

# Inherited retinal degeneration: basic FGF induces phagocytic competence in cultured RPE cells from RCS rats

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**Abstract** In RCS rats, the retinal pigment epithelium (RPE) is defective in phagocytosis of photoreceptor membranes. We have previously shown reduced expression of basic fibroblast growth factor (bFGF) in the RPE of 7–10-day-old RCS rats. This study using primary RPE cultures from rats of this age demonstrates that the phagocytic defect in the mutant RPE can be overcome by treatment with bFGF, by a mechanism involving gene transcription and that normal RPE phagocytosis, also requiring transcription, is blocked by a bFGF neutralizing antibody. The combined data point to a role for bFGF in the normal mechanism of RPE phagocytosis and the RCS defect.

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**Key words:** Basic fibroblast growth factor; RPE cell; Phagocytosis; RCS rat; Inherited retinal degeneration

## 1. Introduction

In the retina, synchronized phagocytosis of several hundred packets of photoreceptor outer segment membranes is a daily task of each cell of the retinal pigment epithelium (RPE), necessitated by the ongoing renewal and rhythmic shedding of disc membranes by the neighboring visual cells [1–3]. The Royal College of Surgeons (RCS) rat is a rodent model of hereditary photoreceptor degeneration in which an unidentified genetic defect (*rdy*) known to be expressed in the RPE renders these cells defective in the phagocytic process. As a result, membranous debris accumulates in the subretinal space from postnatal day 12 (P12) onward, and the photoreceptors degenerate and disappear from the retina between P20 and P60, although the mutant RPE cells remain in place for many months [4–9]. The phagocytic defect is retained in primary cultures of RPE cells isolated from 6 to 12-day-old RCS rats, following phagocytic challenge with isolated rod outer segments (ROS) [10]. Previous studies with the mutant RPE cells have determined that this defect is specific to ROS and involves impaired ROS ingestion subsequent to binding to the cell surfaces [10,11]. It is widely accepted that normal RPE phagocytosis is a receptor-mediated process involving a glycoprotein interaction but the identity of the cell surface receptor(s) participating in ROS binding and ingestion is currently a subject of ongoing investigation. (For a comprehensive review of literature up to 1994, see [12].) To date, two known receptors, i.e. the macrophage mannose receptor [13] and the

scavenger receptor CD36 [14], as well as other unknown proteins of RPE plasma membranes [15,16], have been proposed as candidates for the ROS receptor.

Basic FGF (FGF-2) is a widely distributed heparin-binding growth factor with a remarkably diverse range of actions critical in normal development, reproduction, angiogenesis and responses of the nervous system to injury [17–23]. Although intraocular injection of several different neurotrophic factors including bFGF has been shown to protect normal rat photoreceptors from insults such as light damage [24] and physical trauma [25], only bFGF has been shown to delay the demise of the photoreceptors in the RCS model of inherited retinal degeneration [26]. We have recently shown that bFGF mRNA and protein expression are markedly down-regulated in the RPE of RCS rats during the developmental period (P7–P12) when expression of the phagocytic defect first occurs in vivo [27]. Since the primary genetic defect resides in the RPE in this model [7], the present investigation tested whether exogenously added bFGF could also improve the phagocytic capability of the mutant RPE cells, using an in vitro paradigm. Due to the marked phenotypic heterogeneity characteristic of rat primary RPE cultures, and profound differences in kinetics of phagocytosis by cells of different phenotypes [28], analyses described here were performed over cells within the cultures having morphology and phagocytic behavior (i.e. synchronous ingestion of multiple ROS per cell) closest to that of RPE in vivo. Here we demonstrate (1) that mutant rat RPE cells can be stimulated to ingest bound ROS at the same rate as normal RPE cells following treatment with bFGF, by a mechanism requiring gene transcription and (2) that ROS ingestion by normal RPE cells, which is also under transcriptional control, is inhibited by a neutralizing antibody to bFGF. This is the first demonstration of reversal of the RCS defect by any agent, and of the involvement of bFGF in the normal mechanism of RPE phagocytosis.

## 2. Materials and methods

### 2.1. Primary RPE cultures

RPE cells were isolated from 7–11-day-old normal (RCS *rdy*<sup>+</sup>*p*<sup>+</sup> or RCS *rdy*<sup>+</sup>) and dystrophic (RCS *p*<sup>+</sup>) rats as described [29]. To minimize cell spreading and dedifferentiation in vitro [30], RPE cell sheets were incompletely dispersed following isolation, and the resultant mixture of single cells and epithelial patches was plated at high density in a very small volume ( $3 \times 10^4$  cells in 50  $\mu$ l of medium per well;  $1.5 \times 10^5$ /cm<sup>2</sup>) in 8-well glass chamber slides as previously described [28]. The primary cultures were grown to confluence for 6–9 days in minimum essential medium with Earle's salts, MEM (Gibco)+20% FBS+antibiotics (500  $\mu$ l/well), at which time the pigmented cultures were visible as dark spots of uniform diameter ( $\approx 5$  mm) centered in the wells of the chamber slides.

### 2.2. Pretreatment with growth factors and inhibitors

Preliminary experiments, in which bFGF at various concentrations

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was added at several intervals prior to ROS addition, revealed that 24 h pretreatment of dystrophic RPE cells with 100–200 ng/ml of this growth factor reproducibly resulted in a pronounced acceleration of ROS ingestion by the cells, whereas addition of bFGF at the time of ROS feeding had much less effect. For all experiments reported here, cultures were pretreated with all growth factors for 24 h prior to commencement of the phagocytosis assays. For dose–response experiments replicate RCS p<sup>+</sup> cultures were pretreated with bFGF at concentrations of 0, 0.001, 0.01, 0.1, 1.0, 10.0 and 100.0 ng/ml, and fixed at either 11 or 19 h after addition of ROS. In other experiments, bFGF concentration was 100 ng/ml unless otherwise indicated. The selected concentrations of EGF and NGF (100 ng/ml) were based on previous demonstrations at this dosage of mitogenic or functional responses by cultured RPE cells to mouse or recombinant human EGF [31–33] and by rat pheochromocytoma and chromaffin cells to mouse NGF [34]. Replicate cultures (8–20/experiment) were pretreated in low-serum control medium alone (MEM+5% FBS) or control medium+bFGF, aFGF, EGF or NGF. Bovine pituitary bFGF was obtained from three sources: Collaborative Research (Bedford, MA), Sigma (St. Louis, MO) and R&D Systems (Minneapolis, MN). Bovine brain aFGF and recombinant human EGF were from Collaborative Research; NGF from mouse submaxillary gland was from Upstate Biotechnology (Lake Placid, NY). In some experiments, cells were pretreated for 24 h, with or without 10 ng/ml bovine bFGF, in media containing a goat anti-bFGF antibody (100 µg/ml) that neutralizes the biological activity of human and bovine bFGF (both reagents from R&D Systems). In experiments to investigate transcriptional control of phagocytosis, actinomycin D (Act D; Sigma, 1 µg/ml) was included in the culture medium 3 h prior to ROS addition. At the end of the 19 h phagocytosis assay, viability of the Act D-treated cells was assessed by Trypan blue exclusion, and by ability of the cultures to thrive for several days after removal of Act D.

