Overexpression of Human γC-crystallin 5 bp Duplication Disrupts Lens Morphology in Transgenic Mice

Zhiwei Ma,1,2 Wenliang Yao,1,2,3 Veena Theendakara,1,4 Chi-Chao Chan,5 Eric Wawrousek,6 and J. Fielding Hejtmancik,1

PURPOSE. To delineate the molecular mechanisms underlying autosomal dominant congenital cataracts caused by a 5 bp duplication in human CRYGC.

METHODS. c.119_123dup (CRYGC5bpd) and wild-type human γC-crystallin (CRYGC) were expressed in transgenic mouse lenses by the chicken βB1-crystallin promoter. Lenses were characterized histologically, by real-time PCR, SDS-PAGE, and Western blot. pET and Tet-on expression systems were used to express human CRYGC and CRYGC5bpd proteins in Escherichia coli and HeLa cells, respectively.

RESULTS. Transgenic expression of CRYGC5bpd mutant γC-crystallin results in nuclear cataracts in which lens fiber cells begin to show variable degrees of degeneration and vacuolization by postnatal day 21. By 6 weeks of age all CRYGC5bpd lenses exhibit abnormalities of varying severity, comprising large vacuoles in cortical fiber cells, swelling and disorganization of fiber cells, and defective fiber cell migration and elongation. Levels of CRYGC5bpd mRNA are 3.7- and 14.1-fold higher than endogenous Cryg mRNA in postnatal day 1 and 6-week CRYGC5bpd mouse lenses, respectively. Cryg, Crygb, Crybb2, and Crybh3 mRNA levels are decreased in CRYGC5bpd mice compared with wild-type and CRYGC mice. Both wild-type and mutant human γC-crystallin are uniformly distributed in the cytosol of HeLa cells, but CRYGC5bpd is degraded when expressed in E. coli BL21(DE3).

CONCLUSIONS. Transgenic expression of mutant CRYGC5bpd γC-crystallin at near-physiological levels causes lens opacity and fiber cell defects, confirming the pathogenicity of this mutation. These results further suggest that HGCG5bpd γC-crystallin causes cataracts through a direct toxic or developmental effect on lens cells causing damaged microstructure rather than through formation of HMW aggregates with resultant light scattering. (Invest Ophthalmol Vis Sci. 2011;52:5369–5375) DOI:10.1167/iovs.11-7168

From the 1Ophthalmic Genetics and Visual Function Branch, 2Histology Core, and 3Laboratory of Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, Bethesda, Maryland; 4Department of Medicine, Johns Hopkins University School of Medicine, Baltimore, Maryland; and 5Jules Stein Eye Institute, University of California, Los Angeles, California. 2These authors contributed equally to this work. Supported by the National Eye Institute Intramural Program. Submitted for publication January 3, 2011; revised March 3, 2011; accepted March 9, 2011.

Disclosure: Z. Ma, None; W. Yao, None; V. Theendakara, None; C.-C. Chan, None; E. Wawrousek, None; J.F. Hejtmancik, None

Corresponding author: J. Fielding Hejtmancik, OGVFB/NEI/NIH, 5635 Fishers Lane, Room 1127, Rockville, MD 20852; f3h@helix.nih.gov.

Materials and Methods

Generation of Transgenic Mice

This work abided by the ARVO statement for the use of animals in ophthalmic and visual research. The chicken βB1-crystallin/
**Reverse Transcription and Quantitative Real-Time PCR**

