**HIF-1: An Age-Dependent Regulator of Lens Cell Proliferation**

**Ying-Bo Shui**, **Jeffrey M. Arbeit**, **Randall S. Johnson**, and **David C. Beebe**

1. **Department of Ophthalmology and Visual Sciences, Washington University, St. Louis, Missouri**
2. **Department of Surgery, Washington University, St. Louis, Missouri**
3. **Division of Biological Sciences, University of California San Diego, La Jolla, California**

**Abstract**

**Purpose**—The lens grows throughout life, and lens size is a major risk factor for nuclear and cortical cataracts. A previous study showed that the hypoxic environment around the lens suppressed lens growth in older rats. The present study was conducted to investigate the mechanism responsible for the age-dependent decline in lens cell proliferation.

**Methods**—Transgenic mice expressing Cre recombinase in the lens were bred to mice containing floxed *Hif1a* alleles. Transgenic mice expressing oxygen insensitive forms of HIF-1α in lens epithelial cells were exposed to room air or 60% oxygen. Proliferation was measured by BrdU labeling and cell death by using the TUNEL assay. Morphology was assessed in histologic sections. HIF-1α and p27KIP1 levels were determined by Western blot. The expression of HIF-regulated genes was assessed on microarrays.

**Results**—Lenses lacking *Hif1a* degenerated, precluding study in older animals. Breathing 60% oxygen reduced HIF-1α levels and HIF-1-regulated transcripts in lens epithelial cells from young and older lenses. Overexpression of oxygen-insensitive HIF-1α had no effect on lens size, but suppressed increased proliferation in response to oxygen. Systemic injection of the iron chelator, 1,10-phenanthroline prevented the degradation of HIF-1α and reduced oxygen-induced proliferation. Increasing oxygen decreased levels of p27KIP1 in the epithelial cells of older mice, which was prevented by expressing oxygen-insensitive forms of HIF-1α.

**Conclusions**—HIF-1α is present and HIF-1 is transcriptionally active throughout life, but suppresses growth only in older lenses. Maintaining elevated levels of p27KIP1 in older lenses requires HIF-1. p27KIP1 and other growth regulators may selectively suppress the proliferation of older lens epithelial cells.

The human lens grows continuously throughout life.1–3 Epidemiologic studies found that older subjects with small lenses are at increased risk of cortical cataracts and those with larger lenses are more likely to have nuclear cataracts.4–6

A recent study showed that increasing the level of oxygen in the eyes of older rats increases the rate of lens cell proliferation and lens growth.7 Based on this observation, it has been suggested that the hypoxic environment around the lens normally inhibits the rate of lens growth. It was not clear from these studies whether hypoxia directly inhibits proliferation by altering intracellular factors that control lens cell proliferation, by decreasing the growth-
promoting activity of factors in the aqueous humor, or by decreasing the ability of the lens cells to respond to endogenous growth factors. The present experiments were designed to answer this question.

Hypoxia is known to inhibit cell proliferation in cultured cells.8–10 Most cells respond to hypoxia by stabilizing the hypoxia-dependent transcription factor HIF-1, which, in cell culture models, is essential for hypoxia-induced inhibition of cell proliferation.9,11 To test whether HIF-1 contributes to hypoxia-dependent inhibition of cell proliferation in older lenses in vivo, the gene encoding the oxygen-regulated subunit of HIF-1, Hif1α, was deleted in the lens. We also overexpressed mutated versions of HIF-1α in the lens epithelial cells of transgenic mice or stabilized HIF-1α in vivo by chelating iron. The results of these experiments showed that HIF-1 is essential for normal lens development and suggested that endogenous HIF-1α mediates most of the hypoxia-dependent growth inhibition in the lens. Surprisingly, levels of the cyclin-dependent kinase inhibitor (CKI) p27\(^{KIP1}\) were high in the germinative zone of young and older lens epithelial cells, but were regulated by HIF-1 only in older animals. HIF-1 may suppress cell proliferation by maintaining high levels of CKIs and, perhaps, other molecules that suppress lens cell proliferation.

### Materials and Methods

All studies adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Washington University Animal Studies Committee.

#### HIF-1α Conditional Knockout Mice

Mice (129 strain) harboring floxed alleles of Hif1α\(^{12}\) were mated to LeCre transgenic mice (FVB strain), which express Cre recombinase in the lens under the control of the Pax6 P0 enhancer beginning on embryonic day 9 (E9).\(^{13}\) Offspring were genotyped by PCR. The eyes of Cre-positive and Cre-negative animals that were heterozygous or homozygous for the floxed allele were compared in histologic sections.

#### K14-HIF-1α Transgenic Mice

Mice expressing human HIF-1α cDNA lacking its oxygen-dependent degradation domain (ODD) or mice in which the prolines at positions 402 and 564 of human HIF-1α were replaced by alanine and glycine were overexpressed under control of the keratin-14 promoter.\(^{14,15}\) These mice, which were on a FVB background, are referred to as K14-HIF-1α-ΔODD and K14-HIF-1α-Pro\(^{402A/564G}\), respectively.

