Zinc is likely to be a promising compound for the treatment of age-related macular degeneration (AMD), as demonstrated by previous clinical studies. The Age-Related Eye Disease Study (AREDS) demonstrated that high doses of oral zinc supplementation, with or without concomitant antioxidants, significantly reduced the risk for progression from intermediate to advanced AMD. An earlier clinical trial with a smaller number of patients showed that oral zinc supplementation decreased the risk for visual loss in patients with AMD.

It is well known that zinc homeostasis is essential for cellular functions. Zinc, with the atomic number 30, is the 23rd most abundant element on earth. It is an essential element, necessary for sustaining all life, and it is estimated that 3000 proteins in the human body contain zinc prosthetic groups. Zinc plays a crucial role in many catalytic activities and is an activator for enzymes such as glutathione synthase, superoxide dismutase, membrane dipeptidase, and heme oxygenase. It also stabilizes the conformation of certain zinc-dependent structural domains, such as zinc fingers and zinc clusters, that are commonly found in transcriptional regulatory proteins.

In mammalian cells, two families of zinc transporters are encoded by the solute-linked carrier (SLC) genes. The ZnT proteins (SLC30A) mediate the zinc efflux, and the Zip (SLC39A) proteins mediate the uptake and the vesicular transport of zinc. At least 14 Zip and 9 ZnT proteins have been identified in humans. The complexity, and perhaps redundancy, of the transporters suggest tissue- and cell type-specific mechanisms controlling the local zinc concentration. Zip1 (SLC39A1), Zip2 (SLC39A2), and Zip4 (SLC39A4) are the major importer carriers of zinc, whereas ZnT1 mainly controls the zinc export. Expression patterns and functions of these transporters have not been well characterized in the retina or the RPE.

Oxidative damage to the RPE has been suggested to play a critical role in the pathogenesis of AMD. Results from our previous study showed that zinc can increase GSH synthesis through the upregulation of glutamate-cysteine ligase (GCL) expression in an antioxidant response element (ARE)-dependent pathway in cultured RPE cells. In the present study, we characterized the expression and function of the zinc transporter protein Zip2. Results indicated that zinc is transported into ARPE cells by an active transport system. Overexpression of Zip2 resulted in increased zinc uptake and consequently decreased the risk for visual loss in patients with AMD.
FIGURE 1. Flow cytometry measurement of intracellular zinc concentration in cultured ARPE-19 cells. Cells were exposed to either ZnCl₂ (A) or TPEN (B) at indicated concentrations. A zinc-sensitive fluorescence probe was used to stain the cells, followed by flow cytometry. Data are representative of five separate experiments. MF, geometric mean fluorescence.

TABLE 1. Sequence of Primers Used for RT-PCR Amplification of Human Zip Genes in ARPE-19 Cells