Total RNA was extracted from lenses of different age transgenic and littermate control mice by using a RNA isolation kit (RNasy Mini Kit; Qiagen, Valencia, CA), with the addition of DNase I digestion to prevent DNA contamination. Total RNA concentrations were estimated by spectrophotometer at 260 nm, and then cDNA was synthesized (ThermoScript RT-PCR system; Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. As described previously, primers and probes were designed by commercially available software (Primer Express; ABI) and are shown in Table 1. PCR reactions were performed on a real-time PCR system (Prism 7900HT; ABI). The PCR program consisted of incubation for 2 minutes at 50°C, denaturation for 10 minutes at 95°C, followed by 40 cycles of denaturation for 15 seconds at 95°C, and 1 minute of annealing and elongation at 60°C. Due to the similarity of gene sequences in this study, sequence analyses were performed to confirm the specific amplicon from each gene. TaqMan MGB probes were used to detect the specific PCR product as it accumulates during PCR. All reactions were performed in a 20 μL reaction volume with 1 ng cDNA template from P1D lens or 10 ng cDNA from P6W. The amount of amplified PCR product was calculated from standard curves. PCR efficiencies of the CRYGC5bp, Crygc, Crygb, Crybb2, and Crybb3 sequences are all higher than 96%. The housekeeping gene Gapdh served as an endogenous control for quantitation, the absolute amount of each target mRNA being normalized to the amount of Gapdh mRNA.

**Morphologic and Histologic Analysis**

For gross observation, the lenses from mice at different ages were isolated under a dissecting microscope and photographed by a digital camera (Gel Doc XR System; Bio-Rad, Hercules, CA). For histologic analysis, mouse embryo heads at 15-day (E15D), 1-day (P1D), and 8-day (P8D) postnatal or 21-day (P21D) and 6-week (P6W) postnatal eyes were removed and fixed overnight in 10% neutral buffered formalin. After dehydration and clarification, they were embedded in methyl methacrylate and sectioned serially at 4 μm thickness via the pupill-optic nerve plane, then stained with hematoxylin and eosin by standard histologic technique. The sections were examined by light microscope, and then images were obtained by means of a scanning camera equipped with a screening-capture program (AxioCam HRC and AxioVision; Carl Zeiss, Thornwood, NY). For transmission electron microscopy, the tissues were fixed in 4% glutaraldehyde-formalin. The lenses were embedded in epoxy resin (Ladd LX-112; Ladd Research, Williston, VT). Six 1 μm thick sections stained with toluidine blue were examined under light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate for examination under a microscope (JEM-100B; JEOL, Tokyo, Japan).

**Table 1. PCR Primers and Probes Used to Quantify mRNA Levels**

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Forward Primer (5’–3’)</th>
<th>Reverse Primer (5’–3’)</th>
<th>MGB Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRYGC5bp</td>
<td>AAGCACCACAAATTCTTCTTCGG</td>
<td>CTTGGATGTTGCTCAGCTTTC</td>
<td>ACCATCTTGGAGAAAGA</td>
</tr>
<tr>
<td>Crygc</td>
<td>CATGCGCCCCATTGAGGTCC</td>
<td>AAGGCCGCTTGAATACAGTCAA</td>
<td>AGCGCTTTCAAGATCG</td>
</tr>
<tr>
<td>Crygb</td>
<td>GCCCTACATCGCCACATTGT</td>
<td>AGAGCCGAAATTTGGCATT</td>
<td>AAGCGCCGAAATTT</td>
</tr>
<tr>
<td>Crybb2</td>
<td>TGCCGTGAGAGGATTTACT</td>
<td>GAGCCGCTTCCAGCCAAAAA</td>
<td>CAGGCCTGATCG</td>
</tr>
<tr>
<td>Crybb3</td>
<td>TGGGCGTGGAGAGGAATCT</td>
<td>TGATGAGCCCTTCCACAAT</td>
<td>CGCAATCTGGCATG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAGCCGCTGGCTGAGTATC</td>
<td>GAGCCGCTTCCAGCCAAAAA</td>
<td>CAGGCCTGATCG</td>
</tr>
</tbody>
</table>

