Activation of Unfolded Protein Response in Transgenic Mouse Lenses

Lixing W. Reneker,1 Huiyi Chen,1 and Paul A. Overbeek2

PURPOSE. Overloading of unfolded or misfolded proteins in the endoplasmic reticulum (ER) can cause ER stress and activate the unfolded protein response (UPR) in the cell. The authors tested whether transgene overexpression in the mouse lens would activate the UPR.

METHODS. Transgenic mice expressing proteins that either enter the ER secretory pathway or are synthesized in cytosol were selected. Activation of the UPR was assessed by determining the expression levels of the ER chaperone protein BiP, the spliced form of X-box binding protein-1 (Xbp-1) mRNA, and the transcription factor CHOP. Changes in the ubiquitin-proteasome system in the mouse lens were detected by ubiquitin immunofluorescence.

RESULTS. BiP expression was upregulated in the fiber cells of transgenic mouse lenses expressing platelet-derived growth factor-A (PDGF-A), dominant-negative fibroblast growth factor receptor (DN-FGFR), or DN-Sprouty2 (DN-Spy2). BiP upregulation occurred around embryonic day 16.5, primarily in the fiber cells adjacent to the organelle free zone. Fiber cell differentiation was disrupted in the PDGF-A and DN-Sprouty2 lenses, whereas the fiber cells were degenerating in the DN-FGFR lens. High levels of UPR activation and ubiquitin-labeled protein aggregates were found in the DN-FGFR lens, indicating inefficient disposal of unfolded/misfolded proteins in the fiber cells.

CONCLUSIONS. This study implies that overexpression of some transgenes in the lens can induce ER or overall cell stress in fiber cells, resulting in the activation of UPR signaling pathways. Therefore, investigators should assess the levels of UPR activation when they analyze the downstream effects of transgene expression in the lens. (Invest Ophthalmol Vis Sci. 2011;52:2100–2108) DOI:10.1167/iovs.10-5650

In eukaryotic cells, secretory and transmembrane proteins enter the endoplasmic reticulum (ER) as unfolded polypeptide chains, which fold and mature in the lumen of the ER in a highly assisted process. The ER lumen contains molecular chaperones, folding enzymes, and quality control factors that assist in folding and trafficking of newly synthesized polypeptides.1,2 If the influx exceeds the folding or processing capacity of the ER, the normal physiological balance of the ER is perturbed, a condition called ER stress. Physiologically, ER stress can be caused by the synthesis of mutated proteins, the absence of cofactors, or a drastic increase in the amount of otherwise normal cargo proteins during cell differentiation. A range of environmental stresses and pathogenic conditions (e.g., nutrient deprivation and viral infection) can also trigger ER stress and result in the accumulation of unfolded proteins in the ER.

In response to ER stress, cells activate adaptive intracellular signal transduction pathways, known as the unfolded protein response (UPR), so that the cells can recover from the stress.3–5 Three ER-localized transmembrane protein sensors can initiate the UPR. These are inositol requiring kinase 1 alpha (IRE1alpha), protein kinase-like ER kinase (PERK), and activating transcription factor 6alpha (ATF6alpha). The combined activation of IRE1alpha, PERK, and ATF6alpha initially produces cytoprotective outputs, such as enhanced ER protein folding capacity, reduced protein translation, and clearance of misfolded ER proteins.6 For instance, the activated IRE1alpha endonucleases splices out a 26-nucleotide intron of Xbp-1 mRNA, leading to a frameshift in codon reading and consequently creating an additional potent transactivation domain in the C terminus of the Xbp-1 protein.7 Xbp-1 controls a large group of UPR-response genes, including the ER chaperone BiP, to cope with the unfolded protein load in the ER.8,9 However, when cells undergo prolonged and severe ER stress and ER homeostasis cannot be reestablished, proapoptotic signals are triggered, presumably to protect the organism from these irreversibly damaged cells.10,11 The molecular mechanisms of ER stress–induced cell death are not completely understood. The UPR signaling pathway induced by PERK can contribute to cell death. PERK phosphorylates eukaryotic translation initiation factor 2alpha (eIF2alpha), which results in the enhanced translation of ATF4, a b-ZIP transcription factor. One of the important UPR-specific target genes of ATF4 is CHOP/GADD153, a b-ZIP transcription factor, which plays a role in apoptosis during ER stress.12–14

