**INTRODUCTION**

In recent years, it has become clear that many polypeptide growth factors, identified by their ability to promote mitosis in specific cell types in vitro, play important roles in embryonic development. For example, members of the fibroblast growth factor (FGF) family and members of the transforming growth factor β (TGFβ) superfamily are capable of (Kimelman et al., 1992; Smith, 1989), and necessary for, mesoderm induction during gastrulation (Amaya et al., 1991; Zhou et al., 1993). It is likely that these same growth factors are involved in ... loss of one member of a gene family may be compensated for by overlapping expression of another, related gene (Schneider et al., 1994). An alternative way to explore the developmental potential of a given gene is to alter its pattern of expression during embryogenesis. We have chosen this approach to explore the activity of FGF-1 during ocular development.

The lens is an attractive developmental model because cellular proliferation and differentiation occur in this tissue throughout the vertebrate life cycle. The lens develops ... elongate into primary fiber cells which fill the lumen of the vesicle. The anterior cells of the lens remain as epithelial cells, which exhibit cuboidal morphology and retain the ability to undergo mitosis. The continued growth of the lens ... can be distinguished from lens epithelial cells in that they are elongated, amitotic and express differentiation-specific proteins such as β-crystallin (McAvoy, 1978). Terminally dif-

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**SUMMARY**

The vertebrate ocular lens undergoes a spatially defined pattern of differentiation which may be regulated by the ocular distribution of proteins from the fibroblast growth factor (FGF) family. The ability of altered FGF-1 (acidic FGF) distribution to disrupt the normal pattern of lens differentiation was evaluated by the production of transgenic mice which express FGF-1 under the control of the lens-specific αA-crystallin promoter. Since FGF-1 lacks a classical signal peptide consensus sequence, transgenic mice were also produced with a chimeric construct containing the signal peptide sequence of the FGF-4 gene fused in frame to the coding sequences of the FGF-1 cDNA in order to obtain extracellular expression of the transgene. The presence of transgenic mRNA and protein was confirmed by in situ hybridization, Western analysis and immunohistochemistry. The ocular histology of newborn and young adult transgenic mice expressing FGF-1 without a signal peptide appeared normal. In contrast, mice expressing secreted FGF-1 exhibited lens abnormalities including the elongation of anterior epithelial cells. Epithelial cell elongation was accompanied by expression of the fiber cell differentiation marker, β-crystallin. These observations provide an in vivo demonstration that FGF-1 can induce anterior lens epithelial cells to express characteristics consistent with the onset of fiber cell differentiation. The transgenic induction of differentiation confirms that normal lens morphology reflects an asymmetric distribution of inductive factors within the eye.

Key words: fibroblast growth factor, lens, eye, differentiation, transgenic mice, FGF-1

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**EXTRACELLULAR FGF-1 acts as a lens differentiation factor in transgenic mice**

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ferentiated fiber cells also lose their nuclei and the majority of subcellular organelles. The molecular mechanisms that regulate the conversion of lens epithelial cells into lens fiber cells in the developing animal are unknown. The normal polarity of the lens is likely to be related to spatial environmental differences within the eye. The epithelial cells are only found in the anterior of the lens which is bathed by the aqueous humor, while the bow region, where epithelial cells differentiate into fiber cells, is found near the boundary between the aqueous and vitreous humor (Fig. 1). When embryonic lenses are surgically rotated such that the epithelial cells face the vitreous, the lens epithelial cells are found to elongate into structures resembling normal lens fiber cells (Coulombre and Coulombre, 1963; Yamamoto, 1976). Therefore differentiation of epithelial cells into fiber cells may be regulated by the presence of differentiation-inhibiting factors present in the aqueous fluid, differentiation inducing factors found in the vitreous or some combination of these mechanisms.

FGF molecules are likely candidates for lens differentiation signals in the vitreous. Acidic (FGF-1) and basic FGF (FGF-2) have each been shown to induce proliferation and differentiation of rat lens epithelial cells in culture at physiological concentrations (McAvoy and Chamberlain, 1989). FGF-1 immunoreactivity is continuously present in the rat lens from its earliest stages of development (de Jongh and McAvoy, 1993). Cell-associated immunoreactivity for FGF-1 in the rat lens has been demonstrated to be highest in the nuclei of differentiating lens epithelial cells near the lens equator (Lovicu and McAvoy, 1993). Furthermore, the FGF-1 concentration in bovine eyes has been measured to be 8.5 times higher in the vitreous humor, which is adjacent to the fully differentiated fiber cells of the lens, than in the aqueous humor, which bathes the undifferentiated epithelial cells of the lens (Caruelle et al., 1989). Lens cells have also been shown by in situ hybridization to express at least four specific FGF receptors, FGFR1, FGFR2 (Orr-Urtreger et al., 1991), FGFR3 (Peters et al., 1993) and FREK (Marcelle et al., 1994). Taken together, these data indicate that the localized concentration of FGF-1 or other members of the FGF family may regulate lens development in vivo.