### 2.3. Phagocytosis assays

Except as noted, all steps of our double fluorescent vital assay of ROS phagocytosis were performed as described in detail elsewhere [35]. The assay takes advantage of phagosome–lysosome fusion to enable ROS bound to the cell surface to be distinguished from internalized ROS. Briefly, 24–48 h prior to ROS addition, primary lysosomes in the RPE cells are vitally stained with sulforhodamine (SR), a red fluorescent lysosomotropic dye. The phagocytic particles, i.e. rat ROS are isolated from retinas of adult Long Evans rats by homogenization followed by sucrose density gradient centrifugation, and are then fluorescently stained with fluorescein isothiocyanate (FITC). The FITC-ROS are fed to the SR-stained RPE cultures. Following ingestion, phagosome–lysosome fusion rapidly occurs and SR is transferred to the resultant phagolysosomes. Thus by fluorescence microscopy, the internalized ROS in phagolysosomes appear double-labeled with FITC and SR, whereas ROS remaining bound to the cell surfaces are labeled only with FITC [35]. In all experiments described here, 24 h before addition of ROS, primary RPE cultures were switched to low-serum control medium (MEM+5% FBS) containing 40 µg/ml SR (Molecular Probes, Eugene, OR) along with added bFGF or other growth factors in some wells. Growth factors were added as necessary to all subsequent changes of media until the time of fixation. On the day of the assay, isolated rat ROS were freshly prepared and FITC stained as described [35]. Meanwhile, SR-containing media were replaced with several changes of fresh SR-free media, MEM+5% FBS (450 µl/well). Cells were allowed to condition the final change of medium for 3 h before FITC-ROS, suspended in MEM+5% FBS, were added to each well (10<sup>8</sup> ROS/ml; 50 µl/well). Cultures were incubated with FITC-ROS for durations of 3–19 h; at 2 h intervals, ROS-containing media were rapidly removed by aspiration, cultures were washed several times with fresh media, then fixed in buffered 2% paraformaldehyde for quantitation of bound and ingested ROS. Effects of pretreatment with growth factors, anti-bFGF antibody and actinomycin D were compared with control (MEM+5% FBS) in 16 separate experiments (9 dystrophic, 7 normal), using replicate cultures fixed at 2–5 different time points after ROS addition. Using a Zeiss Photomicroscope III equipped as previously described [28], culture morphology was assessed by phase contrast microscopy and quantitation of bound and ingested ROS was performed by fluorescence microscopy using a high resolution 25× water immersion objective. Counts were made over central regions of confluent cultures, in fields with comparable morphology, comprised of patches of heavily pig-

mented, unspread cells close in size and appearance to RPE *in vivo* (previously defined as 'subtype 1' RPE [28]), or of a mixture of RPE subtype 1 and immediately adjacent unspread daughter cells (RPE subtype 2), arranged in a compact monolayer with cobblestone morphology. Bound and ingested ROS were counted at 40× using a 0.5×0.5 cm ocular grid over 10–20 such fields/culture (field size: 0.021 mm<sup>2</sup>; RPE density: 25–30 cells/field). For each treatment, mean counts per field were compared with those of the untreated replicate culture. Statistical significance of differences was assessed among treatments by analysis of variance, and by Student's *t*-test for paired data.

Representative untreated and bFGF-treated cultures, showing mainly bound or ingested ROS respectively, as assessed by the double fluorescent technique, were also examined by fluorescence microscopy using a Noran scanning laser confocal microscope. Sixty serial optical sections, 0.3 µm apart, were obtained through typical fields of cells and the images were then processed using the Noran InterVision 3-D analysis software program, to produce vertical ('Z') sections through the thickness of the cultures.

### 3. Results

We have recently demonstrated in primary RPE cultures from normal rats that whereas highly spread (subtype 3) RPE cells rapidly bind and continually ingest ROS after short (30 min to 3 h) incubation times in MEM+5% FBS, RPE subtypes 1 and 2, with unspread cuboidal phenotype, bind very few ROS during 3–9 h incubations, then exhibit avid binding and synchronous ingestion of multiple ROS per cell between 9 and 11 h [28]. As also described previously, in this study RPE subtype 1 and 2 cells from dystrophic rats demonstrated steadily increasing total ROS counts during the first 11 h of incubation with ROS, with most of this increase representing ongoing ROS binding with minimal ingestion (Fig. 1A). Between 11 and 19 h, the total ROS counts/field (bound+ingested) declined slightly and during this interval, the numbers of ingested ROS/field rose steadily, accounting for 15.2%, 34.9% and 51.4% of total ROS respectively, after 11, 15 and 19 h incubations (Fig. 1A). Replicate cultures from these experiments, pretreated for 24 h with bFGF and fixed at the same times after ROS addition, revealed an apparent reduction in ROS binding and a profound increase in ROS ingestion, starting at 5 h (Fig. 1B). At the 7 h timepoint, mean numbers of ingested ROS/field were more than 13-fold higher than control, and the percentage of ingested ROS was increased to 53.6, compared with only 3.0 in the control well. At 11 h, numbers of ingested ROS in the bFGF-treated cultures were 4-fold higher than control, and 91.8% of the ROS were ingested, vs. 15.2% in control. By 15 h, ingestion was approximately double that of control; thus at this time the cells in the control cultures appeared to be catching up. At 19 h, counts of ingested ROS in control cultures continued to increase; however, at that time, ingestion in the bFGF-treated cells was 3.6× that of control (Fig. 1A,B). Thus at all times after 3 h, ROS ingestion in the bFGF-pretreated wells was clearly ahead of replicate untreated controls (*P* < 0.005). Fig. 1C–E shows fluorescence micrographs from a phagocytosis assay, showing typical results obtained with replicate untreated and bFGF-pretreated mutant RPE cultures (see legend for details). The bFGF-mediated ingestion of the bound ROS was also confirmed by an independent method, i.e. scanning laser confocal microscopy (see below).