Recently, ER stress has been found to play a fundamental role in the pathogenesis of many human diseases, including neurodegenerative diseases and diabetes.15 In the eye, common diseases such as retinal degeneration and glaucoma have been implicated as protein misfolding diseases arising from persistent ER stress and activation of UPR signaling.16–18 Recently, Firtina et al.19 reported that the accumulation of collagen chains in the lens can activate the UPR and cause cataract formation in lens fiber cells of transgenic and mutant mice.19 Previous studies20,21 led by Shinohara’s group showed that many cataractogenic stressors can activate the UPR in lens cell culture systems. For instance, both glucose deprivation and hyperglycemia can activate the UPR, which results in apoptosis in cultured lens epithelial cells.21 The mechanism of UPR activation under these conditions is unclear but is likely due to overall cellular stress.

Despite extensive investigations on the molecular pathways of the UPR, many questions remain to be explored regarding...
the in vivo impact of the UPR in tissue development, physiology, and homeostasis. The ocular lens serves as a good model system for such analyses. Studies from past years have demonstrated that lens cells are highly susceptible to ER stress by environmental factors and mutations.\textsuperscript{22–24} During the past few years, transgenic mice have been used as an in vivo model to study gene function in lens development and cataractogenesis.\textsuperscript{25–28} We took advantage of the availability of various types of transgenic mice to ask the following questions: Could over-expression of a transgene in the mouse lens induce ER stress and activate the UPR? What are the potential biological effects of UPR activation on lens development?

**Materials and Methods**

**Animals**

Transgenic mice expressing the following minigenes were used: PDGF-A,\textsuperscript{29} the active form of transforming growth factor β1 (TGFβ1),\textsuperscript{30} DN-FGFR (also called tR1 for truncated FGFR1),\textsuperscript{31} DN-Ras,\textsuperscript{26} and DN-Spry2.\textsuperscript{32} We generated the Spry2 transgenic mouse by constructing a minigene using the same modified α-crystallin promoter vector as the DN-Spry2 minigene. The DN-Spry2 transgenic mice (lines LR40 and LR44) were bred to homozygosity for the transgene to increase expression levels. All procedures used in the study were approved by the University of Missouri Animal Care Committee and were conducted in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Reverse Transcriptase-PCR and Real-Time PCR**

Newborn mouse lenses were removed, and total RNA was extracted using an RNA purification kit (RNeasy Mini; Qiagen, Valencia, CA). RNA integrity was confirmed by gel electrophoresis to visualize the 28S and 18S ribosomal RNA bands. Approximately 800 ng total RNA was reverse transcribed into cDNA using a synthesis kit (iScript cDNA; Bio-Rad Laboratories, Hercules, CA). The gene-specific PCR primers are listed in Table 1. Reverse transcriptase (RT)-PCR products of Xbp-1 and Chop genes were separated by electrophoresis using 10% TBE gel (Novex; Invitrogen, Carlsbad, CA) and visualized under UV light after staining with ethidium bromide.

To quantify the relative expression levels of Spry2, Bip, and Chop, real-time PCR reactions were performed (iCycler; Bio-Rad Laboratories Inc.) in triplicate. Expression levels were normalized to β-actin mRNA from the same samples. Statistical differences among groups were analyzed by using one-way ANOVA, and Tukey’s posttest was used for comparisons between two groups (Prism; GraphPad, San Diego, CA). $P < 0.05$ was considered significant.

**Histology and Immunofluorescence**

Tissues were fixed in 10% formalin, embedded in paraffin, and sectioned at 5-μm thickness. After deparaffinization and rehydration, the sections were stained with hematoxylin and eosin (H&E) or were processed for immunofluorescence staining as described.\textsuperscript{33,34} We used the following antibodies: anti-BiP (Cell Signaling, Danvers, MA), anti-ubiquitin (MBL Medical and Biological Laboratory, Nagoya, Japan), and fluorescein-conjugated secondary antibody (Invitrogen).

**TUNEL Assay**

DNA fragmentation was detected using an in situ apoptosis detection kit (ApopTag Red; Chemicon International, Billerica, MA). Sections were counterstained with DAPI and examined under fluorescence microscopy.

