the inhibitory effects of UO126 on FGF-induced fibre differentiation led us to examine the expression of filensin, an intermediate filament comprising the cytoskeleton of lens fibre cells (Blakenship, 2001). We report for the first time that filensin expression is upregulated in response to FGF, as epithelial cells elongate into fibre cells. Consistent with our earlier findings, in the presence of UO126, the increased expression of filensin accompanying FGF-induced cell elongation can be blocked as cells retain their epithelial-like characteristics. This accords with recent findings by Le and Musil (Le and Musil, 2001), who reported a role for ERK activation in FGF-induced upregulation of CP49 expression (another cytoskeletal fibre differentiation marker) in chick lens cells. While our *in vitro* result indicates that FGF-induced filensin expression may be directly mediated via ERK signalling, it should be noted that our *in vivo* labelling of phosphoERK (see Fig. 10) shows that labelling diminishes in the elongating cortical fibres which is where filensin expression is initiated (Blakenship et al., 2001). This raises the possibility that the maintenance, and even perhaps the induction, of filensin expression may be an indirect effect of ERK-mediated FGF signalling.

The expression of  $\beta$ -crystallin is another well established

fibre-specific marker that we have adopted in this study. As reported in many earlier studies, accompanying the morphological changes characteristic of fibre differentiation,  $\beta$ -crystallin expression is upregulated in explants cultured with a high dose of FGF. In the present study, we have shown that we can uncouple the fibre differentiation process by blocking the activation of ERK. In the presence of both UO126 and FGF, although the cells retain an epithelial-like morphology, they continue to express  $\beta$ -crystallin, indicating that FGF-induced  $\beta$ -crystallin expression is independent of ERK activation. Le and Musil (Le and Musil, 2001) also showed that FGF-induced  $\delta$ -crystallin (a chick fibre differentiation marker) expression in chick lens cells is ERK independent. Earlier indications that crystallin gene expression and fibre elongation could be uncoupled arose from experiments where explants were pulsed with FGF for a short period and then exposed to insulin or IGF for the remainder of the culture period. Cells in these explants upregulated the expression of the fibre-specific  $\beta$ - and  $\gamma$ -crystallins but did not elongate, instead they became large and swollen and formed clumps (Leenders et al., 1997; Klok et al., 1998). Similarly, when explants were cultured in the presence of a low (proliferating) dose of FGF in combination with PDGF (another lens epithelial cell mitogen), they upregulated expression of  $\beta$ -crystallin but did not present the elongate morphology normally associated with fibre differentiation, rather they appeared as large rounded cells and formed clusters throughout the explant (A. Kok, F. L., C. Chamberlain and J. M., unpublished). In these studies, it appears that the fibre differentiation process has been uncoupled by application of a combination of factors that predominantly induce an increase in  $\beta$ -crystallin expression, rather than the morphological changes normally accompanying this differentiation event. Taken together with results from the present study, these findings indicate that FGF-induced lens fibre differentiation may involve several independent signalling pathways, with some pathways (e.g. the ERK signalling pathway) important for initiating the morphological changes associated with this process and other, yet to be identified pathways, regulating the expression of the fibre-specific crystallins.

The *in vivo* expression of phosphorylated ERKs in the neonatal rat lens lends further support to our *in vitro* findings that the processes of lens cell proliferation and the early morphological changes associated with secondary fibre differentiation are dependent on ERK activation. In fresh frozen lens sections, we detected immunoreactivity throughout the proliferative population of epithelial cells, extending into the transitional zone where cells elongate and undergo the earliest morphological changes associated with fibre differentiation (see Fig. 10). This is consistent with the increased labelling for the phosphorylated ERKs observed in elongating cells induced by FGF in our explants. As the cells continue to elongate and are displaced slightly deeper in the lens cortex where they express  $\beta$ -crystallin, a marked reduction in phospho-ERK immunolabelling is observed. Such a reduction in ERK phosphorylation in this region of the lens indicates that this signalling pathway may not be essential for the induction and/or maintenance of expression of some differentiation markers, including  $\beta$ -crystallin, and accords with findings from our *in vitro* study. In light of this, the later stages of the fibre differentiation process may be dependent on additional signalling pathways such as those induced by members of the

TGF $\beta$  family. Using a dominant negative approach, recent in vivo studies in transgenic mice have shown that TGF $\beta$  signalling is essential for later stages of the fibre differentiation process (de Jongh et al., 2001).