Dose–response assays were performed in replicate cultures pretreated with MEM+5% FBS alone, or with the addition of bFGF (0.1–100 ng/ml), and the results revealed a clearcut dose–response effect (Fig. 2). In the untreated controls, counts

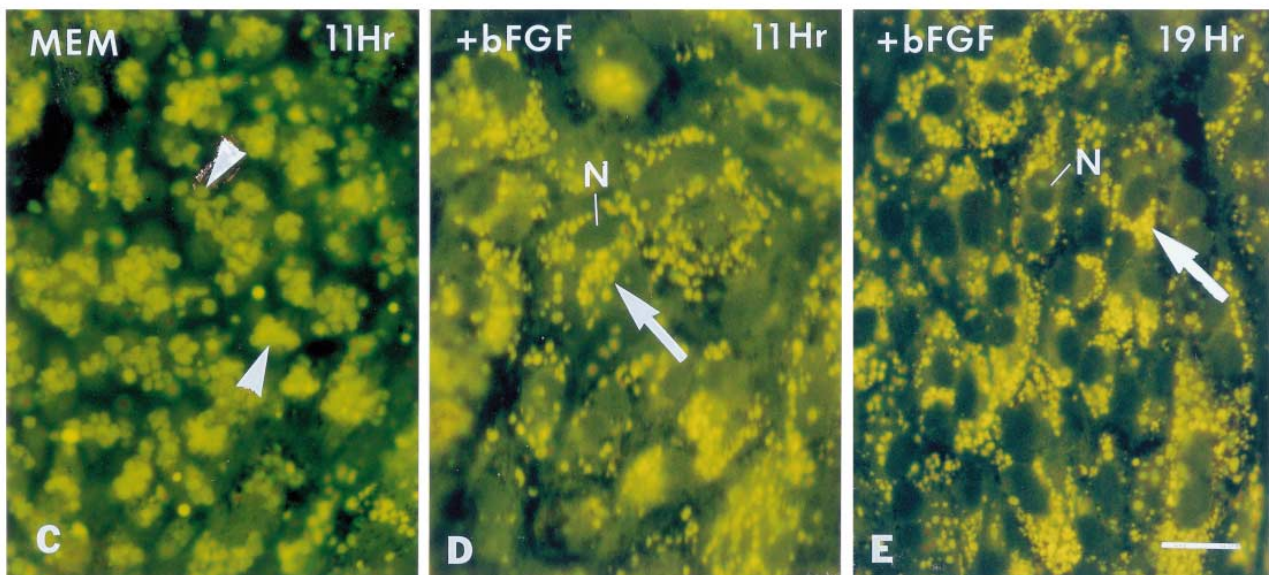
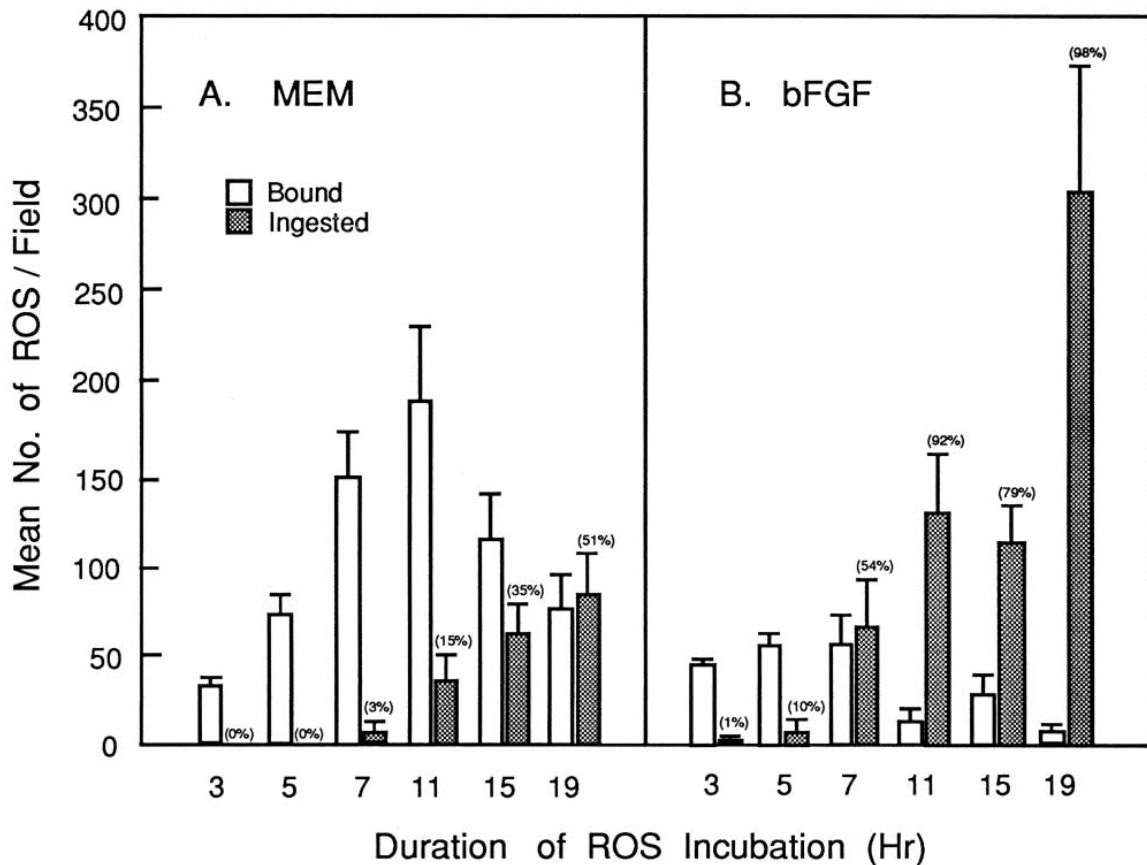
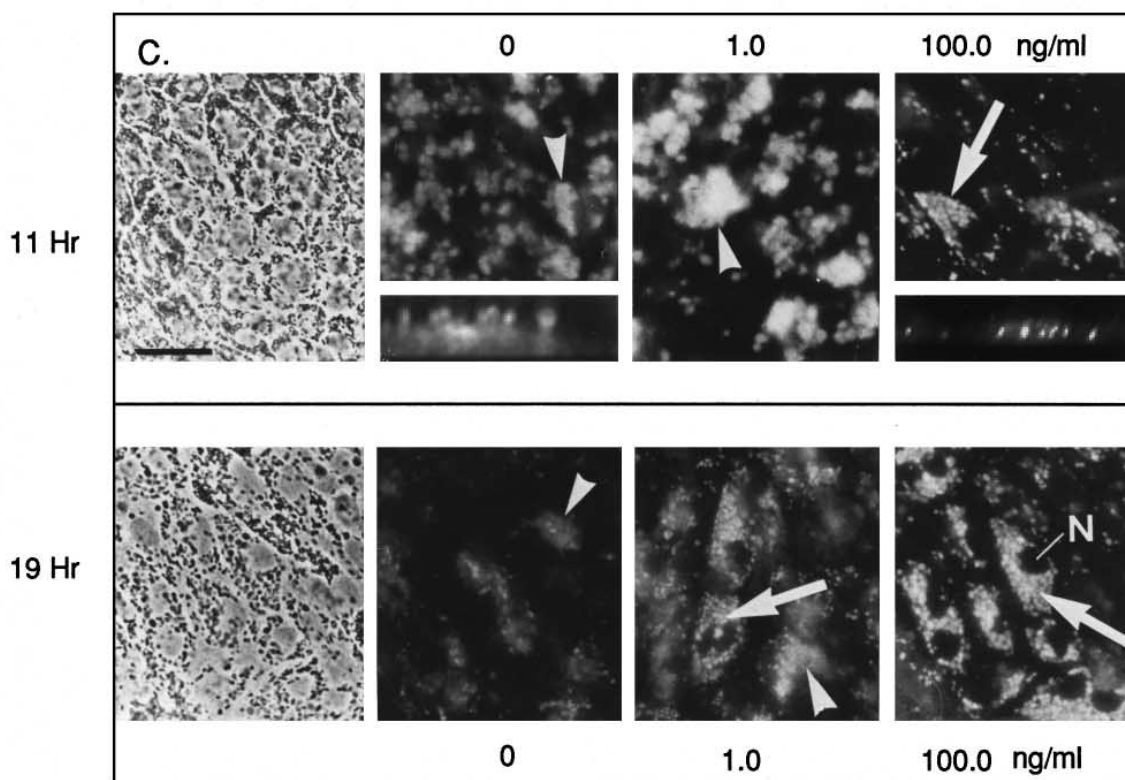
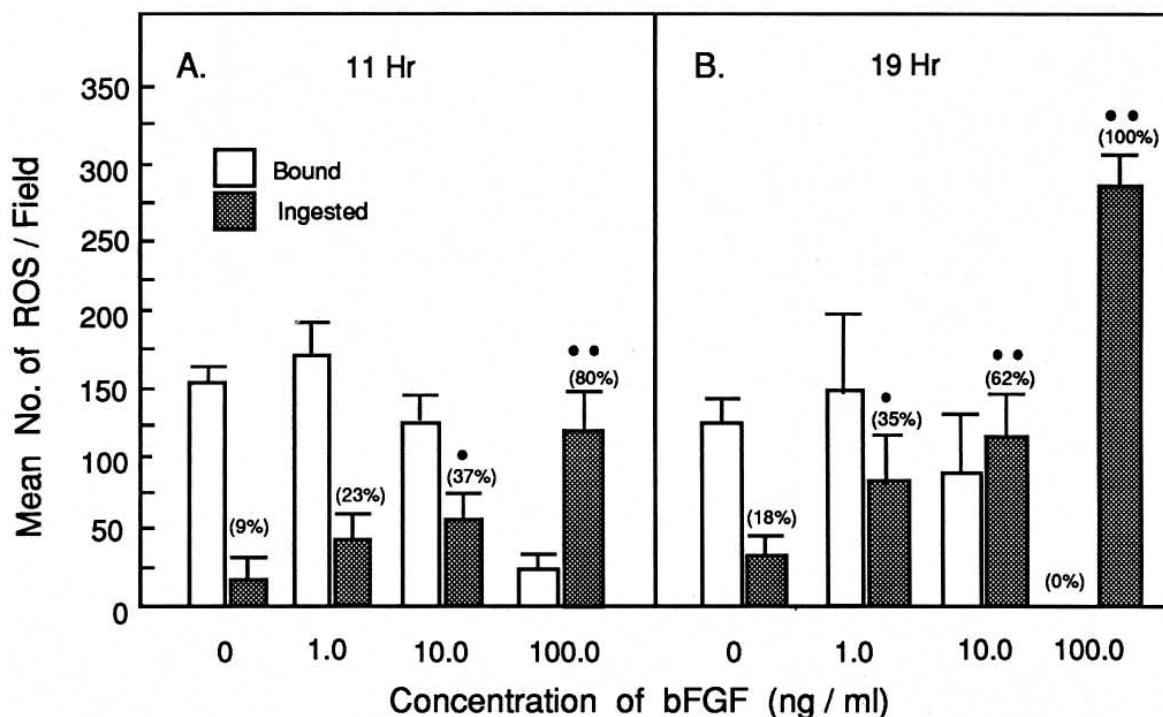