**Results**

**BiP Upregulation in Transgenic Mouse Lenses**

The upregulation of BiP expression is a molecular indicator of ER stress and UPR activation.\textsuperscript{35} To determine whether overexpression of transgenic proteins that enter the ER synthesis pathway can cause ER stress in the mouse lens, BiP immunofluorescence was examined on the transgenic lenses that express either the secretory proteins PDGF-A and TGFβ1 or the transmembrane protein DN-FGFR. Both the wild-type (WT) (Fig. 1A) and the TGFβ1 newborn (postnatal day [P] 0) lenses (Fig. 1C) had little detectable BiP, whereas BiP expression was found in the fiber compartment of both the PDGF-A (Fig. 1B) and the DN-FGFR (Fig. 1D) lenses. Furthermore, increased BiP expression was found in the central fiber cells, suggesting that ER stress occurred in the more differentiated and mature fiber cells.

To investigate whether proteins that are synthesized in the cytosol can also activate the UPR, transgenic mice that express DN-Ras and DN-Spry2 were examined for BiP upregulation in the fiber compartment. BiP expression was found in the fiber cells of the DN-Spry2 lenses (Fig. 1F) but not in the DN-Ras lenses (Fig. 1E), suggesting that UPR activation can also be induced by overexpression of a protein that does not enter the ER pathway.

**BiP Level Correlates with the Transgene Expression Level**

We assessed the impact of the transgene expression level on BiP expression using two DN-Spry2 transgenic lines (LR40 and LR44) that have different expression levels.\textsuperscript{32} The real-time RT-PCR assay (Fig. 2L) confirmed that transgene expression levels were higher in the LR40 line than in the LR44 line. Histologic analysis revealed that fiber cells were defective in the high-expression LR40 line (Fig. 2B). Vacuoles formed in the fiber compartment and the differentiating fiber cells in the cortical region did not elongate or align properly. As a result, the apical margins (Fig. 2F, arrowheads) of the young fibers failed to contact with the anterior epithelium. In contrast to the LR40 lens, the LR44 lens looked normal (Fig. 2C). Using immunofluorescence to localize BiP expression, we found that both fluorescence intensity and number of BiP-positive fiber cells were significantly higher in the LR40 lens than in the LR44 lens (Figs. 2F, 2G), suggesting that BiP levels, which are indicative of UPR activation, correlated with the transgene expression levels. High magnification of the LR44 lens revealed that

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BiP localized around the cell nuclei in the fiber cells adjacent to the organelle-free zone (OFZ; Fig. 2g), where fiber cells undergo terminal differentiation to lose their intracellular organelles and nuclei.36–38 These data imply that the mature fiber cells in the central zone are more sensitive to the stress condition; thus, UPR activation is more likely to occur in these cells than in the young fiber cells in the cortical region of the lens.

**Figure 1.** BiP expression in transgenic mouse lenses. BiP immunofluorescence (green) in newborn (P0) mouse lenses is shown against DAPI-stained cell nuclei (blue). In WT lenses (A), the BiP level was too low to be detected by immunofluorescence. BiP was upregulated in the fiber cells of the transgenic lenses that overexpressed PDGF-A (transgenic line OVE460; B), DN-FGFR (also called tR1; D), and DN-Spry2 (line LR40; F). BiP upregulation was not seen in the transgenic lenses overexpressing either an active form of TGFβ1 (line OVE920A; C) or DN-Ras (line OVE1405; E). epi, lens epithelial cells; fib, lens fiber cells. Scale bars, 100 μm.