FGF-1 and FGF-2, in contrast to the other members of the FGF family, have no signal peptide consensus sequence and their mechanism of extracellular release is unknown. Many cell types, including lens cells, express FGF-1 (Baudouin et al., 1990; de Jongh and McAvoy, 1993; Lovicu and McAvoy, 1993), but it is unknown if these cells secrete the growth factor or if they are able to respond to intracellular stores in an autocrine fashion. Using transgenic mice, we investigated the ability of alterations in the pattern of FGF-1 expression to alter lens and ocular morphogenesis. We further explored the importance of intracellular versus extracellular expression of FGF-1 in eliciting a biological response.

We demonstrate that extracellular FGF-1 is capable of altering normal lens polarity by inducing a differentiation response in the anterior lens epithelium. Our results support the hypothesis, originally proposed by McAvoy (see McAvoy et al., 1991 for review), that FGF molecules play a key role in determining lens polarity and growth patterns in vivo.

MATERIALS AND METHODS

DNA constructs

The alpha crystallin promoter vector used in these studies (CPV2) consists of a plasmid vector (Bluescript KS-, Stratagene) carrying a modified version of the murine αA-crystallin promoter (Overbeek et al., 1985), separated by a polylinker from the small t intron and polyadenylation sequences of the SV40 virus early region (Gorman et al., 1982). To facilitate the cloning of cDNA sequences into CPV2, duplicated restriction enzyme sites in the αA-crystallin promoter were removed by deletion of the 56 bp sequence between the PstI and the EcoRI sites that lie at −344 bp and −288 bp, respectively, upstream from the transcription initiation site. We have observed no differences in transgene expression pattern resulting from this alteration, consistent with the report that the sequences from −88 bp to +46 bp from the transcription initiation site are sufficient to provide lens specificity in transgenic mice (Wawrousek et al., 1990).

Sequences encoding truncated forms of human FGF-1 (amino acids 21-154) with and without a 22 amino acid signal peptide derived from FGF-4 were independently released from the eukaryotic expression vector, pMEXneo (Forough et al., 1993), by double digestion with SalI and EcoRI. The FGF-1 sequences were ligated into the polylinker of CPV2 to produce constructs with (hst/FGF-1α) and without (FGF-1α) a signal peptide (Fig. 2A).

Transgenic mice

DNA fragments for microinjection were isolated from vector sequences by NotI digestion and agarose gel purification using the Qiaex gel extraction kit (Qiagen). DNA was eluted in 10 mM Tris-HCl pH 7.4, 0.1 mM EDTA and microinjected into individual pronuclei of FVB/N embryos (Taketo et al., 1991) at a concentration of 2 ng/µl. Injected embryos were transferred into pseudopregnant ICR females. Potential transgenic mice were screened by isolating genomic DNA (Hogan et al., 1986) from tail biopsies and testing for transgenic sequences using the polymerase chain reaction (PCR) (Saiki et al., 1988).
PCR analyses
Primers used for PCR include: GTGAAAGAACCTTACTTCTGTTGGTG (termed S5), a sense primer that hybridizes to SV40 sequences 5′ to the intron splice site; GTCTCTGGGTCTTC-TACATTTCCTC (termed S3), an antisense primer that hybridizes to SV40 sequences 3′ to the intron splice site; CCCAGAGC-CTCTGTGCTGTACTACT (termed C5), a sense primer for the 5′ untranslated region of the murine β-crystallin transcript (present in the transgenic constructs); and GCCCATATGATGCTCCGAGGACCCGCG (termed F3), an antisense primer which hybridizes to sequences in the human FGF-1 cDNA. The intron-spanning primer pair from the SV40 region (S5 and S3) amplifies a 300 bp band from transgenic genomic DNA and a 236 bp band from transgenic cDNA for both FGF-1αx and hst/FGF-1αx families. The primer pair correspond- ing to the fusion transgene (C5 and F3) amplifies a 428 bp band from FGF-1αx families, and a 494 bp band from hst/FGF-1αx families. (See Fig. 2A,B).