Fig. 1. Enhanced phagocytosis of isolated rat ROS by bFGF-pretreated RPE cells from mutant RCS rats. A,B: Replicate cultures were pre-treated 24 h prior to ROS addition either with low-serum medium (MEM+5% FBS) alone as control (A) or with added bFGF, 100 ng/ml (B). All cultures were fed with FITC-stained ROS at  $t=0$ , then pairs of replicate control and bFGF-pretreated cultures were washed and fixed at the indicated times thereafter. Bound and ingested ROS were counted by fluorescence microscopy over patches of RPE cells with phenotype closely resembling RPE in situ. Ingestion of ROS in the bFGF pretreated cells is significantly enhanced over untreated controls starting at 5 h after ROS addition ( $P<0.005$ ). Data are combined from two separate experiments; counts are means from 10–20 fields per timepoint; error bars represent  $\pm 1$  SD. Percentages in parentheses refer to percent of total ROS (bound+ingested) that are ingested. C–E: Typical fluorescence microscopic data showing bFGF-mediated rescue of phagocytosis in dystrophic RPE cells. C: Untreated (MEM) mutant culture, fixed 11 h after feeding FITC-ROS and viewed with a non-selective FITC filter set as described [35]. D,E: Replicate cultures from this experiment, pre-treated with bFGF and fixed 11 and 19 h, respectively, after FITC-ROS addition, viewed as in (C). In the fixed cultures, FITC-ROS remaining bound to the cell surface appear yellow–green (arrowheads in C), whereas ingested ROS, double stained with FITC+SR, appear as bright yellow spots (arrows in D,E), primarily in focus at the level of the cell nuclei (N). Bar = 37  $\mu$ m.

of total ROS/field (i.e. sum of bound+ingested) were very similar in wells fixed at either 11 or 19 h after ROS addition, and during this period, numbers of ingested ROS approximately doubled (compare Fig. 2A,B; 0 ng/ml). No effect on phagocytosis was observed in cultures pretreated with low concentrations of bFGF (0.001, 0.01 and 0.1 ng/ml; data not shown), but with 1.0 ng/ml, total ROS/field were increased

about 40% above control levels both at 11 and 19 h, and counts of ingested ROS were increased approximately 250% above control at both times (Fig. 2A,B; 1.0 ng/ml). Thus bFGF at a concentration of 1 ng/ml seemed to both enhance ROS binding and accelerate ingestion of the bound ROS, since ingestion must be preceded by binding. In the cultures pretreated with 10 ng/ml bFGF, total ROS/field were slightly