**Western Blot Analysis**

Whole lenses were harvested from P6W CRYGC5bp and wild-type (WT) mice and homogenized in 300 μL of lysis buffer containing 1XPBS, 1mM PMSF, 1% Triton X-100, 1 mM DTT, and a protease inhibitor cocktail (Sigma, St. Louis, MO) for 30 minutes at 4°C. Lysates were then centrifuged at 1400 rpm (MIKRO 200 R; Hettich, Beverly, MA) rotor (18,000g) for 20 minutes at 4°C. The protein concentration of the supernatant was measured (BCA Protein Assay Kit; Pierce, Rockford, IL) using BSA as a standard. Equal amounts of water-soluble lens proteins from CRYGC5bp and WT (40 μg for electrophoresis, 2 μg for Western blot) were mixed with 30 μL buffer containing 2% SDS, 10% sucrose, 1.5X sodium dodecyl sulfate (SDS) loading buffer, and boiled for 5 minutes. The samples were loaded onto polyacrylamide gels and separated by SDS-PAGE (10–20% gradient gels). The gels were then transferred to nitrocellulose membranes and probed with specific antibodies recognizing CRYGC5bp (1:5000 dilution; Santa Cruz, Santa Cruz, CA). Membranes were incubated with the appropriate secondary antibodies (1:2000 dilution; Santa Cruz, Santa Cruz, CA) and visualized by chemiluminescence.
Expression of the CRYGC5bpd mutant γC-crystallin in transgenic mice resulted in nuclear cataracts of varying severities. Gross morphologic analysis of isolated lenses from P20W CRYGC5bpd mice shows a total cataract with dense opacities in the lens core. However, with respect to opacities and histologic and morphologic abnormalities, lenses from transgenic mice that express wild-type human γC-crystallin are indistinguishable from age-matched nontransgenic control mice (Fig. 1B). The size of CRYGC5bpd lenses is not obviously different from the lenses of CRYGC and WT mice.

RESULTS

Phenotype and Lens Morphology

Expression of the CRYGC5bpd mutant γC-crystallin in transgenic mice resulted in nuclear cataracts of varying severities. Gross morphologic analysis of isolated lenses from P20W CRYGC5bpd mice shows a total cataract with dense opacities in the lens core. However, with respect to opacities and histologic and morphologic abnormalities, lenses from transgenic mice that express wild-type human γC-crystallin are indistinguishable from age-matched nontransgenic control mice (Fig. 1B). The size of CRYGC5bpd lenses is not obviously different from the lenses of CRYGC and WT mice.

Electron microscopic examination was performed at 5 weeks, just before major disruption of the lens architecture.

Electron microscopic examination was performed at 5 weeks, just before major disruption of the lens architecture.
becomes apparent on hematoxylin and eosin stain. There are subtle ultrastructural differences between the control and 5bpd lenses with early bladder cell formation (swollen and nucleated lens fiber cells) in CYRG5bpd compared with WT lenses (Figs. 3A, 3B) and swelling of the lens epithelial cells in CYRG5bpd (Figs. 3C, 3D).

### Reverse Transcription and Quantitative Real-Time PCR

Real-time PCR was performed to determine the expression level of the CYRG5bpd gene. After normalization against GAPDH in each sample, levels of CYRG5bpd mRNA in CYRG5bpd lenses are 3.7-fold higher than those of the endogenous mouse γC-crystallin mRNA at P1D, and 14.7-fold higher at P6W (Fig. 4A; Table 2). To investigate the effect of CYRG5bpd transgenic expression further, expression of additional genes in the β-crystallin family (γB-, βB2-, and βB3-crystallin) were examined in P6W mice. Quantitative RT-PCR reveals that γC- and γB-crystallin mRNAs are decreased by approximately 11.5- and 14.1-fold, respectively, in CYRG5bpd lenses compared with controls. In addition, there is a similar but somewhat milder decrease of βB2- and βB3-crystallin mRNA levels in CYRG5bpd relative to control mouse lenses, 4.4- and 3.7-fold, respectively (Table 3; Fig. 4B). However, the level of CYRG5bpd mRNA in the CYRG5bpd transgenic mouse lenses is roughly similar to intrinsic crystallin mRNA levels in control mouse lenses, being 139%, 112%, 137%, and 49% of γC-, γB-, βB3-, and βB2-crystallin levels, respectively.