Total ocular RNA from newborn transgenic animals was isolated using RNA STAT-60 from Tel-Test 'B', Inc. (Chomczynski and Sacchi, 1987). The RNA was treated with DNase I (Pharmacia) and reverse transcribed into cDNA using random primers (Promega) and Moloney Murine Leukemia Virus reverse transcriptase (Gibco-BRL). The resultant cDNA was amplified by PCR (Lago-Deependalayan et al., 1993). PCR conditions were 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl2, 0.01% gelatin, 10% DMSO, 2.5 mM each dNTP, 800 nM each oligonucleotide primer, 25 units of Taq DNA polymerase (Perkin-Elmer) in a 50 μl reaction volume. The conditions included an initial melt at 94°C for 3 minutes followed by 25 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 60 seconds. A final two minute extension at 72°C was also performed. 15 μl of each PCR reaction was run on a 0.8% agarose gel and stained with ethidium bromide.

In situ hybridization
An EcoRI-XbaI fragment of CP2V containing the entire 847 bp SV40 intron and polyadenylation sequence was subcloned into Bluescript KS-(Stratagene) to generate a riboprobe vector. Sense and antisense transcripts were produced by in vitro transcription of 1 μg linearized riboprobe vector with 20 units of T3 (Stratagene) and 20 units of T7 (Pharmacia) RNA polymerase respectively for 3 hours at 30°C using 35S-labeled UTP (Sundin et al., 1990). Hybridizations were performed on embryos collected from timed pregnancies (morning of copulation plug = day 0.5). The embryos were fixsed in 4% paraformaldehyde, dehydrated and embedded in PolyFin embedding wax (Tribe Biomedical Sciences). Tissue sections (5 μm) were collected on poly-L-lysine (Sigma)-coated slides, deparaffinized in xylene and rehydrated in a decreasing ethanol series. Sections were treated with 0.2 N HCl for 15 minutes, rinsed in phosphate-buffered saline (PBS) and incubated in 20 μg/ml proteinase K (Boehringer) in 50 mM Tris pH 7.5; 5 mM EDTA for 7 minutes at 25°C. Slides were rinsed in 0.4% glycine/PBS and acetylated with 0.25% acetic anhydride made in 0.2 M triethanolamine-HCl pH 8.0. Hybridizations were carried out overnight at 50°C in 0.3 M NaCl/10 mM Tris-HCl pH 7.4/10 mM NaH2PO4/5 mM EDTA/0.2% Ficoll 400/0.2% polyvinyl pyrrolidone/50 mM dithiothreitol/0.5 mg/ml polyadenylic acid/50 mg/ml yeast tRNA/10% dextran sulfate/50% formamide/0.25 mM alpha-S-thio ATP. Sigma). Approximately 20 ng of 35S-labeled sense or antisense riboprobe/slide was added to the hybridization mixture. Slides were washed in: F5M (50% formamide/0.3 M NaCl/50 mM citric acid pH 8.0/20 mM β-mercaptoethanol) at 65°C twice for 30 minutes; STE (0.6 M NaCl/60 mM citric acid pH 8.0/20 mM Tris-HCl pH 7.4/1 mM EDTA) at 37°C twice for 10 minutes; STE supplemented with 6 μg/ml RNase A at 37°C for 30 minutes; STE supplemented with 20 mM β-mercaptoethanol at 37°C for 10 minutes; F5M at 65°C twice for 45 minutes; 0.3 M NaCl/50 mM citric acid pH 8.0 at 37°C for 10 minutes and 15 mM NaCl/1.5 mM citric acid pH 8.0 at 25°C for 5 minutes.

Hybridized slides were air dried, dipped in Kodak NTB-2 emulsion and exposed for 4 days at 4°C before developing with Kodak D-19 developer. Slides were counter-stained with Harris hematoxylin.

Western blotting
Heparin-Sepharose, affinity-extracted murine ocular proteins (Forough et al., 1993) were fractionated by SDS-polyacrylamide (15%) gel electrophoresis (SDS-PAGE) and evaluated by western analysis (Forough et al., 1991) using an affinity-purified antibody specific for FGF-1 (Sano et al., 1990). Bound antibodies were visualized using the Lumiglo kit (Kirkegaard & Perry), which employs the use of secondary antibodies coupled to horseradish peroxidase and a chemiluminescent substrate. Luminol, according to manufacturer's suggestions. E. coli produced FGF-1α (amino acids 21-154) and FGF-1β (amino acids 1-154), (Forough et al., 1991), were used as molecular weight standards.