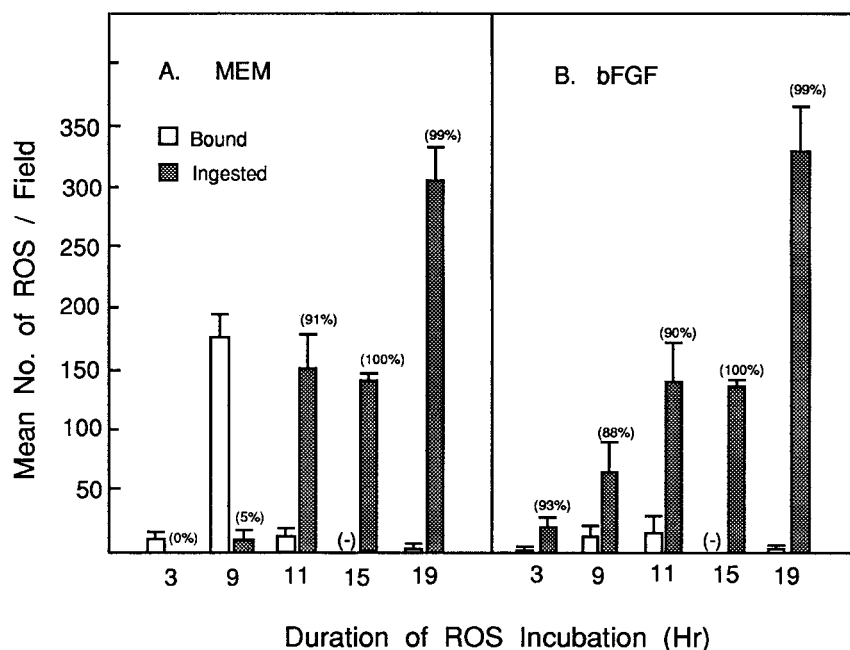


Fig. 3. Biphasic effect of bFGF pretreatment on ROS phagocytosis by cultured RPE from normal (RCS congenic control) rats. A,B: Normal RPE cultures were pretreated for 24 h with control medium alone or with added bFGF (100 ng/ml), and assayed for phagocytic ability at various intervals after ROS challenge, as described for the mutant RCS RPE cultures. A: Normal subtype 1 and 2 RPE cells, pretreated only with control medium, are slow to bind ROS during the first 9 h of ROS incubation, binding ROS in clusters on the cell surface, then synchronously ingesting multiple bound ROS within the next 2 h. A second ingestion peak is observed at 19 h. B: bFGF-pretreated normal cultures show apparent reduced binding but accelerated ingestion during the first 9 h of incubation, with no subsequent effect on total numbers of ingested ROS after 11 or more hours.

higher than control levels at both 11 and 19 h, and the percent ingested ROS increased from 37.3% at 11 h to 61.9% at 19 h (compare Fig. 2A,B; 10 ng/ml). At the highest bFGF concentration tested, over 80% of the ROS were ingested at 11 h, compared with 9% for untreated replicate cultures (Fig. 2A) and at 19 h, total ROS per field were double that of control, and 100% of the ROS, vs. 18% in the control, were ingested (Fig. 2B). The results with 100 ng/ml bFGF, showing 80–100% ingestion at both times thus suggested that these cells had undergone a second round of binding after the 11 h time-point, followed by a second ingestion peak observed at 19 h.

The distribution of bound and ingested ROS in cultures from the dose-response assays is shown in Fig. 2C. In the untreated cultures, the surface-bound ROS became increasingly clumped and patchy between 11 and 19 h, but most of the clustered ROS remained uningested (Fig. 2C, 0 ng/ml; compare at 11 and 19 h). In the cultures treated with 1 ng/ml bFGF, enhanced binding and clumping of the bound ROS,

relative to the untreated control, was observed at 11 h (Fig. 2C, upper panel; compare bound ROS, indicated by arrowheads, at 0 and 1.0 ng/ml). After 19 h, significantly increased ingestion was evident in these cultures (Fig. 2C, lower panel; compare 0 and 1.0 ng/ml, ingested ROS indicated by arrow). In cultures pretreated with 100 ng/ml bFGF, avid ingestion was evident in many cells at 11 h, and a strikingly high level of ingestion was observed after 19 h (Fig. 2C, 100 ng/ml, 11 and 19 h). Thus from the observed patterns of ROS distribution in these cultures, it appeared that the normal sequence of events leading to ingestion, i.e. ROS binding, followed by ROS clustering and finally by ingestion, was progressively accelerated in mutant cultures treated with increasing concentrations of bFGF. To provide independent evidence that ROS ingestion was indeed enhanced in the bFGF-treated wells, representative replicate cultures from this experiment were also analyzed by confocal microscopy to produce vertical ('Z') sections through typical fields, as described in Section

Fig. 2. Dose-dependent rescue of ROS phagocytosis by bFGF in mutant rat RPE cells. A,B: Quantitation of bound and ingested ROS in phagocytosis assays. Replicate cultures were pretreated for 24 h with MEM+5% FBS alone, or with the addition of one of several concentrations of bFGF, then fixed at 11 h (A) or 19 h (B) after continuous ROS feeding. With increasing concentrations of bFGF, mutant RPE cells acquire the ability to ingest bound ROS. Symbols denote significance of differences in ingestion levels between bFGF-pretreated and untreated controls (●)  $P < 0.05$ ; (●●)  $P < 0.005$ . C: Appearance of ROS-fed mutant cultures from a dose-response experiment. Upper and lower panels on the left are phase contrast images showing culture morphology after fixation. Panels on the right are fluorescence micrographs showing distribution of bound and ingested ROS in replicate cultures pretreated with the indicated concentrations of bFGF and fixed at either 11 h (upper panels) or 19 h (lower panels) after addition of isolated FITC-stained rat ROS. In black and white photographs of the double fluorescent phagocytosis assay, individual bound FITC-ROS, in focus at the level of the cell surfaces, appear as weakly fluorescent round masses, often grouped in clusters (arrowheads). Ingested ROS are distinguishable as smaller bright fluorescent spots within the cell bodies, in focus at the level of the cell nuclei, N (arrows). Insets in the upper panels show bound and ingested ROS in vertical sections, reconstructed by 3-D analysis of serial optical sections through the corresponding cultures, obtained by scanning laser confocal microscopy. In cultures pretreated with increasing concentrations of bFGF, the mutant cells compact and subsequently ingest the clusters of bound ROS, the great majority of which in untreated cultures remain bound to the cell surfaces due to the ingestion defect. The phase contrast image in the upper panel is of the field shown in the untreated control culture (0 ng/ml bFGF); lower panel phase image is of the field of highly phagocytic cells on the right, treated with 100 ng/ml bFGF. Bar = 42  $\mu$ m.