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Primer Sequence</th>
<th>Length of PCR Product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zip1</td>
<td>Forward: 5′-CCAGGAACCTAAGAGTGCAAGGCTC-3′&lt;br&gt;Reverse: 5′-ATGGCCAGTGAACTCTCTGGAG-3′</td>
<td>194</td>
</tr>
<tr>
<td>Zip2</td>
<td>Forward: 5′-TGGGCTTTCTCCATCTGTCTCCTCA-3′&lt;br&gt;Reverse: 5′-CTTGAAAAAGATCTGCTCTCTCGA-3′</td>
<td>153</td>
</tr>
<tr>
<td>Zip3</td>
<td>Forward: 5′-CTTTTTCTCCAGGCTGCTCTGTA-3′&lt;br&gt;Reverse: 5′-CGAGGAACGCTGTCAGAGAAG-3′</td>
<td>187</td>
</tr>
<tr>
<td>Zip4</td>
<td>Forward: 5′-GAGAAGCTTCGTCGATCTGSGCTTCA-3′&lt;br&gt;Reverse: 5′-CATATGAGGTGACCTACATG-3′</td>
<td>249</td>
</tr>
<tr>
<td>Zip5</td>
<td>Forward: 5′-ACCTGAATGAGATTCTCTCAGA-3′&lt;br&gt;Reverse: 5′-ACTCTGAAAGGCAGAGATAGTAG-3′</td>
<td>191</td>
</tr>
<tr>
<td>Zip6</td>
<td>Forward: 5′-TTTACAGCTTTCTGGCAGAGATAG-3′&lt;br&gt;Reverse: 5′-CTTGAAAAAGATCTGCTCTCTCGA-3′</td>
<td>204</td>
</tr>
<tr>
<td>Zip7</td>
<td>Forward: 5′-TGGGCTTTCTCCATCTGTCTCCTCA-3′&lt;br&gt;Reverse: 5′-CTTGAAAAAGATCTGCTCTCTCGA-3′</td>
<td>229</td>
</tr>
<tr>
<td>Zip8</td>
<td>Forward: 5′-CCAGGTTCTTATCTCTCTGTA-3′&lt;br&gt;Reverse: 5′-ACCTGAATGAGATTCTCTCAGA-3′</td>
<td>153</td>
</tr>
<tr>
<td>Zip9</td>
<td>Forward: 5′-GAGAAGCTTCGTCGATCTGSGCTTCA-3′&lt;br&gt;Reverse: 5′-CATATGAGGTGACCTACATG-3′</td>
<td>155</td>
</tr>
<tr>
<td>Zip10</td>
<td>Forward: 5′-GTGAGGACATTTCTCAGAGAGG-3′&lt;br&gt;Reverse: 5′-CTTGAAAAAGATCTGCTCTCTCGA-3′</td>
<td>219</td>
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<tr>
<td>Zip11</td>
<td>Forward: 5′-TGGGCTTTCTCCATCTGTCTCCTCA-3′&lt;br&gt;Reverse: 5′-CTTGAAAAAGATCTGCTCTCTCGA-3′</td>
<td>164</td>
</tr>
<tr>
<td>Zip12</td>
<td>Forward: 5′-GTTGAAGAATAGGCTTCGTA-3′&lt;br&gt;Reverse: 5′-TTTACAGCTTTCTGGCAGAGATAG-3′</td>
<td>159</td>
</tr>
<tr>
<td>Zip13</td>
<td>Forward: 5′-GTGAGGACATTTCTCAGAGAGG-3′&lt;br&gt;Reverse: 5′-GCTGATAATGATGAGCTCGAG-3′</td>
<td>203</td>
</tr>
<tr>
<td>Zip14</td>
<td>Forward: 5′-GTTGAAGAATAGGCTTCGTA-3′&lt;br&gt;Reverse: 5′-TTTACAGCTTTCTGGCAGAGATAG-3′</td>
<td>221</td>
</tr>
</tbody>
</table>
mixture was then diluted fivefold using nuclease-free water. Primer sequences used to amplify 14 Zip family genes are listed in Table 1. Each primer had a melting temperature (Tm) of approximately 60°C. PCR products were between 150 and 250 base pairs and were resolved by 2% agarose gel electrophoresis.

Quantitative RT-PCR reaction was performed as described, using primers specific for the GCL catalytic subunit (Hs00155249_m1), GCL modulatory subunit (Hs00157694_m1), or 18S ribosomal RNA (rRNA, 4308529; Applied Biosystems). Average threshold cycle ($C_\text{t}$) values were used to determine the relative difference between control and treated groups and were normalized to 18S rRNA.

Cloning and Overexpression of Human Zip2 Gene in the RPE

A full-length, open-reading frame of the human Zip2 gene was amplified by RT-PCR using RNA isolated from ARPE-19 cells. Primers used were 5'-CTACACCCAGAGATGGAGCA-3' (forward) and 5'-CATCAGAAAAGCCAGGATCT-3' (reverse). The PCR product was gel-purified and cloned into vector (TOPO PCR2.1; Invitrogen, Carlsbad, CA). After sequence verification by the Vanderbilt DNA Sequencing Core Facility, Zip2 was subcloned into retroviral vector (pQCXIP; Clontech, Mountain View, CA). Retrovirus encoding Zip2 was then produced from 293FT viral packaging cells and used to infect the ARPE-19 cells. After selection with puromycin at 1 μg/mL for 3 days, surviving cells were pooled for further functional analyses.