Overall, in the present study we have shown that FGF can stimulate activation of ERK in a dose-dependent manner, with a fibre differentiating dose of FGF stimulating increased activation of ERK compared with a lower proliferation dose of FGF. Consistent with the fact that growth factor-induced cell proliferation in many cell types is dependent on ERK signalling, we report for the first time a similar dependency of FGF-induced lens cell proliferation on ERK activation. Our findings also indicate that FGF-induced lens cell proliferation utilises a similar signalling pathway to that required for cell elongation, but not for specialisation of crystallin gene expression, during the process of lens fibre differentiation. The differential cell responsiveness induced by different concentrations of FGF may be regulated by the intensity and/or duration of the activated ERK signalling pathway (Marshall, 1995). Furthermore, the uncoupling of the fibre differentiation process demonstrated in this study leads us to conclude that other yet to be identified signalling pathways are likely to also be involved in the lens fibre differentiation process.

The authors thank Lena Caruso and Louise van der Weyden for their invaluable technical assistance, and the Electron Microscope Unit at the University of Sydney for use of its facilities. We also thank Dr Paul FitzGerald from the University of California, Davis, for providing the anti-filensin antibody. This study was supported by funding from the NHMRC (Australia), the NEI (USA), the Sydney Foundation for Medical Research and the NSW Department of Health, Australia.