Immunohistochemistry
Eyes were surgically removed, fixed in 10% phosphate-buffered formaldehyde (pH 7.0) and embedded in PolyFin wax (Triangle Biomedical Sciences). Thin sections (5-6 μm) were cut and applied to poly-L-lysine-coated glass slides. Slides were deparaffinized with xylene and rehydrated using a decreasing ethanol series. For histochemical analysis, sections were stained with hematoxylin and eosin. For immunohistochemical evaluation slides were exhausted of endogenous peroxidase activity by a 30 minute preincubation in 10% methanol with 3% H2O2 in phosphate-buffered saline (PBS). Sections were incubated in 1% 5,5′-dioneamine sodium phosphate buffer pH 7.3 for 1 hour to reduce nonspecific staining. Sections were then incubated 30-120 minutes with the appropriate primary antibody diluted in 1-5% preimmune serum. The remaining secondary incubations and staining procedures were carried out using universal horseradish peroxidase kits according to manufacturer’s specifications (DAKO or Vector Laboratories). The substrate, diaminobenzidine tetrahydrochloride (DAB), which yields a brown reaction product, was used for visualization of antigen-antibody complexes. In all cases, negative controls included incubations without the primary antibody. Sections were lightly counter-stained with hematoxylin. The affinity-purified antibody to FGF-1 is described in Sano et al. (1990). Rabbit polyclonal antibodies to murine β-crystallin were provided by Dr James F. Hejtmancik, (National Eye Institute, Bethesda, MD).

Production and analysis of aggregation chimeras
FVB/N (albino) female mice 6-12 weeks of age were superovulated by injection of 5 IU of pregnant mare serum gonadotropin (Calbiochem) followed 46 hours later by the injection of 5 IU of human chorionic gonadotropin (HCG, Sigma). After HCG injections, superovulated females were placed individually overnight with either homozygous hst/FGF-1αx males from family OVE 371 (albino) or with homozygous (pigmented) Rasaβ-geo26 (Friedrich and Soriano, 1991) males. Females were examined the next morning for the presence of a copulation plug. Early morula stage embryos were collected from the females at 55-60 hours postcopulation, and acid tyrode was used to remove the zona pellucidae (Hogan et al., 1986). Embryos were aggregated in pairs and cultured in 20 μl of Brinster’s B10C-3 medium (Gibco-BRL) under paraffin oil in microwell plates. Aggregated embryos that reached the blastocyst stage were transferred to the uteri of pseudopregnant ICR females, which were mated one day later than the donor females. For controls, non-transgenic FVB embryos were aggregated with Rasaβ-geo26 embryos.

Neonatal mice were assessed by PCR (to detect hst/FGF-1αx sequences) and by visual inspection for eye pigmentation (to detect the presence of Rasaβ-geo26 derived cells). The relative contribution of the two strains was estimated based on the amount of eye pigmentation. Eyes were removed from chimeric, non-transgenic and inbred Rasaβ-geo26 mice and fixed for 30 minutes at 4°C in 2%
paraformaldehyde in 0.1 M phosphate buffer pH 7.3. Eyes were then rinsed twice in PBS for 5 minutes and placed in a staining media consisting of: 5 mM potassium ferricyanide (Sigma)/5 mM potassium ferrocyanide (Sigma)/0.1% deoxycholate (Sigma)/0.2% Nonidet P-40 (Sigma)/2mM MgCl₂ (Sigma) in PBS. Eyes were incubated in the staining media for 10 hours in the dark at room temperature. Eyes were then postfixed in 10% formalin for 2 hours before paraffin embedding and sectioning. Tissues and sections were cleared in Histo-clear (National Diagnostics, Atlanta) instead of xylene, and were counter-stained with nuclear fast red (Sigma).