#### Stabilization of HIF-1α by Injecting the Iron Chelator, 1,10-Phenanthroline

The compound (Sigma-Aldrich, St. Louis, MO) was dissolved in sterile 0.9% saline at 3 mg/mL and nontransgenic, mixed sex littermates of the HIF-1 transgenic mice were injected intraperitoneally with 20 mg/kg twice within a 2-hour interval before beginning a 24-hour treatment with room air or 60% oxygen. Mice received one additional injection approximately 18 hours after beginning oxygen treatment. Animals were injected with BrdU 1 hour before the end of treatment. From each animal, the epithelium from one lens was prepared as a flatmount, to determine the BrdU labeling index and the epithelium from the other lens was lysed in 2× electrophoresis sample buffer for determination of HIF-1α levels.

#### Microarray Analysis

One- or 8-month-old, female BALBc mice were exposed to room air or 60% oxygen for 24 hours, and the peripheral regions of epithelial explants were dissected as described in the section on p27\(^{KIP1}\) Western blot analysis. Total RNA was extracted from the peripheral cells.
(MagMAX-96 for Microarrays Total RNA Isolation Kit; Ambion, Austin, TX). The quality of the RNA was checked (2100 Bioanalyzer; Agilent Technologies, Inc., Santa Clara, CA), RNA was amplified (MessageAmp aRNA kit; Ambion), labeled, and hybridized to beads (Illumina Sentrix Mouse-6 Whole Genome Expression BeadChips; Illumina, Inc., San Diego, CA). After normalization with the software (Illumina), expression levels of transcripts known to be regulated by HIF-1 or hypoxia were compared between mice exposed to normal or high oxygen at each age. The statistical significance of differences between expression levels was evaluated by algorithms provided in the software (Bead Station; Illumina).

BrdU Labeling and Oxygen Exposure

BrdU labeling of lens epithelial cells was performed as described previously. In brief, mice were injected intraperitoneally with a 10:1 mixture of 5′-bromodeoxyuridine (BrdU) and fluorodeoxyuridine (50 and 5 mg/kg, respectively; Sigma-Aldrich, St. Louis, MO). One hour after injection, lens epithelial wholemounts were dissected, fixed, and stained with monoclonal antibody to BrdU and the fluorescent nucleic acid stain TOTO-1. Explants were examined with a confocal microscope. Labeled and total nuclei in the germinative zone of the lens epithelium were counted with NIH Image software. The boundaries of the germinative zone were determined manually by drawing a box around the region of the epithelium that contained BrdU-positive cells, as described previously.

To expose mice to high or low oxygen, cages were placed in a large, plastic enclosure where the mice were exposed to room air (~21% oxygen), 11% or 60% oxygen for 24 hours. Oxygen levels were regulated with an oxygen controller (Pro-Ox 110; Biospherix, Redfield, NY). One hour before the end of the exposure, animals were injected with BrdU and the BrdU labeling index was determined.

Western Blot Analysis

To detect the low levels of HIF-1α typically found in tissues in vivo, samples of lens epithelium were dissected rapidly in medium kept on ice and lysed directly in 2x electrophoresis sample buffer, to minimize exposure to room air. After electrophoresis in 4% to 12% gradient gels, the proteins were transferred to nitrocellulose membranes for ≥4 hours and probed with a rabbit polyclonal antibody to HIF-1α (Bethyl Laboratories, Inc., Montgomery, TX) diluted 1:500 or 1:1000. Detection was with horseradish peroxidase (HRP)-labeled anti-rabbit secondary antibody diluted 1:5000 (Santa Cruz Biotechnology, Santa Cruz, CA) and chemiluminescence (SuperSignal West Dura; Pierce Chemical) with exposures of 1 to 30 minutes.

To determine the relative levels of p27KIP1, flatmounts of the lens epithelium were prepared and the central, mitotically quiescent zone was separated from the peripheral, germinative zone with a trephine (1.5 mm for epithelia from 1-month-old animals and 2.0 mm for epithelia from 9-month-old animals). Epithelial cells were lysed in RIPA buffer. Loading of the gels was equalized by determining the DNA content of a 10- to 15-μL aliquot of each sample (PicoGreen dsDNA Quantitation Kit; Invitrogen-Molecular Probes, Eugene, OR). Protein samples representing equal amounts of DNA were loaded, and Western blot analysis was conducted. Blots were probed with a rabbit polyclonal antibody to p27KIP1 (C-19, sc-528; Santa Cruz Biotechnology). The intensity of the stained bands was normalized to the level of β-actin on the same blots by using a mouse monoclonal antibody (MAB 1501R, 1:2000; Chemicon, Temecula, CA) and HRP-labeled anti-mouse secondary antibody (1:10,000; Santa Cruz). Blots were exposed to chemiluminescence (Hyperfilm ECL; GE Healthcare, Buckinghamshire, UK) and band intensities were recorded and quantified with a gel documentation system (EDAS 290; Eastman Kodak, Rochester, NY). Normalization was performed by adjusting the intensity of the p27KIP1 band, based on the level of β-actin, then setting the highest normalized intensity...
The results of three experiments, with duplicate blots for each experiment, were normalized, averaged, and plotted ± SEM.