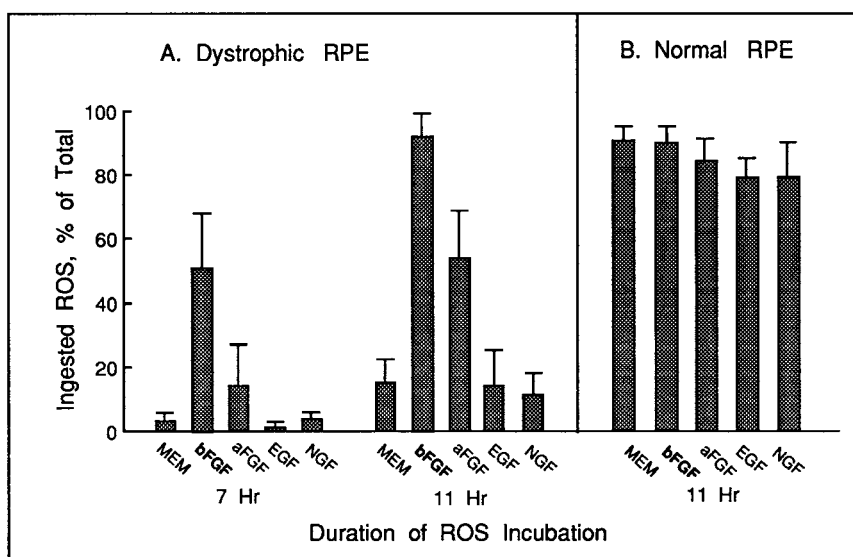


Fig. 4. Comparison of ROS ingestion rates in mutant and normal rat RPE cells pretreated with bFGF and other growth factors. A,B: Replicate RCS dystrophic (A) and normal congenic control (B) RPE cultures from experiments shown in Figs. 1 and 3 were pretreated for 24 h with control medium alone, or with added bFGF, aFGF, EGF or NGF (all at 100 ng/ml), and assayed for phagocytic ability at 2 h intervals from 3 to 19 h after ROS challenge. A: Representative results, shown here at the 7 and 11 h timepoints, show that bFGF, and to a lesser extent aFGF, but not other growth factors are effective in accelerating ROS ingestion in the mutant cells. B: None of the tested growth factors significantly alters total numbers of ROS ingested by normal cells, except as previously noted for bFGF in Fig. 3B.

2. This analysis confirmed that in untreated controls, the majority of the ROS were on the cell surfaces whereas following bFGF treatment they were internalized (Fig. 2C, insets, 0 and 100 ng/ml).

To examine the effect of bFGF pretreatment on normal RPE cells, similar experiments were conducted in which phagocytosis was assessed from 3–19 h after ROS addition in replicate cultures pretreated in MEM+5% FBS with or without 100 ng/ml bFGF. As previously described for subtype 1 and 2 normal RPE cells [28], few ROS were bound or ingested after brief (3 h) incubations, but after prolonged (7–11 h) incubations, multiple ROS were bound in large clusters on the surfaces of these cells, and then ingested en masse during the subsequent 2 h (Fig. 3A). With further incubation, i.e.

from 13–17 h, the total numbers of ROS per field declined somewhat, and at all times after 9 h, over 90% of the total ROS were ingested. At 19 h, a second peak of ingestion occurred (Fig. 3A). Comparison of bFGF-pretreated normal cultures with replicate untreated controls fixed 3 and 9 h after ROS addition revealed decreased numbers of bound ROS and increased numbers of ingested ROS at both times (compare Fig. 3A,B). At the 9 h timepoint, the decrease in bound ROS was more than 10-fold, and the increase in ingested ROS was  $6.5 \times$  higher than control ( $P < 0.005$ ). Thus in the normal cells, bFGF pretreatment seemed to reduce the time spent by ROS on the cell surfaces, and to hence accelerate the ROS ingestion rate during the 3–9 h interval. However, at the 11 h point, and at all times thereafter, numbers of bound

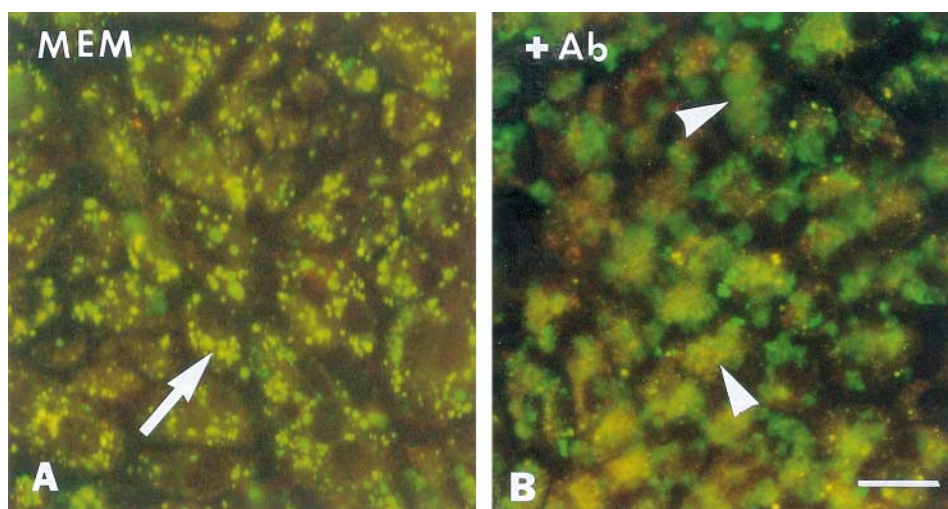


Fig. 5. Inhibition of ingestion in normal RPE cells by a neutralizing anti-bFGF antibody. Fluorescence micrographs of replicate normal RPE cultures fixed 11 h after ROS feeding. Image in (A) shows typical ingestion (arrow) in an untreated culture (MEM). In the replicate culture pretreated with an anti-bFGF neutralizing antibody (Ab) shown in (B), most ROS remain in clusters on the cell surfaces (arrowheads), and ingestion is markedly inhibited. Bar = 37  $\mu$ m.