**Figure 2.** Morphologic analysis of transgenic lenses and localization of BiP and Spry2. (A–D) Hematoxylin and eosin–stained sections of representative transgenic mouse lens. The DN-Spry2 LR40 lens (B) developed more severe defects than the DN-Spry2 LR44 lens (C). Vacuoles were formed in the fiber compartment, and the central fiber cells were defective in the LR40 lens. The LR44 lens was similar to the WT lens (A). The transgenic lens overexpressing WT-Spry2 (LR33) was microphthalmic, and the fiber cells were defective (D). (E–H) BiP localization. BiP immunofluorescence (green) was merged with DAPI nuclear staining (blue) in F–H. The BiP level was undetectable in the fiber cells of the WT lens (E), whereas BiP was present in the fiber cells of both the LR40 (F) and the LR44 (G and g) lenses. (F, arrowheads) An apical margin of elongating fiber cells. In the LR44 lens, BiP immunofluorescence was found in the fiber cells adjacent to the OFZ (G). Under higher magnification (g), BiP was localized around the fiber cell nuclei (arrow point to the circular shape of BiP immunofluorescence). BiP expression was barely detected in the fiber cells of the transgenic lens expressing WT-Spry2 (H). (I–L) Spry2 localization (I–K) and Spry2 mRNA level were determined by real-time PCR (L). Spry2 protein was found in lens epithelial cells and in the fiber cells at a low level in the WT lens (I). Transgene expression contributed to the increase of Spry2 immunofluorescence in the fiber cells of LR40 (J) and LR33 (K) lens. Spry2 immunoreactivity was higher in the LR33 than in the LR40 lens. For real-time PCR, Spry2 mRNA level was normalized to β-actin mRNA (L). Transgene expression level was higher in the heterozygous LR33 lens than in either homozygous LR40 or LR44 lenses (*P < 0.001).
Effects of Spry2 Mutation on UPR Activation

DN-Spry2 has a single amino acid mutation, substituting phenylalanine for tyrosine at amino acid position 55 of Spry2.39,40 To determine the effect of mutation on BiP expression and UPR activation, transgenic mice expressing the WT Spry2 were generated using the same expression vector as for the DN-Spry2 mice. Among the two established Spry2 transgenic lines, we chose a high-expression line (LR33) for the study. The LR33 transgenic lens was significantly smaller than either the WT or the DN-Spry2 lenses (Fig. 2D); it also had severely defective fiber cells and developed cataracts. Localization of BiP expression in the LR33 lens revealed that only a few fiber cells in the central zone were weakly positive (Fig. 2H). Using immunofluorescence with an antibody that recognized both the WT and the DN-Spry2 proteins, we compared Spry2 protein levels and found that Spry2 immunoreactivity was higher in the LR33 lens than in LR40 (Figs. 2J, 2K). The higher transgene expression level in the LR33 lens was confirmed at the mRNA level by using real-time RT-PCR (Fig. 2L). Thus, our data suggested that the mutant DN-Spry2 is a more potent activator of the UPR than the WT Spry2, probably because of the cellular stress induced by a protein folding deficiency caused by a phenylalanine-to-tyrosine substitution. The data also indicated that fiber cell differentiation can be altered without inducing a dramatic increase in BiP, as shown in the Spry2 lens (Fig. 2D).

BiP Upregulation in Transgenic Lenses during Development

The onset of BiP upregulation was examined by using the DN-Spry2 (LR40) and DN-FGFR lenses at different developmental ages (Fig. 3). By embryonic day (E) 14.5, weak BiP expression was found in the lens epithelial cells but not in the fiber cells in both the WT (Fig. 3A) and the DN-Spry2 lenses (Fig. 3B). Only a few fiber cells in the DN-FGFR lens were weakly positive for BiP at this age (Fig. 3D). BiP expression increased significantly in the fiber cells of both the DN-Spry2 lens and the DN-FGFR lenses after E16.5 (Figs. 3C, 3E, 3F), suggesting that the mature fiber cells are sensitized to ER stress.

Accumulation of the Transgene Product PDGF-A Chain in Lens Fiber Cells

To assess the contribution of intracellular accumulation of the transgene product to UPR activation in the lens fiber cells, we examined the localization of the PDGF-A chain in the eyes of PDGF-A transgenic mice (Fig. 4). Immunostaining for the PDGF-A chain showed strong signal in the vitreous cavity of transgenic mouse eyes from E16.5 to P0 (Figs. 4B, 4C, asterisks), indicating that the transgene product was secreted from the lens cells. PDGF-A immunofluorescence was not detected in the age-matched WT eyes probably because of its low level (data not shown). Accumulation of the PDGF-A chain inside the fiber cells was first observed on E16.5 (Fig. 4B), and the level increased significantly in the P0 transgenic lens (Figs. 4C, 4D). In the central area where strong PDGF-A retention was
found (Fig. 4D, arrows), intense BiP immunofluorescence was detected (Fig. 4F, arrows), suggesting that the accumulation of unfolded or misfolded transgene product induced BiP upregulation through UPR activation. Strong PDGF-A retention was also found where BiP expression was not detected (Figs. 4C, 4E), suggesting that factors other than BiP can also contribute to the accumulation of PDGF-A in lens fiber cells.