### Apoptosis Assay

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick-end-labeling (TUNEL) was performed (Aptoptag kit; Chemicon). Lenses were fixed in 10% neutral buffered formalin (Fisher Scientific, Pittsburgh, PA), washed in PBS, dehydrated, embedded in paraffin, and sectioned at 4 μm. Deparaffinized slides were treated with 3% H2O2 in methanol for 30 minutes, followed by proteinase K treatment (20 μg/mL) for 15 minutes. Epitope retrieval was performed in 0.01 M citrate buffer (pH 6.0) either at 100°C for 20 minutes using a water bath or by placing slides in a decloaking chamber (Biocare Medical, Walnut Creek, CA) for 3 minutes. The slides were incubated with TdT enzyme in equilibration buffer for 1 hour at 37°C. The reaction was terminated with wash buffer provided by the manufacturer for 10 minutes at room temperature. Anti-digoxigenin peroxidase conjugate was added for 30 minutes at room temperature, followed by color development with diaminobenzidine and hydrogen peroxide. The slides were counterstained with hematoxylin.

### Statistical Analyses

Differences between samples were evaluated with Student’s t-test. The Bonferroni adjustment was applied for multiple comparisons.

### Results

#### Regulation of HIF-1α Levels by Oxygen in Lens Cells In Vivo

The effect of hypoxia on cells is often mediated by the oxygen-regulated transcription factor, HIF-1, which is composed of an oxygen-sensitive subunit, HIF-1α, and a constitutively expressed, oxygen-independent subunit, HIF-1β (ARNT). In hypoxia, HIF-1α is stabilized and associates with the β-subunit. The active complex is translocated to the nucleus, where it increases the expression of genes encoding glycolytic enzymes, glucose transporters, and proteins that enhance the delivery of oxygen, such as VEGF-A and erythropoietin. HIF-1 may also mediate the suppression of cell proliferation that occurs when cells are cultured in hypoxic conditions.

To determine whether HIF-1 regulates lens cell proliferation in response to the hypoxic conditions that are normally present in the eye, we first determined whether HIF-1α was detectable in lens epithelial cells and could be regulated by oxygen. Western blot analysis demonstrated that HIF-1α was present in lens epithelial cells immediately after their removal from the eye. Levels were markedly lower if 1- or 8-month-old animals were first exposed to 60% oxygen (Fig. 1).

#### Hypoxia-Regulated Gene Expression in 1- and 8-Month-Old Mice

Our previous studies of rat lenses showed that higher-than-normal oxygen levels did not affect lens cell proliferation in 1-month-old rats, but that intraocular hypoxia suppressed lens cell proliferation at older ages. Since HIF-1α was present in 1- and 8-month-old lens epithelial cells, we tested whether the expression of genes that are known to be regulated by HIF-1 is altered in 1- and 8-month-old lenses. Microarray analysis of transcripts isolated from the peripheral lens epithelial cells of animals that had breathed normal or elevated oxygen for 24 hours showed that levels of many transcripts that are known to be regulated by HIF-1 and/or hypoxia were decreased significantly when animals breathed 60% oxygen (Table 1). Although the rate of proliferation of the epithelial cells from 1-month-old lenses did not change in response to changes in oxygen (see below), the levels of oxygen-regulated transcripts decreased at least as much in these cells as in the epithelial cells of 8-month-old lenses. Therefore, under
normal (hypoxic) intraocular conditions, HIF-1 is likely to promote the expression of hypoxia-regulated genes in young and older lens cells.

**Deletion of Hif1a in the Lens**

To directly test whether HIF-1 is responsible for the hypoxia-dependent inhibition of lens cell proliferation in older animals, we examined the lenses of mice in which the HIF-1α gene was disrupted by conditional gene targeting. Transgenic mice that express Cre recombinase in the lens (LeCre)13 were mated to mice carrying a floxed Hif1a allele12 to generate mice that did not produce functional HIF-1α protein in the lens. Lenses from newborn Hif1a conditional knockout mice had levels of HIF-1α mRNA that were at least 1000 times lower than wild-type (Garcia et al., manuscript submitted). At postnatal day 3 (P3), CKO lenses had normal-appearing epithelial cells and elongating fiber cells, although a few degenerating fiber cells were present deeper in the fiber mass (Figs. 2A, 2B). By 1 month of age, HIF-1α CKO lenses were smaller than normal, with swollen and disorganized fiber cells, which had numerous apoptotic nuclei (Figs. 2C–2F). Lens morphology became progressively more disrupted in older CKO animals and the lenses eventually degenerated (not shown). This precluded analysis of the role of HIF-1 in regulating the proliferation of older lens epithelial cells. Together with the results of the microarray data, these results suggest that, as the lens ages postnatally, HIF-1 regulates many genes needed for the lens to function in a hypoxic environment and is essential for the survival of lens fiber cells.