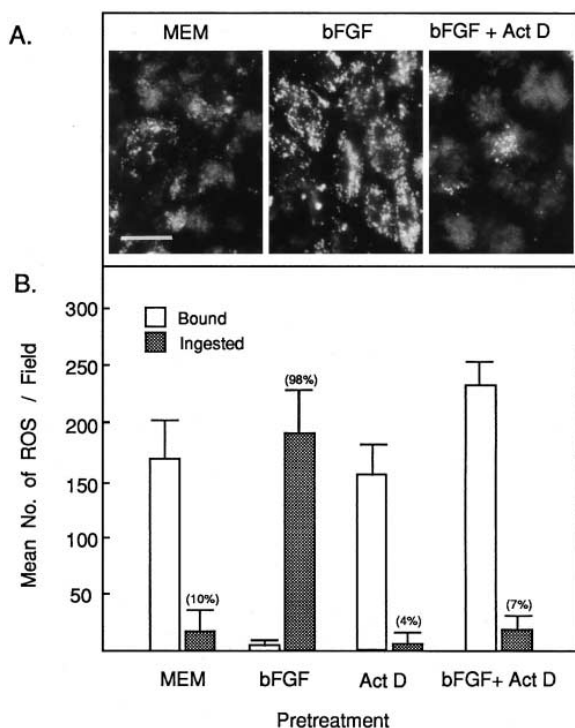


Fig. 6. Transcriptional control of bFGF-mediated rescue of ROS phagocytosis in mutant RPE cells. A,B: Replicate mutant RPE cultures were pretreated with MEM+5% FBS alone (control, MEM) or with addition of bFGF for 24 h prior to addition of ROS (bFGF). Three hours prior to ROS addition, actinomycin D (1  $\mu$ g/ml) was added to half of the cultures receiving each pretreatment (Act D, bFGF+Act D). All cultures were fixed after a 19 h ROS incubation. Avid ingestion of surface bound ROS is observed in the bFGF-pretreated cells (bFGF), whereas ROS remain bound in clumps on the cell surfaces in cultures receiving bFGF and Act D, as in untreated controls. Bar = 40  $\mu$ m.

and ingested ROS were essentially identical in untreated and bFGF-treated replicate cultures (Fig. 3A,B). Thus the initially accelerated ingestion rate did not appear to significantly alter the overall numbers of ROS that bound, nor the percent ingestion at the 11 or 19 h peaks. When the data from comparably treated normal and dystrophic cultures were compared, it was seen that ingestion rates of bFGF-pretreated normal or dystrophic cells were not significantly different except at the 3 h timepoint (compare Figs. 1 and 3). Thus it appeared that following bFGF pretreatment, dystrophic RPE cells acquired the ability to ingest bound ROS at the same rate as normal RPE cells with the same phenotype.

In replicate cultures from the above experiments pretreated with EGF, NGF or aFGF and fixed concurrently, patterns of ROS binding and ingestion were analyzed at all timepoints; quantitative data from several representative incubation times are shown in Fig. 4A. In the dystrophic cultures, at no time was ingestion increased over untreated control levels in cells pretreated with EGF or NGF. As shown above in Fig. 1, ingestion was consistently increased in the bFGF-pretreated wells starting at 5 h (typical data from 7 and 11 h timepoints shown in Fig. 4A). In cultures pretreated with aFGF, a slight ingestion increase was seen beginning at 7 h, and this effect was pronounced in cultures fixed from 11 h onward (Fig. 4A). Thus ROS ingestion was enhanced in dystrophic RPE cells by the related peptide growth factors bFGF and aFGF (FGF-2, FGF-1), but not by EGF and NGF. Replicate cultures from

the experiments described above using normal RPE cells were also assayed from 3–19 h after ROS addition, following pretreatment with bFGF, aFGF, EGF or NGF. Except as noted above for bFGF during 3–9 h incubations, no significant differences in ingestion rates were observed with any of these pretreatments (Fig. 4B).

The bFGF-mediated reversal of the ingestion defect in mutant RPE cells, previously shown to be deficient in bFGF [27], as well as the bFGF-mediated acceleration of ROS ingestion in the normal cells, suggested that endogenous bFGF might be involved in the mechanism of phagocytosis by RPE cells. An antibody known to neutralize the biological activity of bFGF (Ab) was used to directly test this hypothesis. Normal RPE cells were pretreated in medium alone (MEM) or MEM+Ab, then tested for their phagocytic ability. In cultures fixed 11 h after ROS feeding, normal levels of ingestion, i.e.  $160 \pm 20$  ingested ROS/field, were observed in the untreated controls, but strikingly, ingestion was reduced to less than 10% of control, i.e.  $15 \pm 3.2$ , in cells pretreated with the neutralizing Ab. Fluorescence micrographs from this experiment are shown in Fig. 5. Typical for untreated normal cells, very few ROS remained bound to the cell surfaces after 11 h, and the cell bodies were filled with ingested ROS (Fig. 5A). In sharp contrast, in the replicate cultures pretreated with the Ab, large clusters of ROS remained bound to the cell surfaces, apparently unable to be internalized (Fig. 5B). This result clearly indicated the need for endogenous bFGF of the normal cells to be functional, in order for ROS ingestion to take place. In parallel experiments conducted with dystrophic RPE cells, as expected, the bFGF-mediated enhancement of phagocytosis was blocked in cultures pretreated with both bFGF+Ab, but there was no effect on these ingestion-deficient cultures pretreated with Ab alone (data not shown).

Finally, experiments were performed to test whether the FGF-mediated phagocytic rescue effect was under transcriptional control. In mutant cultures pretreated with control medium alone (MEM+5% FBS), as expected, the great majority of the ROS remained bound in clumps on the mutant cell surfaces after a 19 h incubation with ROS (Fig. 6A, MEM), and the counts revealed only 10% ingestion at this time (Fig. 6B; MEM). In replicate wells pretreated with bFGF, ROS ingestion was rescued (Fig. 6A, bFGF) with counts of ingested ROS 10-fold greater than control, and ingestion levels at nearly 100% (Fig. 6B; bFGF). In MEM-only pretreated replicate wells to which Act D was added 3 h prior to ROS addition, ingestion was reduced very slightly, but ROS binding was not significantly affected by Act D addition (Fig. 6B; Act D). Thus ROS binding by the dystrophic cells apparently was not dependent upon new transcripts synthesized during the 3 h period prior to ROS addition or during the course of the assay. In contrast, in replicate cultures pretreated with both bFGF and Act D, ingestion was at very low levels (6.7% of total ROS), and ROS binding was increased 37% above control levels (Fig. 6A,B). Again, ROS binding was not reduced by Act D, but the bFGF-induced ability to ingest ROS was completely abolished, clearly indicating that the rescue of ROS ingestion was dependent upon gene transcription ongoing in the bFGF-pretreated, but not untreated mutant cells. Similarly, data from studies with MEM-only and bFGF-pretreated normal RPE cultures showed that ingestion of bound ROS, but not ROS binding itself, was inhibited by treatment with Act D 3 h prior to ROS addition (data not

shown). Viability of the cells was not affected by Act D treatment in either dystrophic or normal cultures.