Measurement of Intracellular Zinc Concentration by Flow Cytometry

A zinc-sensitive fluorescence probe (FluoZin-3; Molecular Probes, Eugene, OR) was used to assess the relative cellular zinc concentration. It can measure changes of zinc concentrations in the range of 1 to 100 nM and has minimal reactivity with other divalent metal ions. RPE cells were washed twice with phosphate-buffered saline (PBS) and detached from the culture plate by gentle trypsinization. Cells were collected by centrifugation, washed with PBS, and stained (FluoZin-3, 15 nM; Molecular Probes) in PBS for 30 minutes at 37°C. After two washes with PBS, samples were analyzed by flow cytometry (FACScan with CellQuest software; Becton Dickinson, San Jose, CA).

HPLC Measure of Cellular GSH Level

ARPE cells were extracted with perchloric acid solution (5% vol/vol) containing 0.2 M boric acid. Acid-soluble extracts were derivatized with iodoacetic acid and dansyl chloride (Sigma). Thiol metabolites in the samples were analyzed with an HPLC system (model 2695 and YMC Pack NH2 Amino column; Waters, Milford, MA).

Measurement of Nrf2 Activity by Dual Luciferase Reporter Assay

An ARE reporter plasmid was cotransfected with pRL-CMV vector (Promega) into ARPE-19 cells using transfection reagent (Fugene 6; Roche, Branchburg, NJ). Detailed plasmid map and transfection procedures of the reporter construct have been described. Eight hours after transfection, cells were treated with either zinc or sulforaphane for another 16 hours. Luciferase activities were measured by using the dual luciferase assay kit (Promega) in a luminometer (FB12; Zylux, Pforzheim, Germany).

**Figure 2.** Regulation of Zip2 expression by zinc. (A) Measurement of the expression of zip family genes by RT-PCR. The sequence of the primers is listed in Table 1. (B) Negative control reaction for the RT-PCR. RNA was amplified without reverse transcription. (C) Cloning of the full-length coding sequence of the Zip2 gene using RNA isolated from ARPE-19 cells. (D, E) Real-Time RT-PCR measurement of Zip2 levels in RPE cells exposed to zinc and TPEN, respectively. Average threshold values ($C_\text{t}$) were used to quantify Zip2 expression and were normalized to the 18s rRNA. Data presented are the average of three separate experiments (mean ± SEM). *P < 0.05; significantly different from control untreated cells; one-way ANOVA and Dunnett post hoc test.
RESULTS

Regulation of Zip2 Expression by Zinc

To determine whether in vitro cultured RPE cells retain the ability to transport zinc, we incubated the ARPE-19 cells in media with different concentrations of zinc for 16 hours and measured the relative intracellular zinc content with a zinc-sensitive fluorescence probe (FluoZin-3; Molecular Probes; Fig. 1A). This probe is an N-iminodiacetate chelator designed for the detection of relative zinc concentrations within 1 to 100 nM, with minimal interference with other divalent metal ions. Results from flow cytometry measurements (Fig. 1A) showed that the fluorescence (FluoZin-3; Molecular Probes) increased in cells exposed to 50 and 100 μM zinc, indicating increased uptake of zinc when its availability is higher.

Next we used a metal chelator, N,N,N′,N′-tetakis-(2-pyridylmethyl)-ethylenediamine (TPEN), to decrease the zinc availability in the culture medium. Results (Fig. 1B) showed that after 4-hour incubation, the intracellular zinc concentration decreased. Thus, cultured human RPE cells can transport zinc, and the cellular zinc concentration is controlled by availability.