**UPR Activation and Changes in Fiber Cell Differentiation and Survival**

The onset and localization of BiP in transgenic lens fibers suggest that ER stress and activation of the UPR may interfere with normal differentiation of the fiber cells. We used DN-Spry2 and PDGF-A transgenic mice to assess the effects of ER stress on fiber differentiation and lens development. The fiber cells in these mice are defective but not degenerating like those in the DN-FGFR lens. Histologic analyses revealed that fiber cell elongation was attenuated in both DN-Spry2 and PDGF-A lenses (Figs. 5D, 5G). The elongating fibers in the WT lens aligned along the anterior-posterior pole and packed in a highly ordered concentric manner (indicated by the lines in Fig. 5A), whereas the DN-Spry2 and PDGF-A transgenic lens fibers were disorganized, did not align properly, and did not form the normal curvature as found in the WT lens (Figs. 5D, 5G).

To determine whether UPR activation interferes with late-stage fiber maturation, we examined the distribution patterns of PDI (an ER marker) and cell nuclei in the WT, DN-Spry2, and PDGF-A lenses. Mature fiber cells in the WT lens underwent demucleation and degradation of intracellular organelles, resulting in the formation of the OFZ at the lens core. This was confirmed by the lack of PDI and nuclear staining in the OFZ of the WT lens. In contrast, PDI and nuclear staining in the DN-Spry2 (Figs. 5E, 5F) and PDGF-A (Figs. 5H, 5I) lenses were found across the entire fiber compartment, and the OFZ was missing at the center of the lens in both transgenic mice. These data suggest that ER stress and UPR activation may interfere with the normal differentiation process and prevent the terminal differentiation of fiber cells. Alternatively, the defects in the mature fiber cells in PDGF-A and DN-FGFR lenses could result from the abnormalities in the lens epithelial layer and cortical fiber cells and may not be directly caused by UPR activation.

Prolonged and severe ER stress is known to induce apoptosis. Cell death was reported to have occurred in the DN-FGFR transgenic lenses. To investigate whether persistent ER stress could also lead to cell death in DN-Spry2 and PDGF-A lenses, TUNEL assays were performed on newborn (P0) lenses (Fig. 6). Many fiber nuclei were apoptotic in the E18.5 DN-FGFR lens (Fig. 6B). TUNEL-positive nuclei were not detected in the WT, DN-Spry2, or PDGF-A lenses (Figs. 6A, 6C, 6D). Thus, ER stress does not inevitably result in apoptosis in lens fiber cells.

**Levels of UPR Activation in Transgenic Lenses**

Activation of the three UPR sensors—IRE1α, ATF6α, and PERK—results in transcriptional activation of Xbp-1, ATF-6α, and ATF-4, either to help cells adapt to persistent stress or to initiate apoptotic signaling cascades if the damage is too severe and irreversible. To investigate whether severe lens defects in the DN-FGFR lens correlated with high levels of UPR activation, we examined some of the major transcriptional responses induced by UPR sensors. To demonstrate the activation of the IRE1/Xbp1 pathway in the transgenic lenses, RT-PCR analysis was performed on total RNA from newborn lenses using primers designed to distinguish the spliced and unspliced forms of Xbp-1 mRNA. Figure 7A shows that the unspliced Xbp-1 mRNA was amplified in the WT, DN-FGFR, and DN-Spry2 lenses, whereas the levels of the spliced form were higher in the transgenic lenses, suggesting that the IRE1/Xbp1 pathway is activated in the DN-FGFR and DN-Spry2 lenses. Additionally, compared with the WT lens, both the DN-FGFR and DN-Spry2 transgenic lenses had higher levels of CHOP mRNA (Fig. 7A), suggesting that the CHOP protein may help cells adapt to persistent ER stress or to initiate apoptosis after the damage is too severe and irreversible into a form that may not be directly caused by UPR activation.
was used as an internal control. To investigate degraded by the ubiquitin-proteasome system (UPS), a process proteins in the ER lumen are transported back to the cytosol and Xbp-1, Chop spliced form of Xbp-1, Chop, was determined by real-time PCR (C). BiP mRNA and the transcription factor CHOP. In the transgenic lenses, the spliced fragment (179 bp) was readily detected in both the DN-Spry2 and the DN-FGFR lenses but at a very low level in the WT lens, whereas the unspliced Xbp-1 (205 bp) was present in all three genotypes. Chop mRNA was detected in WT and transgenic lenses, but the level was increased in the transgenic lenses. β-Actin was used as an internal control. (B) Relative expression levels of BiP and Chop were determined by real-time PCR (C). BiP and Chop mRNA levels, normalized to β-actin mRNA, were higher in the transgenic lenses than in the WT lens (*P < 0.05, **P < 0.001).