**Transgenic Mice Overexpressing Stable Forms of HIF-1α**

As an alternative means to assess whether HIF-1 inhibits lens epithelial cell proliferation, we analyzed the lenses of transgenic mice in which oxygen-insensitive forms of HIF-1α were expressed in the epithelial cells. HIF-1α is targeted for degradation by the hydroxylation of two prolines in a region of the protein called the oxygen-dependent degradation domain (ODD). Under normoxic conditions (high-oxygen conditions in the eye), HIF-1α protein lacking the entire ODD or the target prolines within the ODD is not recognized by the von Hippel-Lindau protein, protecting it from degradation.42,43 The keratin-14 promoter was used to express forms of HIF-1α that were not degraded by oxygen in the epidermis and in other epithelial tissues, including the lens epithelium.44 The HIF-1α transgenes used were human HIF-1α cDNAs in which prolines 402 and 564 were mutated to alanine and glycine, respectively, or in which the entire ODD was deleted (K14-HIF-1α-Pro402A/564G and K14-HIF-1α-ΔODD, respectively).14,42,45 Both 1- and 8-month-old transgenic mice had elevated levels of HIF1α in lens epithelial cells, compared with their nontransgenic littermates (Fig. 3A). Lenses expressing these transgenes were normal in size and appearance at all ages examined.

Transgenic mice and their nontransgenic littermates were exposed to room air or 60% oxygen for 3 days and the BrdU labeling index of germinative zone epithelial cells was determined. Nontransgenic mice responded to oxygen exposure in a manner similar to rats.7 One-month-old nontransgenic mice had a relatively high BrdU labeling index that was not altered after treatment with 60% oxygen. Lens epithelial cell proliferation decreased progressively with age, but increased markedly after animals were exposed to 60% oxygen (Figs. 3B, 3C). Unlike rats,7 the oxygen-treated epithelial cells of older mice did not reach the same high level of proliferation as that in the epithelial cells of 1-month-old animals.

The higher levels of HIF-1α in 1- and 7-month-old K14-HIF-1α-Pro402A/564G and K14-HIF-1α-ΔODD transgenic mice did not alter the normal decline in epithelial cell proliferation that occurs with age (Figs. 3B, 3C). However, unlike their nontransgenic littermates, exposure of 4-month-old K14-HIF-1α-Pro402A/564G transgenic mice to 60% oxygen did not increase the BrdU labeling index of germinative zone epithelial cells (Fig. 3B). In 7-month-old K14-HIF-1α-Pro402A/564G transgenic animals, oxygen exposure did modestly increase BrdU
labeling, but by a significantly smaller amount than in nontransgenic littermates (Fig. 3B). At 1 month of age, lens epithelial cells from K14-HIF-1α-ΔODD transgenic mice and their nontransgenic littermates had similar BrdU labeling indexes that were not altered by exposure to 60% oxygen. Seven-month-old K14-HIF-1α-ΔODD transgenic mice responded in a manner that was similar to the K14-HIF-1α-Pro402A/564G transgenic mice. Transgene expression significantly reduced the oxygen-stimulated increase in the BrdU labeling index, compared to nontransgenic littermates (Fig. 3C). These data suggest that HIF-1 can suppress the proliferation of lens epithelial cells in vivo and may be responsible for much or all the slower growth seen in older lenses.

Stabilization of HIF-1α by Injection of 1,10-Phenanthroline

Oxygen-dependent degradation of HIF-1α is mediated the hydroxylation of prolines 402 and 564 of human HIF-1α. In addition, hydroxylation of arg-803 inhibits the activity of one of the transcriptional activation domains of HIF-1α. Prolyl and arginyl hydroxylases require iron as a cofactor.46–48 Depleting iron stabilizes HIF-1α and maintains its transcriptional activity by preventing the hydroxylation of proline and arginine. Treatment of mice overnight with the iron chelator, 1,10-phenanthroline increased HIF-1α levels in 1.5- or 8-month-old mice exposed to 21% or 60% oxygen (Fig. 4A). In the lenses of 1.5-month-old mice, breathing 60% oxygen slightly increased the BrdU labeling index of germinative zone epithelial cells. Phenanthroline treatment significantly suppressed this oxygen-stimulated proliferation (Fig. 4B). In 8-month-old mice, injection of phenanthroline suppressed the oxygen-stimulated increase in proliferation, but did not significantly affect the basal rate of proliferation in animals breathing room air. As with the overexpression of stable forms of HIF-1α, phenanthroline did not return proliferation to basal levels, suggesting that there may be a minor, HIF-1-independent mechanism that contributes to the oxygen-mediated increased in lens cell proliferation.

Levels of p27KIP1 in the Germinative Zone of Older Mice in the Presence of Hypoxia and HIF-1α

Results of studies have suggested that the inhibition of cell proliferation under hypoxic conditions involves the ability of HIF-1 to stimulate the accumulation of the CKIs p21CIP1 and/or p27KIP1. Expression of p21CIP1 protein is low in the mouse lens.50,51 Another CKI, p57KIP2 is expressed at high levels as epithelial cells withdraw from the cell cycle in the transition zone.52 The expression of other CKIs in the lens has not been examined. Because others have shown that p27KIP1 levels can be increased by HIF-1, we determined whether changes in intraocular oxygen altered p27KIP1 protein levels in wild-type lenses and lenses over expressing stable HIF1α. The specificity of the p27KIP1 antibody used in these studies was demonstrated by showing that the ~27-kDa band that it recognized was absent from the lenses of p27KIP1 knockout mice (data not shown). For these studies, proteins extracted from the peripheral, germinative zone epithelial cells were examined separately from proteins of the central, mitotically quiescent region of the lens epithelium (Fig. 5A).