RESULTS

In order to alter the normal pattern of FGF-1 distribution in the eye, transgenic mice were produced with constructs containing a human FGF-1 cDNA (amino acids 21-154) driven by the αA-crystallin promoter which has been shown to direct transgene expression to the ocular lens (Overbeek et al., 1985). One construct, FGF-1α (Fig. 2A), was expected to encode an FGF-1 peptide that would accumulate intracellularly. The truncation of the first 20 amino acids of FGF-1 has been shown to have no effect on the mitogenic activity of the growth factor in vitro (Burgess et al., 1985), and provides a convenient way to distinguish transgenic from endogenous, full length, FGF-1. To promote extracellular presentation of FGF-1, a chimeric construct, hst/FGF-1α, was produced by fusing the signal peptide sequence of FGF-4 (hst/KS) in-frame to the coding region of FGF-1α (Fig. 2A). The FGF-4 signal peptide fused to FGF-1 coding sequences leads to the secretion of recombinant FGF-1 in stably transfected NIH 3T3 cells (Forough et al., 1993). Not I fragments (Fig. 2A) were microinjected into FVB/N inbred mouse embryos and transgenic founders were identified by polymerase chain reaction (PCR) using amplimers specific to the fusion transgenes (C5 and F3) or to the SV40 fragment at the 3' end of both constructs (S5 and S3) (Fig. 2A). Two transgenic families (OVE 401 and 402) were established from the FGF-1α construct, and five transgenic families (OVE 369, 370, 371, 372 and 373) were established from the hst/FGF-1α construct. Two of these five families (OVE 372 and 373) were generated by a co-injection of the hst/FGF-1α transgene and a tyrosinase minigene, TyBS, which leads to a restoration of pigmentation in albino mice (Overbeek et al., 1991).

Expression of FGF-1α and hst/FGF-1α mRNAs was determined by RT-PCR analysis of total ocular RNA extracted from newborn mice (Fig. 2B). PCR amplification with primers S5/S3 demonstrated that the 64 bp SV40 intron was efficiently spliced out of the transgenic transcripts (compare the transgenic cDNA in lanes 2 and 4 to the transgenic genomic DNA in lane 3, Fig. 2B). PCR amplimers C5/F3, designed to amplify the 5' untranslated portion of the murine αA-crystallin transcripts (present in CPV2) fused to the human FGF-1 cDNA, were able to distinguish the size difference between the hst/FGF-α and FGF-1α transcripts, which corresponds to the 66 bp FGF-4 signal peptide sequence present in the chimeric hst/FGF-α construct (lanes 6 and 7, Fig. 2B). Non-transgenic ocular cDNA, was not amplified by either amplimer pair (lanes 5 and 9, Fig. 2B). The absence of genomic DNA contamination in isolated ocular RNA samples was confirmed by PCR on ocular RNA which had not been reverse transcribed into cDNA (lane 8 Fig. 2B).
exhibited approximately 10 ng of full length FGF-1 per eye (Fig. 3 lane 2), while FGF-1 immunoreactivity was not detected in non-transgenic lenses by our assay (Fig. 3 lane 5). In contrast, eyes from transgenic families contained readily detectable levels (200-400 ng/eye) of the recombinant FGF-1α or hst/FGF-1α proteins, the majority of which was localized to the lens (lanes 3, 4, 6 and 7, Fig. 3). Both FGF-1α and hst/FGF-1α exhibited similar apparent molecular weights suggesting accurate proteolytic cleavage and secretion of hst/FGF-1α. Previous in vitro experiments (Forough et al., 1993) have confirmed the biological activity of these eukaryotically expressed recombinant proteins.

In situ hybridization analysis confirmed that both the FGF-1α and the hst/FGF-1α transgenes were expressed at high levels exclusively in the developing lens (Fig. 4). The entire SV40 portion of the transgenic constructs was used to generate a riboprobe which would specifically detect transgenic transcripts (Fig. 2A). Negative controls included an antisense transgene-specific riboprobe hybridized to non-transgenic eyes and a sense strand riboprobe for FGF-1α and hst/FGF-1α eyes (Fig. 4E-H). No specific hybridization was seen in any of the negative controls. At embryonic day 13.5 (Fig. 4) and embryonic day 15.5 (data not shown), the highest level of transgene transcripts is found in the differentiating secondary fiber cells, an observation consistent with the midgestation expression of the endogenous murine αA-crystallin gene (Trèton et al., 1991). Also, the level of expression of the FGF-1α transgene appears, by in situ hybridization, to be lower than that of the hst/FGF-1α transgene. Although message levels for FGF-1α may be lower than hst/FGF-1α, both transgenes produce similar levels of ocular FGF-1 protein according to western analysis (Fig. 3). These results may reflect differences in the in vivo protein half-life between secreted and non-secreted transgenic FGF-1.