Figure 6. Cell death detection by TUNEL assay. Fiber cell nuclei at the central zone in the newborn (P0) WT lens had degraded, and TUNEL labeling was negative (A). TUNEL-positive nuclei were intensely labeled in the E18.5 DN-FGFR lens (B). Very few nuclei were weakly labeled in the DN-Spry2 lens (C), and no positive nuclei were found in the PDGF-A lens (D).

Figure 7. Expression levels of the spliced form of Xbp-1, Chop, and BiP in DN-Spry2 and DN-FGFR lenses. (A) RT-PCR to detect two forms of Xbp-1 mRNA and CHOP mRNA. The spliced fragment (179 bp) was readily detected in both the DN-Spry2 and the DN-FGFR lenses but at a very low level in the WT lens, whereas the unspliced Xbp-1 (205 bp) was present in all three genotypes. Chop mRNA was detected in WT and transgenic lenses, but the level was increased in the transgenic lenses. β-Actin was used as an internal control. (B) Relative expression levels of BiP and Chop were determined by real-time PCR (C). BiP and Chop mRNA levels, normalized to β-actin mRNA, were higher in the transgenic lenses than in the WT lens (*P < 0.05, **P < 0.001).

**Discussion**

We investigated the possibility that transgene expression in the mouse lens can induce ER stress and activate the UPR in lens fiber cells. We chose transgenic mice expressing proteins that either enter the ER secretory pathway, including PDGF-A, TGFβ1, and DN-FGFR, or are synthesized in the cytosol such as DN-Ras, Spry2, and DN-Spry2. The results are summarized in Table 2. BiP was upregulated in the fiber cells of transgenic lenses that express PDGF-A, DN-FGFR, and DN-Spry2. Activation of the UPR in these transgenic lenses was confirmed by the expression of both the spliced form of Xbp-1 mRNA and the transcription factor CHOP. In the transgenic lenses that express PDGF-A and DN-FGFR, activation of the UPR is likely a direct result of ER stress from protein overload. For DN-Spry2, which is synthesized in the cytosol, the mechanism of UPR activation in the fiber cells is unclear but probably results from overall cellular stress. The impact of UPR activation on lens fiber cells is difficult to evaluate. The fiber defects in the BiP-upregulated lenses probably resulted from combined effects of the transgenic proteins and UPR activation. Our two general outcomes include are these: fiber cell elongation was attenuated, fiber alignment and curvature were abnormal, and the hallmarks of fiber terminal differentiation, such as denucleation and intracellular organelle degradation, were blocked in both the PDGF-A and the DN-Spry2 lenses; ubiquitin-labeled protein aggregates were formed, and the apoptosis pathway was activated in the fiber cells from the most severe case of ER stress in the DN-FGFR lens.

**Activation of the UPR by Transgenic Proteins That Enter the ER Secretory Pathway**

The PDGF-A, TGFβ1, and DN-FGFR minigenes were constructed using the mouse α-crystallin promoter and encoded proteins that enter the lens secretory pathways. When a large amount of transgene-derived polypeptide enters the ER, it increases the demand on protein folding and assembly. If the transgenic protein influx exceeds the ER capacity, the normal
physiological balance of the ER is perturbed, thereby causing ER stress in the fiber cells. Recently, Firtina et al.\textsuperscript{19} showed that expression of normal or mutant collagen IV (an extracellular matrix protein) in the transgenic mouse lens also caused ER stress and activated UPR in the lens fiber cells. Together the data suggest that it is important to determine UPR activation levels in lens fiber cells when a transgene encoding for a secretory/membrane protein is expressed at a high level in the mouse lens.