Exposure of 1-month-old nontransgenic or K14-HIF-1α-ΔODD-transgenic mice to 60% oxygen for up to 24 hours had no appreciable effect on the relative levels of p27KIP1 in the central region of the lens epithelium (Figs. 5B, 5C). In the peripheral region of the epithelium of 1-month-old nontransgenic animals, oxygen treatment caused p27KIP1 to decrease transiently 6 hours after exposure, but levels returned to normal by 24 hours. This transient decline in p27KIP1 levels was not seen in the peripheral epithelial cells of 1-month-old K14-HIF-1α-ΔODD transgenic lenses. Even though they proliferate rapidly at this age, levels of p27KIP1 were higher in peripheral than in central lens epithelial cells. Therefore, the relatively
high levels of p27KIP1 found in the germinative zone cells do not appreciably inhibit proliferation at this age. Factors other than the level of p27KIP1 are likely to be responsible for the very low rate of proliferation in central epithelial cells.

In contrast to 1-month-old lenses, breathing 60% oxygen caused a progressive decrease in p27KIP1 levels in the peripheral cells of the lens epithelium from 9-month-old nontransgenic animals, reaching approximately 40% of the initial level by 24 hours of oxygen exposure (P < 0.001; Figs. 5B, 5C). The level of p27KIP1 in the central zone cells from these same lenses was not significantly altered by oxygen treatment. In 9-month-old animals that overexpressed K14-HIF-1α-ΔODD, breathing 60% oxygen did not reduce the levels of p27KIP1 in the central or peripheral lens epithelial cells, showing that, in the periphery of these older lenses, HIF-1 is necessary to maintain the high level of p27KIP1 that is normally present in vivo.

Discussion

The results of the present work show that HIF-1α is normally expressed and is sensitive to oxygen in the epithelial cells of 1-month-old and older lenses. HIF-1α is needed for normal postnatal lens development and genes known to be regulated by HIF-1 are regulated by the level of hypoxia in young and older lenses in vivo. Increased expression of mutated forms of HIF-1α that are resistant to degradation in the presence of oxygen had no effect on basal rate of lens cell proliferation, but suppressed the increase in epithelial cell proliferation stimulated by oxygen. One of the targets of HIF-1 may be proteins that regulate the stability of the cyclin-dependent kinase inhibitor p27KIP1 and, possibly, other cell cycle regulators, in the germinative zone epithelial cells. It remains to be tested whether the oxygen-dependent decline in p27KIP1 levels is sufficient to account for the oxygen-dependent changes in the rate of proliferation of germinative zone epithelial cells.

Control of Cell Proliferation by Hypoxia and HIF-1 In Vivo

Although most studies of the ability of hypoxia to block cell proliferation have used cultured cells, hypoxia, acting through HIF-1, can suppress cell proliferation in vivo. Targeted deletion of Hif1α in chondrocytes in the growth plate of long bones, a normally hypoxic tissue, leads to increased cell death, increased BrdU incorporation, and decreased accumulation of the cyclin-dependent kinase inhibitor p57KIP2.6 Conversely, conditional deletion of the von Hippel-Lindau gene (Vhl) in chondrocytes stabilizes HIF-1α protein and reduces the rate of chondrocyte proliferation.5 Chondrocytes lacking both Vhl and Hif1a have a phenotype that is similar to the Hif1a knockouts, showing that the effect of Vhl deletion is primarily through its effect on HIF-1α levels. This implies that hypoxia, acting through HIF-1α, inhibits chondrocyte proliferation in vivo.5 In contrast to its function in cartilage or in older lens cells, hypoxia sometimes promotes cell proliferation in vivo. Systemic hypoxia stimulates the proliferation of erythrocyte precursors and vascular endothelial cells by increasing HIF-1 levels, leading to the production of mitogens like erythropoietin and VEGF-A.16,18 Similarly, hypoxia increases the proliferation of adult neuronal precursors54 and pulmonary artery smooth muscle cells in vivo, although the mechanisms responsible for these effects are not fully understood.