The expression of human γC-crystallin mRNA (CRYGC), γC-, γB-, βB3-, and βB2-crystallin was also quantitated in 4-week CYRG transgenic and nontransgenic mice. The mRNA level of CRYGC is similar to those of endogenous γC-, γB-, and βB2-crystallins, but fivefold higher than βB3-crystallin. However, the mRNA levels of endogenous γC-, γB-, βB3-, and βB2-crystallin are very similar in CRYGC and WT mice (Table 4).

### Western Blot Analysis

To investigate whether the transgenic CYRG5bpd mRNA is effectively translated in the lens, polyacrylamide gel electro-
TABLE 3. Expression of CRYGC5bpd, Crygc, Crygb, Crybb3, and Crybb2 in the Lenses of 6-Week-Old CRYGC5bpd Transgenic and WT Mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>CRYGC5bpd/Gapdh</th>
<th>Crygc/Gapdh</th>
<th>Crygb/Gapdh</th>
<th>Crybb3/Gapdh</th>
<th>Crybb2/Gapdh</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRYGC5bpd</td>
<td>6.49 ± 0.75</td>
<td>4.6 ± 1.08</td>
<td>4.1 ± 1.12</td>
<td>15.4 ± 1.09</td>
<td>29.8 ± 8.88</td>
</tr>
<tr>
<td>WT</td>
<td>46.7 ± 4.90</td>
<td>57.8 ± 11.34</td>
<td>47.4 ± 4.92</td>
<td>131.7 ± 22.78</td>
<td></td>
</tr>
<tr>
<td>CRYGC5bpd mRNA/Cry mRNA</td>
<td>1.39</td>
<td>1.12</td>
<td>1.37</td>
<td>0.49</td>
<td></td>
</tr>
</tbody>
</table>

WT, wild-type.

Western blots and Western blot analyses were carried out on proteins from P6W mice lenses. The 5 bp insertion in exon 2 of the human CRYGC results in an amino acid sequence frameshift, giving a new protein comprising the first 41 amino acids of γC-crystallin followed by 62 novel amino acids and a new stop codon. Thus the mutated protein is predicted to be composed of 103 amino acids with a calculated molecular weight of 10.7 kDa and a predicted isoelectric point of pH 8.4, compared with the normal γC-crystallin protein, which has a molecular weight of 21 kDa and a PI of 7.1. From the Coomassie stained SDS-PAGE of lens water-soluble extract, a band at approximately 10 kDa is seen only in the CRYGC5bpd lane (Fig. 1C), but not in the control lane, which is identical with the CRYGC transgenic, consistent with the predicted size of mutant CRYGC5bpd protein. The protein profile of the insoluble fractions show some quantitative differences between control and CRYGC5bpd mutant lenses, which have a slight decrease in the intact γC-crystallin bands and some increase in smaller bands, possibly representing degradation products. However, the differences are relatively minor (Fig. 1C). In addition, both insoluble fraction profiles show some additional small bands, probably degradation products, not seen in the soluble fractions. Using a polyclonal antibody against γ-crystallins, the same approximately 10 kDa band is visualized only in the CRYGC5bpd lane. The antibody also recognizes the γ-crystallins around 20 kDa in both the WT and CRYGC5bpd lens extracts as well as a protein of approximately 26 kDa, suggesting that antibody cross-reacts with the β-crystallins to some degree.

Characterization of Recombinant γC-Crystallin in Cells

When E. coli BL21(DE3) was transformed with the constructs pET-CRYGC and pET-CRYGC5bpd, WT γC-crystallin is expressed exclusively in the soluble fraction, but the CRYGC5bpd mutant protein is degraded, which is detected by Western blot with polyclonal antibodies raised to γ-crystallin (data not shown). To further investigate the influence of mutant CRYGC5bpd γC-crystallin on cell growth and properties, we generated HeLa cells in which expression of exogenous CRYGC and CRYGC5bpd proteins can be induced by Dox treatment using the Tet-on system. Expression of neither CRYGC nor CRYGC5bpd in HeLa or lens epithelial cells had a discernable effect on cell growth or survival (data not shown). Transient transfections of cells with recombinant CRYGC and CRYGC5bpd after 48 hours induction with Dox demonstrated that both WT and CRYGC5bpd γC-crystallins are uniformly distributed in the cytosol of transfected cells, which maintain normal morphology. The intensity of staining is somewhat higher for WT CRYGC than CRYGC5bpd. The γC-crystallin expression in some untreated cells derived from a small amount of leakiness of the Tet promoter in the absence of Dox (Fig. 5).