To evaluate developmental changes induced by expression of the FGF-1 transgenes, ocular histology of newborn transgenic animals was analyzed (Fig. 5). The histological structure of newborn FGF-1α transgenic mice did not differ significantly from non-transgenic animals (compare A and E to B and F in Fig. 5). The transgenic lenses in both FGF-1α families (OVE 401 and 402) were transparent and remained cataract-free for at least four months. The retinas in the FGF-1α mice appeared normal and retinal cells did not exhibit atypical distribution of FGF-1 (data not shown). In contrast, transgenic hst/FGF-1α eyes exhibited ocular abnormalities which fell into two general categories. The mildly affected hst/FGF-1α families (OVE 370, 372 and 373) exhibited moderate elongation and/or hyperplasia of lens epithelial cells accompanied by mild lens vacuolization (Fig. 5C,G). The vacuolization in these families is progressive, leading to large cataractous lenses in adulthood (data not shown). Two severely affected hst/FGF-1α families (OVE 369 and 371) exhibited dramatic elongation of the anterior lens epithelium suggesting the induction of fiber cell differentiation. The elongation of the lens epithelium was
accompanied by the loss of the anterior chamber, severe lens vacuolization (Fig. 5D,H) and cataracts. These animals are severely microphthalmic in adulthood with deteriorated lenses (data not shown). Both types of hst/FGF-1α animals demonstrate increased vasculature around the lens which persists into adulthood primarily in the area adjacent to the optic nerve (data not shown). Small and large cysts within the hst/FGF-1α lenses were occasionally filled with red blood cells. Whether these represent degenerating capillaries or hemorrhage into cysts has not been determined. Scattered polymorphonuclear leukocytes were also evident in both the degenerating lenses and the vitreal regions (space between the lens and retina). Lenses of hst/FGF-1α mice occasionally exhibited a persistent lens stalk attaching the lens to the cornea (data not shown). Other ocular abnormalities included unusual folding of the retina, abnormal retinal nerve bundles, and a thickening of the corneal epithelium in some families (Fig. 5D,H).

Immunohistochemical analyses using affinity-purified polyclonal FGF-1 antibodies were performed to specifically localize FGF-1 in transgenic and non-transgenic lenses (Fig. 6). Weak FGF-1 immunoreactivity was detected in the non-transgenic lens epithelium but was absent from the fiber cells. In the FGF-1α transgenic lenses, FGF-1 immunoreactivity was highest in the cortical fiber cells where it exhibited a cytosolic staining pattern (Fig. 6B). Immunoreactivity for FGF-1 was determined in ocular sections in the mildly affected hst/FGF-1α transgenic family OVE 372. Adult lenses in this family have recognizable epithelial cells, fiber cells and a bow region. FGF-1 immunoreactivity in this family was present over the entire lens (data not shown). The lens cells often exhibited exaggerated FGF-1 immunoreactivity on the plasma membrane, particularly in the differentiating cells of the bow region (Fig. 6C). In several cells within the lens perinuclear or nuclear FGF-1 immunoreactivity was detected (Fig. 6C).

The epithelial cells in the hst/FGF-1α lenses exhibit characteristics consistent with the onset of fiber cell differentiation. They often lose their normal cuboidal morphology and become elongated. In the most severely affected hst/FGF-1α transgenic family, OVE 371, elongation of the epithelial cells begins at embryonic day 15.5. Epithelial cell elongation characteristically begins in the most central portion of the lens epithelium (see Fig. 7). Additionally, the elongating lens epithelial cells express β-crystallin (Fig. 7), a protein characteristic of lens fiber cells (McAvoy, 1978). β-crystallin expression by elongating central lens epithelial cells occurs at embryonic day 15.5 in this family and, by embryonic day 17.5, the entire epithelium has elongated and expresses β-crystallin (Fig. 7E,F). The elongation of the lens epithelium results in a loss of certain normal epithelial cell functions such as proliferation. Therefore in the most severely affected hst/FGF-1α families, where the entire lens epithelium differentiates prenatally, the lens is unable to grow, resulting in microphakia and associated microphthalmia. As the lens epithelial cells begin to elongate in the hst/FGF-1α transgenic mice, the primary fiber cells begin to degenerate. The reason for this is not clear, but may relate to the importance of the lens epithelium in maintaining

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Fig. 5. Hematoxylin and eosin histology of newborn non-transgenic (A,E), FGF-1α transgenic (B,F), and hst/FGF-1α transgenic (C,D,G,H) eyes. The histology of the FGF-1α eye does not differ significantly from that of the non-transgenic eye. This is in contrast to that seen in the two different hst/FGF-1α families shown in C,G and D,H, respectively. The lens epithelial cells (arrows) in the newborn hst/FGF-1α eyes have lost the normal cuboidal morphology and have become columnar (G) in the less severely affected family (OVE 370) or have totally elongated and become vacuolated (H) in the more severely affected family (OVE 371). c, cornea. Scale bar, (A-D) 200 µm; (E-H) 50 µm.
the viability of the fiber cells. The fiber cell differentiation induced by hst/FGF-1α does not proceed to completion as evidenced by a failure of the differentiating epithelial cells to dedenature.