Suppression of Proliferation by HIF-1 in Lens Epithelial Cells

Overexpression of stable forms of HIF-1α in older lens epithelial cells did not affect their low, basal rate of proliferation, but reduced the ability of oxygen to increase their proliferation. However, this block was not always complete. The incomplete suppression of proliferation in older lenses under hyperoxic conditions may be accounted for by the dual manner in which oxygen regulates HIF-1 activity. In addition to targeting HIF-1α for degradation, oxygen reduces the ability of HIF-1α to activate transcription by promoting the hydroxylation of an
asparagine in its C-terminal transcription activation domain by the arginyl hydroxylase factor-inhibiting HIF (FIH).\textsuperscript{46,56,57} FIH activity may prevent the overexpressed HIF-1 from completely blocking proliferation by hydroxylating R803 in the HIF-1\(\alpha\) transactivation domain.\textsuperscript{19} This possibility was tested by injecting mice with the iron chelator 1,10-phenanthroline. Proline or asparagine hydroxylation requires iron as a cofactor.\textsuperscript{46–48} Therefore, depleting iron should block the hydroxylation of HIF-1\(\alpha\), preventing its degradation and promoting its transcriptional activity. Intraperitoneal injection of phenanthroline increased the levels of HIF-1\(\alpha\) in young and older lens cells and selectively suppressed the ability of oxygen to stimulate lens cell proliferation in older animals. However, like the stable forms of HIF-1\(\alpha\), phenanthroline was not able to completely block the increase in proliferation caused by increased intraocular oxygen. These observations suggest that, in addition to decreased levels of HIF-1, another factor, perhaps increased reactive oxygen species (ROS),\textsuperscript{58} contributes to the oxygen-stimulated increase in lens epithelial cell proliferation. In the normal, hypoxic eye, where ROS are expected to be low, HIF-1 activity may account for all the inhibition of lens cell proliferation.

Most studies have suggested that exposing cells to acute hypoxia suppresses cell proliferation by increasing transcription of the cyclin-dependent kinase inhibitors p21\textsuperscript{CIP1} and p27\textsuperscript{KIP1}.\textsuperscript{8,9,11,49} In murine embryonic stem cells, Hif1a was required for the increased expression of p21\textsuperscript{CIP1} and the decreased proliferation caused by oxygen and glucose deprivation.\textsuperscript{9} Similarly, hypoxia increased the transcription of p27\textsuperscript{KIP1} and inhibited the proliferation of murine primary fibroblasts.\textsuperscript{49} Consistent with the known function of p27\textsuperscript{KIP1} as an inhibitor of the G\(_1\) to S transition, hypoxia did not inhibit the proliferation of cells lacking Rb. HIF-1\(\alpha\) was also necessary for hypoxia to inhibit the proliferation of primary mouse embryo fibroblasts and primary B lymphocytes.\textsuperscript{11} In this case, hypoxia promoted an HIF-1\(\alpha\)-dependent increase in both p21\textsuperscript{CIP1} and p27\textsuperscript{KIP1}. As expected, HIF-1\(\alpha\) was also required for hypoxia to maintain the hypophosphorylated state of Rb.\textsuperscript{11} Stable forms of HIF-1\(\alpha\) induced cell cycle arrest and increased the expression of p21\textsuperscript{CIP1} in tumor cells and normal human fibroblasts by displacing Myc from the p21\textsuperscript{CIP1} promoter.\textsuperscript{8}

It is possible that, as in most of these previous studies, oxygen and HIF-1 regulate lens p27\textsuperscript{KIP1} levels by stimulating Cdkn1b transcription. However, our microarray studies found no oxygen-dependent change in the accumulation of transcripts encoded by Cdkn1b or any of the other seven mouse CKIs. Therefore, HIF-1 might function by inhibiting the rate of degradation of p27\textsuperscript{KIP1} by decreasing the levels of molecules like Skp2 or KPC, ubiquitin ligases that target p27\textsuperscript{KIP1} for degradation.\textsuperscript{59,60} These possibilities will be examined in future studies.

In the present study, although lower levels of p27\textsuperscript{KIP1} were associated with increased proliferation of germinative zone cells, p27\textsuperscript{KIP1} levels were consistently lower in the mitotically quiescent central epithelial cells than in germinative zone cells. Similarly, p27\textsuperscript{KIP1} levels were high in the germinative zone cells of young lenses, where proliferation was relatively high. These observations suggest that p27\textsuperscript{KIP1} is not the only factor that controls lens cell proliferation in an age-dependent manner.

The low proliferative activity of central epithelial cells may reflect the distribution of growth factors in the ocular fluids; these have been suggested to come from the ciliary epithelial cells adjacent to the germinative zone of the lens.\textsuperscript{61–63} The higher levels of p27\textsuperscript{KIP1} in the germinative zone cells may be related to the observation that, at least in rabbits, oxygen levels are significantly lower in the aqueous humor bathing this region than the more central regions of the lens epithelium.\textsuperscript{64}
The Age-Dependence of HIF-1 Function in the Lens

An important aspect of this study is that HIF-1 was found to be present and functioning in the young lens, but did not inhibit the proliferation of lens epithelial cells until later in life. Evidence that HIF-1 functions in the young lens came from oxygen-dependent changes in transcripts known to be regulated by HIF-1 and from lenses in which Hif1a was disrupted by conditional gene targeting. Transcripts encoding VEGF-A (Vegfa), carbonic anhydrase IX (Car9), prolyl hydroxylase-3 (Phd2), macrophage migration inhibitory factor (Mif), and pyruvate dehydrogenase kinase-1 (Pdk1), all known targets of HIF-1, were among 31 with significantly lower accumulation in lens cells from 1-month-old mice breathing 60% oxygen than in littermates breathing room air. By 1 month of age, lenses lacking Hif1a were small and contained abnormal and degenerating fiber cells, indicating that HIF-1 activity is required for the survival of postnatal lens fiber cells.