Activation of the UPR by Transgenic Proteins Synthesized in Cytosol

For transgenic mice that express DN-Ras, DN-Spry2, and Spry2, the minigenes were constructed using a modified lens promoter containing a chicken \( \alpha \)-crystallin enhancer fused to the mouse \( \alpha \)-crystallin promoter.\textsuperscript{32} The proteins derived from these minigenes do not contain a functional signal peptide; hence, they do not normally enter the secretory pathway.\textsuperscript{45–47} It is puzzling to us that UPR activation was detected in the DN-Spry2 lenses. We found a similar case with an \( \alpha \)-crystallin G98R mutant.\textsuperscript{24} WT \( \alpha \)-crystallin is a water-soluble protein synthesized in the cytosol and abundantly present in the lens. In contrast, \( \alpha \)-crystallin G98R mutant caused severe crystallin aggregation and consequently UPR activation in cultured human lens epithelial cells. We speculate that activation of the UPR in the lens cells expressing either DN-Spry2 or G98R \( \alpha \)-crystallin likely results from overall stress induced by the accumulation of mutant proteins in the cell and not from protein overloading in the ER.\textsuperscript{48,49}

UPR Activation on Fiber Differentiation

Although the overall phenotypic changes were different in the transgenic lenses undergoing UPR activation, this study identified some common features that may be a result of the UPR. In the transgenic lenses expressing PDGF-A or DN-Spry2, cell elongation was attenuated, ER was expanded, and denucleation and intracellular organelle degradation were inhibited. We propose that alterations of the differentiation program could be a part of the adaptive response. The exact mechanisms by which UPR activation inhibits fiber cell differentiation are not known. Our data show that BiP upregulation occurs primarily in the fiber cells adjacent to the OFZ. These fiber cells are in the late stage of fiber differentiation and are about to become mature fiber cells. In these cells, the endogenous differentiation program turns on the cellular degradation machinery required to eliminate cell nuclei and intracellular organelles (including ER).\textsuperscript{38} Therefore, the protein folding and assembly capacity of the ER and Golgi could also be declining. Overexpressing a transgenic protein that enters the ER secretory pathway increases the demand on protein-folding and thus requires fiber cells to expand the ER to alleviate protein overloading. Such adaptive changes may override the endogenous terminal differentiation process. Furthermore, activation of 1

Table 2. Summary of UPR in the Lenses of Different Types of Transgenic Mice

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* BiP upregulation was detected at a very low level.

Increased levels of UPR markers in the transgenic lenses were shown by the (+) scale. No entry means that the study was not performed.
of the 3 UPR sensors, the ER transmembrane protein PERK, would result in a global protein translational attenuation in the transgenic mouse lens, as demonstrated by Firtina et al.19 We have not examined the changes in protein synthesis in any of the UPR-activated transgenic lenses. However, we assume that the PERK-signaling pathway was activated in the DN-Spry2 and DN-FGFR lenses because the expression level of CHOP, which is one of the downstream target genes of PERK, was elevated in these transgenic lenses (Fig. 7A). Because fiber cell elongation is associated with a rapid elevation in protein synthesis for membrane- and fiber cell-specific proteins, activation of the PERK-mediated UPR signaling pathway would have an adverse effect on epithelial-to-fiber differentiation. Thus, we speculate that the UPR signaling pathway can either inhibit or disrupt the normal fiber differentiation program. To determine how UPR activation contributes to the disruption of fiber differentiation and lens development, future experiments will include generating transgenic mice that express UPR-induced genes such as BiP and CHOP in the lens fiber cells. The effects of these proteins on lens differentiation and development may help us to dissect the phenotypes caused by URP activation or the specific action of the transgene protein in future transgenic studies.