The uptake and export of zinc are mediated through the Zip and ZnT proteins, respectively. To characterize the expression of Zip family protein in the RPE, we examined the RNA levels of various zip genes with RT-PCR using gene-specific primers. Results (Fig. 2A) showed that ARPE-19 cells expressed most of the Zip genes except Zip4 and Zip12. Zip1, Zip2, and Zip4 are the major plasma membrane transporter proteins responsible for zinc uptake. Although Zip1 is ubiquitously expressed, the expression of Zip2 was low and was limited to certain human tissues, such as prostate and uterus. To further characterize the functions of Zip2 in the RPE, we cloned a full-length, open-reading frame of the Zip2 gene from ARPE-19 cells (Fig. 2C). The coding sequence was identical with the
the Nrf2/ARE-dependent antioxidant system. Thus, we hypothesized that overexpression of Zip2 would increase the uptake of zinc into the RPE cells and would result in upregulation of the antioxidant functions. To that end, Zip2 was overexpressed in ARPE-19 cells with a retroviral vector. Because no commercial antibody is available, we used RT-PCR analysis to confirm the overexpression (Fig. 3A). Compared with data presented in Figure 2A, which used 36 PCR cycles, only 25 PCR cycles were used for the experiment presented in Figure 3A. Consequently, no visible band was shown in either parental or vector-transduced ARPE cells. To evaluate the function of Zip2, we measured the cellular zinc concentration in parental, vector-transduced, and Zip2-overexpressing cells (Fig. 3B). Compared with vector and ARPE cells, a higher percentage of Zip2-overexpressing cells showed increased fluorescence (FluoZin-3; Promega) with or without 100 μM zinc treatment.

To determine whether overexpression of the Zip2 transporter had any effect on the ARE-Nrf2 pathway in the RPE, we used a dual luciferase reporter assay to compare the activity of Nrf2 in Zip2-overexpressing cells and vector-transduced ARPE cells. Cells with increased Zip2 showed a significantly higher level of Nrf2 activity, and the difference became more apparent when exposed to sulforaphane (Fig. 4A). The latter is an aglycone breakdown product of the glucosinolate glucoraphanin and has been shown to be a potent inducer of phase 2 detoxification enzymes through the Nrf2-ARE pathway.

A number of genes function downstream of Nrf2 and encode proteins involved in the antioxidant system, including the catalytic and modulatory subunits of the γ-glutamyl cysteine ligase (GCLC and GCLM, respectively). Quantitative RT-PCR analyses showed that both GCLC and GCLM were increased with and without zinc treatment in Zip2-overexpressing cells compared with vector-transduced ARPE cells (Figs. 4B, 4C). It has been established that GCL is the rate-limiting enzyme in GSH synthesis. After treatment with 15 to 100 μM zinc for 16 hours, the intracellular GSH content was measured by HPLC in ARPE cells transduced with Zip2 or vector. Results (Fig. 5) showed that cells with increased Zip2 had a significantly higher GSH level when exposed to 50 and 100 μM Zn. The same amount of zinc was insufficient to increase GSH in vector-transduced ARPE cells. Thus, the increased uptake of zinc leads to upregulated Nrf2-dependent antioxidant system in the RPE.

**DISCUSSION**

Despite the fact that AMD is the leading cause of blindness in patients older than 60 years in the United States, no uniformly accepted treatment leads to significant recovery of visual func-