Although HIF-1 was present and functional in lens epithelial cells from 1-month-old mice, it did not appear to regulate proliferation at this age. When young mice breathed 11% oxygen, which reduced intraocular oxygen levels below the levels present in older animals, proliferation was not affected. Similarly, overexpression of stable HIF-1α in young lens epithelial cells or increasing HIF-1α levels in young lenses by treatment with 1,10-phenanthroline did not significantly decrease proliferation.

In the present studies, oxygen levels were increased in the eye by having animals breathe 60% oxygen. This perturbation was necessary to demonstrate that the hypoxic environment that is normally present in the eye suppresses lens cell proliferation. However, breathing 60% oxygen is a nonphysiologic treatment. Pharmacologic treatments that inactivate HIF-1 are being developed for tumor therapy. Supplying these HIF-1-blocking drugs to the lens in vivo could directly test the importance of HIF-1 in suppressing lens cell proliferation without altering oxygen levels in the eye.

These data suggest that the decline in lens growth that occurs with increasing age is caused by a gradual increase in the sensitivity of lens epithelial cell proliferation to inhibition by genes that are regulated by HIF-1, rather than by changes in the level or overall activity of HIF-1 (Fig. 6). The molecular events underlying this change are not known. They may involve changes in the level of specific coregulators of HIF-1 transcriptional activity, changes in chromatin structure around specific HIF-1 targets, or other means of increasing the efficiency with which HIF-1 regulates genes that ultimately inhibit cell proliferation. Identifying the targets and the mechanisms that modulate the growth inhibitory effects of HIF-1 will provide important information about the control of lens size. Given that smaller and larger lenses are associated with increased risk of cortical and nuclear cataracts, respectively, this information may provide clues to the causes of age-related cataracts.

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Figure 1.
Western blot analysis showing that HIF-1α is normally detectable in lens epithelial cells at 1 and 8 months of age. At either age, exposure of animals to 60% oxygen for 3 days led to a substantial decline in the level of HIF-1α. β-Actin levels in the same samples are shown as the loading control.
Figure 2.
Conditional deletion of Hif1a leads to the progressive degeneration of the lens during the first month of life. (A) The normal morphology present in newborn (P3), wild-type lenses (LeCre-negative, Hif1a$^{flox/flox}$). (B) The lens from a P3 eye in which HIF-1α had been conditionally deleted (LeCre-positive; Hif1a$^{flox/flox}$). The morphology of the epithelial cells and superficial fiber cells appeared normal. Some fiber cells deeper in the lens were swollen, with signs of degeneration. (C) Lenses that were heterozygous for Hif1a (LeCre-positive; Hif1a$^{flox/+}$) were normal in morphology. (D) 1-month-old lenses lacking Hif1a (LeCre-positive; Hif1a$^{flox/flox}$) were smaller than normal with swollen and disorganized fiber cells. (E) No TUNEL-positive cells were detected in lenses that were heterozygous for Hif1a (LeCre-positive; Hif1a$^{flox/+}$).