## REFERENCES

- Blankenship, T. N., Hess, J. F. and FitzGerald P. G. (2001). Development- and differentiation-dependent reorganization of intermediate filaments in fiber cells. *Invest. Ophthalmol. Vis. Sci.* **42**, 735-742.
- Brondello, J. M., McKenzie, F. R., Sun, H., Tonks N. K. and Pouyssegur J. (1995). Constitutive MAP kinase phosphatase (MKP-1) expression blocks G1 specific gene transcription and S-phase entry in fibroblasts. *Oncogene* **10**, 1896-1904.
- Browaeys-Poly, E., Cailliau, K. and Vilain, J. P. (2000). Signal transduction pathways triggered by fibroblast growth factor receptor 1 expressed in *Xenopus laevis* oocytes after fibroblast growth factor 1 addition. Role of Grb2, phosphatidylinositol 3-kinase, Src tyrosine kinase, and phospholipase C gamma. *Eur. J. Biochem.* **267**, 6256-6263.
- Browaeys-Poly E., Cailliau, K. and Vilain, J. P. (2001). Transduction cascades initiated by fibroblast growth factor 1 on *Xenopus* oocytes expressing MDA-MB-231 mRNAs. Role of Grb2, phosphatidylinositol 3-kinase, Src tyrosine kinase, and phospholipase C gamma. *Cell Signal.* **13**, 363-368.
- Chamberlain, C. G. and McAvoy, J. W. (1997). Fibre differentiation and polarity in the mammalian lens: a key role for FGF. *Prog. Ret. Eye Res.* **16**, 443-478.
- Chen, Y., Li, X., Eswarakumar, V. P., Seger, R. and Lonai, P. (2000). Fibroblast growth factor (FGF) signaling through PI 3-kinase and Akt/PKB is required for embryoid body differentiation. *Oncogene* **19**, 3750-3756.
- Chow, R. L., Roux, G. D., Roghani, M., Palmer, M. A., Rifkin, D. B., Moscatelli, D. A. and Lang, R. A. (1995). FGF suppresses apoptosis and induces differentiation of fibre cells in the mouse lens. *Development* **121**, 4383-4393.
- de Jongh, R. U., Lovicu, F. J., Overbeek, P. A., Schneider, M. D., Joya, J., Hardeman, E. and McAvoy, J. W. (2001). Requirement for TGF $\beta$  receptor signaling during terminal lens fiber differentiation. *Development* **128**, 3995-4010.
- Fantl, W. J., Johnson, D. E. and Williams, L. T. (1993). Signaling by receptor tyrosine kinases. *Annu. Rev. Biochem.* **62**, 453-481.
- Favata, M. F., Horiuchi, K. Y., Manos, E. J., Daulerio, A. J., Stradley, D. A., Feese, W. S., Van Dyk, D. E., Pitts, W. J., Earl, R. A., Hobbs, F. et al. (1998). Identification of a novel inhibitor of mitogen-activated protein kinase. *J. Biol. Chem.* **273**, 18623-18632.
- Govindarajan, V. and Overbeek, P. A. (2001). Secreted FGFR3, but not FGFR1, inhibits lens fiber differentiation. *Development* **128**, 1617-1627.
- Hulleman, E., Bijvelt, J. J. M., Verkleij, A. J., Verrips, C. T. and Boonstra J. (1999). Nuclear translocation of mitogen-activated protein kinase p42<sup>MAPK</sup> during the ongoing cell cycle. *J. Cell. Physiol.* **180**, 325-333.
- Hyatt, G. A. and Beebe, D. C. (1993). Regulation of lens cell growth and polarity by an embryo-specific growth factor and by inhibitors of lens cell proliferation and differentiation. *Development* **117**, 701-709.
- Klok, E. J., Lubsen, N. H., Chamberlain, C. G. and McAvoy, J. W. (1998). Induction and maintenance of differentiation of rat lens epithelium by FGF-2, insulin and IGF-1. *Exp. Eye Res.* **67**, 425-431.
- Le, A. C. and Musil, L. S. (2001). FGF signaling in chick lens development. *Dev. Biol.* **233**, 394-411.
- Leenders, W. P., van Genesen, S. T., Schoenmakers, J. G., van Zoelen, E. J. and Lubsen, N. H. (1997). Synergism between temporally distinct growth factors: bFGF, insulin and lens cell differentiation. *Mech. Dev.* **67**, 193-201.
- Lewis, T. S., Shapiro, P. S. and Ahn, N. G. (1998). Signal transduction through MAP kinase cascades. *Adv. Cancer Res.* **74**, 49-139.
- Lin, H. Y., Xu, J., Ischenko, I., Ornitz, D. M., Halegoua, S. and Hayman, M. J. (1998). Identification of the cytoplasmic regions of fibroblast growth factor (FGF) receptor 1 which play important roles in induction of neurite outgrowth in PC12 cells by FGF-1. *Mol. Cell Biol.* **18**, 3762-3770.
- Lovicu, F. J. and McAvoy, J. W. (1989). Structural analysis of lens epithelial explants induced to differentiate into fibres by fibroblast growth factor (FGF). *Exp. Eye Res.* **49**, 479-494.
- Lovicu, F. J. and Overbeek, P. A. (1998). Overlapping effects of different members of the FGF family on lens fiber differentiation in transgenic mice. *Development* **125**, 3365-3377.
- Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell.* **80**, 179-185.
- McAvoy, J. W. and Chamberlain, C. G. (1989). Fibroblast growth factor (FGF) induces different responses in lens epithelial cells depending on its concentration. *Development* **107**, 221-228.
- McAvoy, J. W., Chamberlain, C. G., de Jongh, R. U., Richardson, N. A. and Lovicu, F. J. (1991). The role of fibroblast growth factor in eye lens development. *Ann. New York Acad. Sci.* **638**, 256-274.
- McAvoy, J. W., Chamberlain, C. G., de Jongh, R. U., Hales, A. M. and Lovicu, F. J. (1999). Lens development. *Eye* **13**, 425-437.
- Ornitz, D. M. and Itoh, N. (2001). Fibroblast growth factors. *Genome Biol.* **2**, 3005.
- Pagés, G., Lenormand, P., L'Allemain, G., Chambard, J.-C., Meloche, S. and Pouyssegur J. (1993). Mitogen-activated protein kinases p42<sup>MAPK</sup> and p44<sup>MAPK</sup> are required for fibroblast proliferation. *Proc. Natl. Acad. Sci. USA* **90**, 8319-8323.
- Robinson, M. L., Overbeek, P. A., Verran, D. J., Grizzle, W. E., Stockard, C. R., Friesel, R., Maciag, T. and Thompson, J. A. (1995a). Extracellular FGF-1 acts as a lens differentiation factor in transgenic mice. *Development* **121**, 505-514.
- Robinson, M. L., MacMillan-Crow, L. A., Thompson, J. A. and Overbeek, P. A. (1995b). Expression of a truncated FGF receptor results in defective lens development in transgenic mice. *Development* **121**, 3959-3967.
- Sa, G. and Das, T. (1999). Basic fibroblast growth factor stimulates cytosolic phospholipase A2, phospholipase C-gamma1 and phospholipase D through distinguishable signaling mechanisms. *Mol. Cell Biochem.* **198**, 19-30.
- Schaeffer, H. J. and Weber, M. J. (1999). Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol. Cell Biol.* **19**, 2435-2444.
- Shirke, S., Faber, S. C., Hallem, E., Makarenkova, H. P., Robinson, M. L., Overbeek, P. A. and Lang, R. A. (2001). Misexpression of IGF-I in the mouse lens expands the transitional zone and perturbs lens polarization. *Mech. Dev.* **101**, 167-174.
- Stolen, C. M. and Griep, A. E. (2000). Disruption of lens fiber cell differentiation and survival at multiple stages by region-specific expression of truncated FGF receptors. *Dev. Biol.* **217**, 205-220.
- Yamashita, T., Yoshioka, M. and Itoh, N. (2000). Identification of a novel fibroblast growth factor, FGF-23, preferentially expressed in the ventrolateral thalamic nucleus of the brain. *Biochem. Biophys. Res. Commun.* **277**, 494-498.