TABLE 4. Expression of CRYGC, Crygc, Crygb, Crybb3, and Crybb2 in the Lenses of 6-Week-Old CRYGC Transgenic and WT Mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>CRYGC/Gapdh</th>
<th>Crygc/Gapdh</th>
<th>Crygb/Gapdh</th>
<th>Crybb3/Gapdh</th>
<th>Crybb2/Gapdh</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>39.9 ± 5.60</td>
<td>30.0 ± 6.52</td>
<td>43.9 ± 8.54</td>
<td>7.2 ± 1.06</td>
<td>40.4 ± 8.77</td>
</tr>
<tr>
<td>CRYGC</td>
<td>32.2 ± 2.73</td>
<td>47.2 ± 4.73</td>
<td>7.7 ± 0.68</td>
<td>45.3 ± 7.03</td>
<td></td>
</tr>
<tr>
<td>WT/CRYGC</td>
<td>1.07</td>
<td>1.08</td>
<td>1.07</td>
<td>1.12</td>
<td></td>
</tr>
<tr>
<td>CRYGC mRNA/Cry mRNA</td>
<td>1.24</td>
<td>0.85</td>
<td>5.18</td>
<td>0.88</td>
<td></td>
</tr>
</tbody>
</table>

WT, wild-type.
severity of the cataracts among transgenic mice. This variability existed even among mice having the same founder and thus identical insertion sites for the transgene, and presumably similar expression levels. In spite of the high levels of expression of the transgene both before and after birth, lens defects were detected in transgenic mice beginning at 3 weeks of age, and as late as 6 weeks in some mice. Similarly, in some mice expression of the transgene caused degeneration of cells over a large part of the lens accompanied by large protein-filled lacunae and even rupture of the lens, while in others the degeneration was milder with fewer lacunae and with cellular degeneration limited to the bow and superficial nuclear region. This variable severity and onset is similar to the human phenotype, with some family members being completely asymptomatic and having mild or no opacity on slit lamp examination, while others presented with severe congenital cataracts. The variable severity of the phenotype in this inbred strain of transgenic mice raised in a controlled environment cannot be explained by modifying genes or environmental influences but suggests that a stochastic process might be responsible for the phenotypic variability. One attractive possibility is that it might relate to variable efficiency of nonsense-mediated decay of the mutant.

These results also confirm the high activity of the chicken βB1-crystallin promoter in transgenic mice as previously reported. The mRNA from the pcBB2CRYGC5bpd construct is expressed in the lens at levels roughly similar to the endogenous β-crystallins and is sufficiently well translated to cause cataracts. This is in contrast to transgenic mice generated previously using the 425 bp αA-crystallin promoter with an SV40 t-antigen splice site, which did not develop cataracts (data not shown). While the protein product is easily seen on acrylamide gels, the levels appear to be somewhat lower than those of endogenous crystallins, although they were not estimated precisely. This might be due to increased turnover related to the expected instability of the mutant protein relative to endogenous γ-crystallins.

The highly vacuolated and degenerative lens fiber cells and abnormal lens architecture in CRYGC5bpd mouse lenses could be explained by a toxic effect of the CRYGC5bpd protein. We know different β-crystallin mutants may show different unique phenotypes, but the mutant proteins often form large aggregates, which then cause light scattering in the lens with preservation of normal lens morphology. However, the pathogenesis of the cataracts in CRYGC5bpd transgenic mice is not due primarily to light scattering by protein precipitates. Rather the protein appears to be toxic to the cells of the lens as demonstrated by bladder cell formation and vacuolization on histology and ultrastructure. The degeneration begins in the equatorial epithelia and reaches a maximum in the cortical fiber cells, consistent with the activity of the βB1-crystallin promoter. This process eventually culminates in severe disruption of the lens architecture with the formation of large lacunae containing clumps of proteinaceous debris, particularly in the bow region. In most cases epithelial cell changes overlap severely affected areas of the nucleus, suggesting the possibility of a secondary effect. An interesting alternative is that expression of the mutant γ-crystallin might interfere with fiber cell elongation and development, with secondary degeneration of the aberrant cells, although there are no data to support this possibility over a direct toxic effect.