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(F) Many TUNEL-positive cells were seen in the fiber cells of lenses lacking Hif1α (LeCre-positive; Hif1a^fl/fl).
Figure 3.
Inhibition of lens cell proliferation by transgenic overexpression of stable forms of HIF1-α. 
(A) Western blot showing that both human HIF-1α transgenes were overexpressed in lens epithelial cells at 1 and 8 months of age. In lenses that overexpressed HIF-1α-ΔODD (ΔODD), both wild type mouse HIF-1α and the truncated human protein lacking the oxygen-dependent degradation domain were detectable. The HIF-1α-Pro^{402A/564G} mutant protein (Pro) migrated with the wild-type protein. At both ages this resulted in more intense staining of the HIF-1α band. (B) Increased levels of the HIF-1α-Pro^{402A/564G} mutant did not alter the BrdU labeling index in 1-month-old lens epithelia (Tg 1M). Exposure of nontransgenic mice (NTg) to 60% oxygen increased the BrdU labeling index at 4 and 8 months of age. Overexpression of the
stable proline mutant of HIF-1α (Tg) completely suppressed this increase in lens epithelial cells of 4-month-old mice and significantly inhibited it in 7-month-old animals. (C) Overexpression of HIF-1α-ΔODD (Tg) or exposure to 60% oxygen did not significantly alter the BrdU labeling index of germinative zone epithelial cells from the lenses of 1-month-old mice. Transgenic expression of HIF-1α-ΔODD (Tg) significantly inhibited the oxygen-induced increase in BrdU labeling in lens epithelial cells from 7-month-old animals, compared with nontransgenic littermates (NTg).
Figure 4.
Intraperitoneal injection of the iron chelator 1,10-phenanthroline increased HIF-1α levels in the lens epithelial cells of young and older mice and suppressed lens cell proliferation at both ages. (A) Western blot showing that oxygen treatment decreased and intraperitoneal injection with 1,10-phenanthroline increased HIF-1α levels in 1.5- and 8-month-old mice. Each lane contains the lens epithelial cells from one pair of lenses from a single mouse. Results were consistent in more than six pairs of mice for each treatment. (B) In 1.5-month-old mice, breathing 60% oxygen for 24 hours slightly increased lens epithelial cell proliferation, although this increase did not reach statistical significance. Treatment with 1,10-phenanthroline significantly decreased lens epithelial cell proliferation from this higher level in oxygen-treated mice (60%+P), but did not significantly affect proliferation in the lenses of mice maintained in room air (21%+P). (C) In 8-month-old mice, breathing 60% oxygen for 24 hours significantly increased lens epithelial cell proliferation. Intraperitoneal injection of 1,10-phenanthroline significantly decreased lens epithelial cell proliferation in oxygen-treated mice (60%+P), but not in mice maintained in room air (21%+P). However, the BrdU labeling index in the lens epithelial cells of oxygen- and phenanthroline-treated mice was still significantly greater than in mice treated with phenanthroline but not exposed to 60% oxygen. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 5.
Levels of the cyclin-dependent kinase inhibitor, p27\textsuperscript{KIP1}, decreased after 9-month-old mice were exposed to 60% oxygen. Oxygen exposure did not decrease p27\textsuperscript{KIP1} levels in lenses from 1-month-old mice or in 9-month-old mice that expressed a stable form of HIF-1\(\alpha\). (A) A flatmount of the lens epithelium. Gray: area covered by the anterior epithelial monolayer. The clear flaps at the edges represent the cell-free posterior lens capsule. A trephine was used to separate the central (C) from the peripheral (P) regions of the epithelium. To recover a similar proportion of the central and peripheral zones, a 1.5-mm trephine was used in 1-month-old animals and a 2.0-mm trephine in 9-month-old animals. (B) Western blot analysis showing the levels of p27\textsuperscript{KIP1} and \(\beta\)-actin in 1- and 9-month-old nontransgenic and K14-HIF-1\(\alpha\) \(\Delta\)ODD transgenic lens epithelial cells from the center (C) and periphery (P) of the lenses of mice exposed to 60% oxygen for 0, 3, 6, or 24 hours (C0–C24 and P0–P24). Breathing 60% oxygen decreased levels
of p27KIP1 only in 8-month-old, nontransgenic mice. Overexpression of HIF-1α lacking the oxygen-dependent degradation domain nullified the effect of oxygen on p27KIP1. (C) Graphs showing the mean levels of p27KIP1, relative to the levels of β-actin, calculated from densitometer scans of blots such as those shown in (B). Data are the mean ± SEM of four to five blots. *P < 0.05, ***P < 0.001.
Figure 6.
Diagram illustrating the postulated age- and oxygen-dependent relationships between HIF-1 and p27KIP1 and the rate of proliferation of germinative zone epithelial cells. In younger lenses, HIF-1 is not needed to maintain the level of p27KIP1, and the relatively high level of p27KIP1 present in lens cells at this age does not suppress lens cell proliferation. Growth factors in the ocular fluids promote relatively rapid lens cell proliferation. In older animals, HIF-1 is necessary to maintain high levels of p27KIP1, which may inhibit lens epithelial cell proliferation and slow lens growth, even in the presence of growth factors. In older lenses, when oxygen levels increase, HIF-1α is degraded, lowering the level of p27KIP1 and releasing the lens epithelial cells from growth inhibition. Growth factors in the ocular fluids then promote increased proliferation of lens epithelial cells.
## Table 1
Decreases in the Levels of Transcripts Known to Be Regulated by Hypoxia and/or HIF-1 in the Peripheral Lens Epithelial Cells of 1- and 8-Month-Old Mice Breathing 60% Oxygen, Compared with Mice Breathing Room Air

<table>
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<tr>
<th>Gene Name</th>
<th>1-Month-Old Change (x-fold)</th>
<th>P</th>
<th>8-Month-Old Change (x-fold)</th>
<th>P</th>
<th>Ref.</th>
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<td>&lt;0.001</td>
<td>−1.56</td>
<td>&lt;0.001</td>
<td>19</td>
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<td>&lt;0.001</td>
<td>−1.82</td>
<td>&lt;0.001</td>
<td>20</td>
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<td>−1.75</td>
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Data are the mean of results from triplicate samples. Values were omitted for transcripts that did not change significantly at one age.
Table 2
Transcript Levels for CKIs in Mouse Lens Epithelial Cells Were Not Significantly Affected by Breathing 60% Oxygen at 1 or 8 Months of Age

<table>
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<th>CKI</th>
<th>1-Month-Old</th>
<th>P</th>
<th>8-Month-Old</th>
<th>P</th>
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<td></td>
<td>Change (x-fold)</td>
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<td>ND</td>
<td>◆</td>
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<td>Cdkn1c (p57&lt;sup&gt;KIP2&lt;/sup&gt;)</td>
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<td>1.2</td>
<td>&gt;0.05</td>
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<tr>
<td>Cdkn2a (p19&lt;sup&gt;ARF&lt;/sup&gt;)</td>
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<td>◆</td>
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</tr>
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<td>1.1</td>
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</tr>
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<td>&gt;0.05</td>
<td>−1.1</td>
<td>&gt;0.05</td>
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Data are the mean of triplicate samples. ND, not detected; ◆, transcript not detected, no probability calculated.