The ocular phenotypes of the FGF-1α and the hst/FGF-1α mice suggest that the secretion of FGF-1 due to the signal peptide sequence from FGF-4, induces an autocrine or paracrine response in lens epithelial cells. Aggregation chimeras were produced to determine if the biological effect of hst/FGF-1α is cell-intrinsic or extrinsic. Aggregation pairs consisted of one hst/FGF-1α (OVE 371) embryo and one Rosaβ-geo26 embryo. Cells derived from Rosaβ-geo26 mice express bacterial β-galactosidase and produce a blue, intracellular precipitate in the presence of the chromogenic substrate, X-gal (Friedrich and Soriano, 1991). A total of 15 hst/FGF-1α↔Rosaβ-geo26 and 4 FVB↔Rosaβ-geo26 chimeric mice were generated. The ocular histology of the chimeric eyes after staining with X-gal revealed that hst/FGF-1α expressing cells are able to induce a differentiation response in Rosaβ-geo26 lens epithelial cells in a paracrine manner (Fig. 8). This result strongly suggests that the hst/FGF-1α lens cells are indeed secreting transgenic FGF-1.

**DISCUSSION**

The transgenic mice that express hst/FGF-1α are unique in that the overexpression of a single growth factor induces lens cells to differentiate along an apparently normal, but spatially altered, developmental pathway in vivo. The FGF-1α transgenic animals did not exhibit significant alterations in normal lens development, implying that intracellular sequestration limits the inductive potential of FGF-1. In contrast, the hst/FGF-1α transgenic mice exhibit significant ocular malformations, beginning with the global induction of lens epithelial cell differentiation. Although similar amounts of transgenic FGF-1 protein were detected in the western blots from FGF-1α and hst/FGF-1α eyes, the phenotypic outcomes of these constructs were quite different. Our observations are consistent with results obtained with NIH 3T3 cells in which both hst/FGF-1α and f1α transgenes were expressed (Overbeek et al., 1991) which was co-injected with the hst/FGF-1α transgene to generate this transgenic family, OVE 372. Scale bar 30 µm.

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**Fig. 6.** FGF-1 expression in the bow region of non-transgenic (A), FGF-1α transgenic (B) and hst/FGF-1α transgenic (C) lenses probed with an affinity-purified polyclonal antibody to human FGF-1. Positive FGF-1 immunoreactivity is brown. FGF-1 immunoreactivity was detected at low levels in the epithelial cells of the non-transgenic lens (A). The FGF-1 immunoreactivity was cytoplasmic and concentrated in the cortical fiber cells of the FGF-1α transgenic lens (B). In the hst/FGF-1α transgenic eyes immunoreactivity to FGF-1 was most intense on the plasma membrane of cells undergoing differentiation in the bow region (C). Plasma membrane staining, sometimes accompanied by perinuclear or nuclear staining (thin arrow, panel C) was also evident in the lens interior. The star in section A indicates an artifactual folding of the lens capsule. Thick arrows point to the transition between the epithelial cells (above the arrow) and the differentiating fiber cells in the bow region (below the arrow). The pigmented cells in panel C are the result of the tyrosinase minigene (Overbeek et al., 1991) which was co-injected with the hst/FGF-1α transgene to generate this transgenic family, OVE 372. Scale bar 30 µm.
and Coulombre, 1963; Yamamoto 1976). We have confirmed that the ocular distribution of FGF molecules is important for the maintenance of normal lens polarity. Expression of extracellular FGF-1 as a transgene in the ocular lens alters the normal spatial pattern of ocular FGF-1 resulting in elongation and differentiation of central lens epithelial cells.