#### 4. Discussion

The results of this study demonstrate that 24 h of pretreatment with bFGF enabled RCS dystrophic RPE cells with subtype 1 and 2 morphology [28] to ingest as many ROS as their normal counterparts (e.g. about 150 and 300 ROS/field respectively, after 11 and 19 h incubations). Calculated on the basis of 25 cells/field, this represents an average of 6 and 12 ingested ROS/cell, respectively, at these two times, although some cells were seen to ingest many more ROS than this average figure. Thus exogenous bFGF enabled the mutant cells to overcome the ROS ingestion defect, apparent in untreated replicate cultures, which ingested only 1.6 and 3.2 ROS/cell at the same fixation times, despite comparable levels of total ROS. The apparent equalizing effect of bFGF on phagocytosis by normal and dystrophic cells was not attributable however to reduced phagocytosis by the bFGF-pretreated normal cultures, since ingestion rates by normal subtype 1 and 2 RPE cells at the 11 and 19 h ingestion peaks were not significantly different between untreated and bFGF-pretreated replicate normal cultures (Figs. 1 and 3). To our knowledge, the data presented here provide the first demonstration of any agent that can reverse the phagocytic defect expressed *in vitro* in RCS rat RPE cells with type 1 and 2 phenotype, most closely resembling RPE *in situ*. Previously, a modest increase in ROS ingestion was reported to occur following carbachol treatment of cultured dystrophic RPE cells of unspecified morphology, after incubating the cells with ROS for 30 min [36]. However, the overall levels of phagocytosis were very low in those experiments, and the ameliorating effect could not be reproduced in another laboratory [37]. There has also been a recent report that bFGF pretreatment had no effect on phagocytosis by cultured RCS RPE cells (shown to have cuboidal phenotype resembling our subtype 1 and 2 cells), after a 1 h incubation with ROS in media containing 1% FBS [38]. But in that study as well, baseline levels of ROS binding and ingestion, relative to those demonstrated here, were quite low, i.e. 1000 ROS per well containing 30 000 cells, which averages only 0.03 bound ROS/cell. Under such conditions, the effect of bFGF reported here would not have been observed. Indeed, in cells with this phenotype, we have found very low levels of phagocytosis even after ROS incubations of 3–7 h, followed by avid binding and ingestion after 9–11 h [28]. This response is in marked contrast to that of highly spread (subtype 3 cells) in the same cultures, which bind and rapidly ingest ROS shortly after exposure to them. The important relationship between RPE phenotype *in vitro* and rates of ROS binding and ingestion has been addressed only recently in the RPE phagocytosis literature [28], so that in many previous studies, the RPE cell morphology was usually not shown or described, and ROS incubations were carried out using short intervals (30 min to 3 h) most suitable for study of spread RPE cells in lower density cultures.

The results showed that bFGF from three different sources, as well as aFGF, were able to ameliorate the ROS ingestion defect in the mutant cells whereas EGF and NGF, tested on replicate cultures, did not. Basic and acidic FGF are heparin-binding peptide growth factors, known to bind with different affinity to the same FGF receptors [22]. Expression of FGF

receptors on cultured normal and dystrophic rat RPE cells has been previously reported [39]. Cultured RPE cells are also known to respond to, and possess receptors for EGF [31–33] and NGF [40]. The data shown here are consistent with the rescue effect in the mutant cells having been mediated via FGF receptors on the cultured RPE cells. However the identity of the cell surface receptor(s) initiating ROS binding and ingestion in normal and bFGF-treated dystrophic RPE cells is still an essential, but missing piece of the puzzle.

From our various studies with bFGF, evidence is gathering in support of the hypothesis that this factor may play a central role in the mechanism of normal RPE phagocytosis and of the RCS phagocytic defect. First, we have recently demonstrated that *in vivo*, and in freshly isolated RPE sheets, bFGF mRNA and protein expression are markedly reduced in the RPE of neonatal RCS rats at the time when the phagocytic defect first appears *in vivo* [27]. Second, we show here that bFGF has a rescue effect on the phagocytosis-defective mutant RPE cells in culture. Third, the data from the bFGF-pretreated normal cells, showing accelerated ingestion rates after both 3 and 9 h incubations (Fig. 3), provided the first clue that bFGF might be involved in this process in normal RPE cells. This in turn led to more direct support for this hypothesis, i.e. the demonstration that ingestion in normal RPE cells is blocked with a neutralizing antibody to bFGF (Fig. 5). Basic FGF is also known to be expressed by ‘professional’ phagocytic cell types such as macrophages [41,42]; however, despite the large body of knowledge regarding specific receptors and intracellular pathways utilized during phagocytosis of various particles by these cells, the possible involvement of bFGF in their phagocytic mechanism has rarely been considered. Interestingly, there is recent evidence of bFGF up-regulation prior to induction of another specialized type of phagocytosis in the nervous system, i.e. ingestion of the fragmented myelin sheath by Schwann cells during Wallerian degeneration. Acquisition of phagocytic properties by this cell type is proposed to involve *c-fos* induced activation of bFGF and several other downstream genes [43].

The data from the experiments with Act D provide the first evidence that ingestion of bound ROS in both normal and bFGF-rescued dystrophic RPE cells requires ongoing gene transcription. It is possible that bFGF itself may be up-regulated in normal RPE cells following binding of ROS to their cell surface receptors, with subsequent bFGF-mediated induction of downstream transcripts required for ingestion. If so, then the application of exogenous bFGF to normal RPE cells might have had the effect of eliminating the delay required for bFGF induction, thereby enabling extracellular processing and ingestion to begin more rapidly after ROS binding. In the dystrophic cells, the ‘rescue transcripts’ clearly were induced by the bFGF treatment, since untreated replicate cultures were unable to ingest the bound ROS. At present, there is no information regarding FGF-mediated induction of any specific gene product in RPE cells. However, a number of genes known to be expressed in RPE have been shown to be induced by bFGF in other cell types; these include tyrosine hydroxylase [44–47], FGF receptor type 1 [39,45,47], integrin receptors [48–50], urokinase-type plasminogen activator receptor [51,52], matrix metalloproteinases [53–55] and metallothionein [52,56]. In pursuing the molecular mechanisms of normal RPE phagocytosis and of the bFGF-induced rescue effect, it will be of interest to test for possible induction of bFGF, as



well as the above transcripts and those of postulated ROS receptors [13–16], in normal and bFGF-treated dystrophic RPE cells. This task should be made easier with the availability of immortal RPE cell lines from congenic control and mutant RCS rats [57], since RPE cell lines are more suitable than primary cultures for studies of gene expression during phagocytosis [28].

It will also be important to determine if the bFGF-mediated rescue in culture is relevant to the RPE phagocytic defect in vivo. In a previous study in which bFGF was injected into RCS rat eyes on P23, neurotrophic effects on photoreceptors were observed 1–2 months later, without apparent improvement in RPE phagocytosis [26]. By P23 in this mutant, however, there is already substantial accumulation of membranous debris in the subretinal space [4] which might interfere with delivery of bFGF to the RPE. Based on the finding that bFGF mRNA and protein are down-regulated in the mutant RPE in vivo during the developmental period (P7–P12) when normal phagocytosis is established and the RCS defect begins [27], and on observations consistent with improved phagocytosis in vivo, following bFGF injections into eyes of RCS rat pups [58], further studies are in progress to pursue the mechanism of the bFGF-mediated rescue of phagocytosis in vitro and in vivo, in RPE cells from neonatal RCS rats.

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