**Zip2-Mediated Zinc Uptake and Antioxidant Functions in the RPE**

The AREDS results suggested that zinc is effective in protecting the retina and the RPE from age-related pathologic conditions. Our previous study showed that this protection might involve the Nrf2/ARE-dependent antioxidant system. Thus, we hypothesized that overexpression of Zip2 would increase the uptake of zinc into the RPE cells and would result in upregulation of the antioxidant functions. To that end, Zip2 was overexpressed in ARPE-19 cells with a retroviral vector. Because no commercial antibody is available, we used RT-PCR analysis to confirm the overexpression (Fig. 3A). Compared with data presented in Figure 2A, which used 36 PCR cycles, only 25 PCR cycles were used for the experiment presented in Figure 3A. Consequently, no visible band was shown in either parental or vector-transduced ARPE cells. To evaluate the function of Zip2, we measured the cellular zinc concentration in parental, vector-transduced, and Zip2-overexpressing cells (Fig. 3B). Compared with vector and ARPE cells, a higher percentage of Zip2-overexpressing cells showed increased fluorescence (FluoZin-3; Promega) with or without 100 μM zinc treatment.

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**DISCUSSION**

Despite the fact that AMD is the leading cause of blindness in patients older than 60 years in the United States, no uniformly accepted treatment leads to significant recovery of visual func-
tion. Developing prevention strategies for AMD has assumed great importance. It has been established that zinc intake, with or without other supplements, can reduce the progression of intermediate to advanced AMD. However, the safety margin of a heavy metal compound such as zinc is always a serious consideration.

The pharmacokinetics of supplemented zinc compound is determined by the tissue distribution and activity of the zinc transporter proteins ZnT and Zip. Results from our present study suggest that Zip2 is a plasma membrane zinc transporter expressed in cultured RPE cells. Our results indicate that the overexpression of Zip2 led to an increased population of cells with higher intracellular zinc (Fig. 3B). Moreover, when exposed to 100 μM ZnCl2, both the percentage of cells with higher zinc content and the relative zinc content increased markedly (Fig. 3B). Thus, Zip2 is functioning in the RPE and regulates the uptake of zinc.

We have reported that compounds that induce phase 2 detoxification enzymes can protect the RPE from oxidative injury. Results from our recent study further confirmed that zinc is one such compound that can activate the Nrf2-ARE pathway and increase GSH synthesis in the RPE. Cells overexpressing Zip2 had higher basal levels of Nrf2 (Fig. 4A) and higher expression of GCL, the rate-limiting enzyme controlling GSH synthesis (Figs. 4B, 4C). More important, these cells showed more robust response to zinc and sulforaphane treatment (Fig. 4). At 50 and 100 μM, ZnCl2 was not effective in activating Nrf2 in vector-transduced cells, but cells with increased Zip2 responded well to the same concentrations of zinc and showed increased GSH synthesis (Fig. 5). In cultured RPE cells, zinc is toxic at concentrations higher than 150 μM. Thus, by increasing Zip2 expression, the amount of zinc required to activate Nrf2 was decreased by twofold to threefold, and, consequently, the safety margin could be greatly improved.

The expression of Zip2 is altered in response to the increase in or depletion of extracellular zinc levels. Zinc treatment upregulated Zip2, whereas TPEN treatment downregulated it (Fig. 1). Transcriptional regulation of Zip2 indicates that its expression can be modulated in the RPE by interventional strategies. The human Zip2 gene is located on chromosome 14, and its transcriptional regulation has not been well characterized. How the transcription factors function in the context of varying zinc availability remains to be determined.

One limitation of the study is that it was performed in cultured RPE cells. Any cell culture system has limitations, and the results from in vitro studies may not fully reflect the in vivo process of zinc transport in the retina. It will be interesting to measure age-related changes of the zinc transporter proteins in the retina and compare the local responses to zinc supplementation as a function of age. It is possible that in AMD patients, dietary availability of zinc may not be limited; rather, age-related changes to the transporters of zinc in the retina could have occurred. The resultant decrease in tissue concentration of zinc in the retina could compromise the antioxidant activities and may contribute to the degenerative process.

In summary, we have characterized the functions of a major Zn transporter protein in the RPE. Increased Zip2 potentiated the Nrf2-dependent antioxidant response. In addition to direct supplementation of zinc, modulating the expression of the Zn transporter proteins could have potential implications in slowing the process of retinal degeneration.

References