Previous transgenic investigations using the \(\alpha\)-crystallin promoter have demonstrated transgene expression as early as 12.5 days of gestation (Overbeek et al., 1985). Elongation of primary fiber cells begins at approximately 11 days of gestation. Therefore, it is likely that primary fiber cell differentiation is initiated before the expression of the transgene begins. This could explain why the primary fiber cells differentiate normally at first and are well elongated prior to the elongation and differentiation of the epithelial cells. When the transgenic epithelial cells exhibit changes, the central epithelial cells begin the process first (Fig. 7C,D), followed by the adjacent epithelium. The epithelial cells toward the equator seem to be the most resistant to FGF-1-induced differentiation. Perhaps the pattern of differentiation in the lens epithelial cells simply reflects a non-uniform distribution of transgenic FGF-1 protein in the eye. Alternative explanations are possible considering the special properties of lens epithelial cells immediately prior to differentiation. In the normal lens, equatorial epithelial cells would be undergoing their final mitosis before becoming secondary fiber cells. These cells may be resistant to FGF-1-induced differentiation because they have already been committed to replication. The lens equator is also near the developing ciliary system where embryonic plasma proteins may enter the aqueous chamber through permeable blood vessels in the anterior segment (Beebe et al., 1986). In the developing chicken eye, two separate activities have been identified both in embryonic serum and aqueous humor. One of these activities promotes proliferation and the other inhibits differentiation of lens cells (Hyatt and Beebe, 1993). It is conceivable that a similar set of activities exist in the mouse embryo and are present at high enough concentrations at the lens equator to override the differentiation signals from FGF-1.

In the hst/FGF-1\(\alpha\) transgenics, there are defects in denucleation and terminal differentiation of the anterior epithelial cells suggesting that intact lens epithelial cells may be necessary for the completion of normal fiber cell differentiation in vivo. Alternatively, FGF-1 may act only to initiate lens differentiation. More terminal features of fiber cell differentiation, such as denucleation, may require factors other than FGF.

Based on the experiments with chimeric mice, the hst/FGF-1\(\alpha\) lens epithelial cells are apparently provided with a paracrine system that promotes extracellular interactions of the growth factor with high-affinity, cell surface receptors, followed by activation of a biological cascade.

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**Fig. 7.** Expression of the lens fiber cell-specific protein, \(\beta\)-crystallin in the lens epithelial cells of OVE 371 hst/FGF-1\(\alpha\) transgenic eyes. Tissue sections of non-transgenic (A,B) and hst/FGF-1\(\alpha\) transgenic (C-F) eyes were stained with a polyclonal antibody to murine \(\beta\)-crystallin. The brown positive staining can be seen specifically in the fiber cells of the non-transgenic embryo (A,B). The \(\beta\)-crystallins are not expressed in the normal lens epithelium (e) or cornea (c). In the hst/FGF-1\(\alpha\) lens at embryonic day 15.5 (C,D), the fiber cells as well as the basal portion of the elongating epithelial cells contain detectable \(\beta\)-crystallin. By embryonic day 17.5, the lens epithelial cells of the hst/FGF-1\(\alpha\) fetus show extensive elongation and \(\beta\)-crystallin expression (E,F). In the hst/FGF-1\(\alpha\) eyes, differentiation of the lens epithelial cells is accompanied by the deterioration of the primary fiber cells (C,E).

Scale bar, (A,C,E) 200 \(\mu\)m; (B,D,F) 50 \(\mu\)m.
FGF-induced lens differentiation in vivo

signaling a specific developmental pathway. The fact that lens epithelial cells respond to FGF-1 by initiating the fiber cell differentiation pathway in vivo suggests that an FGF family member is likely to initiate endogenous lens differentiation. The FGF-1α transgenic mice presumably do not secrete FGF-1 and therefore are unable to stimulate cell surface FGF receptors. In order to respond to the extracellular FGF signal, the lens epithelial cells are predicted to express one or more of the FGF receptors. In situ hybridizations have previously shown that FGFR1, FGFR2, FGFR3 and FREG are expressed by lens cells (Orr-Urteger et al., 1991; Peters et al., 1993; Marcelle et al., 1994). Additional experiments will be needed to determine whether these FGF receptors play independent or overlapping roles in the induction of epithelial cell differentiation.

Development of specific tissues and organs is likely to involve a series of growth factor inductions that occur in a spatially, temporally and concentration-dependent manner. Our experiments provide strong evidence that this is the case for the lens. We have altered the spatial concentration of FGF-1 in the eye during embryonic development at a time when the entire lens epithelium is competent to respond by differentiating. Earlier inductive events, perhaps supplied by other growth factors, restrict the response of the lens epithelial cells to that of fiber cell differentiation. The elucidation of intracellular responses which are elicited by FGF receptor stimulation and which lead to lens differentiation is a topic for further investigation.

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Stripes of blue cells in the retina and lens confirm that this eye is chimeric (A). High magnification of the anterior lens (B) reveals that the entire lens epithelium has elongated, including the blue lens epithelial cells (arrow) that carry the Rosaβ-geo26 transgene. A


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