At present, knowledge of the impact of UPR activation on tissue homeostasis and physiology in vivo is limited. A recent report by Firtina et al.19 provided in vivo evidence that the UPR played a role in cataract formation. Ectopic expression of extracellular matrix protein collagen IV in the lens of transgenic mice caused an accumulation of collagen chains in the secretory pathway and activated UPR signaling pathways. As a result, lens fiber cell differentiation was disrupted and cell death was induced. Another in vivo example comes from the study in chondrocytes during endochondral bone formation.34 Like lens fiber cells, chondrocytes undergo terminal differentiation during normal development. Expression of mutant collagen X in the differentiating hypertrophic chondrocytes (HCs) causes accumulation of the misfolded mutant protein inside the cells and activation of the ER stress signaling pathway. HCs survive ER stress by altering the differentiation program to a less mature state, similar to what we have seen in the PDGF-A and DN-Spry2 lenses. Overall, the combined data from both systems suggest that altering the differentiation program may represent a general adaptive response to ER stress in vivo.

Cell Death in DN-FGFR Transgenic Lens

Our results show that cell death occurred in the DN-FGFR lens but not in the DN-Spry2 or PDGF-A lenses. A recent publication by Firtina et al.19 suggested that extremely high levels of UPR activation can cause cell death in the mouse lens, but the onset was much delayed as seen in the DN-FGFR. FGF signaling is known to be essential for lens fiber cell differentiation and survival.51,52 Based on previous studies,31,53,54 it was concluded that defects in fiber differentiation and cell death in the DN-FGFR lens resulted from reduced FGF signaling activity. The present study shows that expression of DN-FGFR in the lens fiber cells causes ER stress and activates the UPR signaling pathway (Fig. 7). The UPR is known to facilitate both adaptation to stress and initiation of apoptosis, depending on the nature and severity of ER stress. Upregulated expression of CHOP was shown to play an important role in UPR-induced cell death.12–14 Real-time RT-PCR data showed that CHOP and Bip expression were higher in the DN-FGFR lens than in the DN-Spry2 lens, suggesting that ER stress was more severe in the DN-FGFR lens. Therefore, apoptotic signals in the DN-FGFR lens can be initiated by both reduction in FGF signaling and activation of the UPR.

To address the issue of whether reduction of FGF signaling would induce ER stress and activate the UPR signaling pathway, we examined the Bip expression patterns in the FGF2-deficient mouse lenses. Garcia et al.55 found that loss of FGF2 induced apoptosis in both lens epithelial cells and fiber cells. Using the same Cre transgenic line (LeCre) to delete FGF2 in the lens, we found cell death in the E16.5 and P0 FGF2-deficient lenses, but BiP was not upregulated in the fiber cells (Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5650/-/DCSupplemental), suggesting that the loss of FGF2 does not induce ER stress in the lens fiber cells. The lens expresses several FGFRs.56 Whether the loss of additional or multiple FGFRs would induce ER stress in the lens fiber cells is a subject for future investigation.

We examined the ubiquitin distribution pattern in the transgenic lenses. Ubiquitin-labeled protein aggregates were found in the DN-FGFR lens but not in the PDGF-A or the DN-Spry2 lens, suggesting that the severe ER stress in the DN-FGFR lens may have a result of inefficient disposal of unfolded/misfolded protein by the UPS. We also examined ubiquitin expression patterns in the FGF2-deficient mouse lenses and did not detect any ubiquitin-positive aggregates in the fiber compartment (Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.10-5650/-/DCSupplemental), suggesting that the depletion of FGF2 alone does not result in the formation of ubiquitin aggregates. Therefore, it is possible that DN-FGFR is an aggregation-prone protein. Failure to remove the aggregation-prone DN-FGFR protein may “choke-up” the UPS and activate apoptotic signals in the lens fiber cells.

In summary, our study indicates that overexpression of a transgenic protein that either enters the secretory pathway or is synthesized in cytosol has the potential to induce ER or overall cellular stress and to activate UPR signaling pathways in the lens fiber cells. Although the phenotypic outcome varies among the transgenic lenses, there is a correlation between UPR activation and disruption of normal fiber differentiation and maturation. Despite the useful application to study gene function in lens development and cataractogenesis, transgenic techniques may inadvertently activate the UPR signaling pathways, which can alter the gene expression profile during lens development. Because there is no direct way to predict which transgene will elicit ER or cellular stress, we recommend that the levels of UPR activation in lens fiber cells should be assessed when using transgenic techniques.

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References

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