The reduction in mRNA levels of endogenous β-crystallins initially might suggest a regulatory effect of the CRYGC5bpd protein. However, the reduction of mRNA level of endogenous γβ-crystallins in CRYGC5bpd transgenic mice also could be explained by the toxic effect of the CRYGC5bpd protein. Degeneration of cortical and central fiber cells would exert an extreme effect on mRNA stability, causing the remarkable decrease of mRNA levels of γC-, γβ-, βB2-, and βB3-crystallin seen in the CRYGC5bpd lenses. However, levels of these mRNAs are similar in the lenses of mice transgenic for CRYGC and WT mice (Table 3; Table 4), which argues against feedback of the γ-crystallin protein or mRNA on endogenous crystallin transcription. In lenses transgenic for WT CRYGC the levels of the transgenic mRNAs are similar to those of the endogenous β- and γ-crystallins (Table 4), although the human and mouse γ-crystallin could not be distinguished on gel electrophoresis. Finally, the γ-crystallins tend to be expressed more specifically in the central embryonic and fetal nuclear fiber cells than the β-crystallins, which are also expressed in the equatorial epithelium and cortical fiber cells, and this could explain their greater decrease in CRYGC5bpd mouse lenses.

The most obvious early effect of CRYGC5bpd overexpression is variable vacuolization of lens fiber cells, followed by degeneration of the cortical fiber cells (bladder cell formation) resulting in large lacunae with proteinaceous debris, suggesting a toxic effect of the mutant protein on lens fiber cells. In contrast, transfection of HeLa cells with recombinant CRYGC and CRYGC5bpd after a 48-hour induction with Dox showed

**FIGURE 5.** Fluorescence confocal microscopic pictures. The red fluorescence in the first column is Alexa 555 Fluor from the secondary antibody, whereas the nuclei of cells were counterstained with DAPI and are seen blue in second column. The third column shows merged images of both stains. The top two rows show the Tet-on advanced HeLa cells transfected with CRYGC5bpd. γC-crystallins were expressed by adding Dox in (A–C). (D–F) show the transfected cells growing without Dox. The bottom two rows show the WT CRYGC transfectants. Dox-induced expression (G–I) and without Dox γC-crystallins were not expressed (J–L). There is no significant difference between WT and mutant crystallins, although the levels of WT CRYGC appear to be somewhat higher than those of CRYGC5bpd in these experiments.
no obvious deleterious effect on the cells, and both WT and CRYGC5bpd γC-crystallin were uniformly distributed in their cytosol but not their nuclei. While we do not have a definitive explanation, this might represent either greater sensitivity of the lens cells to the mutant crystallin or a mitigating dilutional effect of cell growth in the HeLa cells. Taken together, these data suggest that the CRYGC5bpd crystallin mutant protein causes cataract through a toxic effect on lens fiber cells, thus disrupting the cellular architecture and order rather than by aggregating and precipitating within otherwise normal fiber cells and then scattering light, although a combination of both mechanisms cannot be excluded.

In summary, these results demonstrated that abnormal lens phenotype was developed by overexpression of mutant CRYGC5bpd on the background of WT endogenous mouse crystallins, including γC-crystallin. This confirms the association of this mutation with cataracts in humans. The mechanism of cataract formation appears to be a toxic effect of the CRYGC5bpd crystallin mutant protein affecting the growth and organization of lens fiber cells and eventually causing cellular degeneration, rather than primarily causing cataract by aggregating and precipitating and then scattering light.

References