Engineered Zinc Finger Nuclease–Mediated Homologous Recombination of the Human Rhodopsin Gene

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PURPOSE. Novel zinc finger nucleases (ZFNs) were designed to target the human rhodopsin gene and induce homologous recombination of a donor DNA fragment.

METHODS. Three-finger zinc finger nucleases were designed based on previously published guidelines. To assay for ZFN specificity, the authors generated human embryonic retinoblast cell lines stably expressing a Pro23His rhodopsin, the most common mutation associated with autosomal dominant retinitis pigmentosa in North America. They report quantification of these rhodopsin-specific ZFNs to induce a targeted double-strand break in the human genome, demonstrate their ability to induce homologous recombination of a donor DNA fragment, and report the quantification of the frequency of ZFN-mediated homologous recombination.

RESULTS. Compared with endogenous homologous recombination, the authors observed a 12-fold increase in homologous recombination and an absolute frequency of ZFN-directed homologous recombination as high as 17% in the human rhodopsin gene.

CONCLUSIONS. ZFNs are chimeric proteins with significant potential for the treatment of inherited diseases. In this study, the authors report the design of novel ZFNs targeting the human rhodopsin gene. These ZFNs may be useful for the treatment of retinal diseases such as retinitis pigmentosa, one of the most common causes of inherited blindness in the developed world. Herein, they also report on several aspects of donor fragment design and in vitro conditions that facilitate ZFN-mediated homologous recombination. (*Invest Ophthalmol Vis Sci.* 2010; 51:6374–6380) DOI:10.1167/iops.10-5781

Zinc finger nucleases (ZFNs) are chimeric, engineered transcription factors fused to the nonspecific endonuclease domain of restriction enzymes such as *FokI*. These recombinant proteins can be designed to recognize virtually any DNA sequence in the human genome and to stimulate homologous recombination by creating a targeted double-strand break (DSB).^{1,2} This strategy can be used to permanently repair a pathogenic nucleotide sequence and thus has significant potential for the treatment of genetic diseases.

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ZFNs bind DNA by virtue of their zinc finger transcription factor DNA-binding domain and induce a DSB through their nuclease domain (Fig. 1A). To induce a DSB, two *FokI* nuclease domains must dimerize in close proximity. Therefore, two separate ZFNs are required to generate a DSB. Given that each ZFN may contain, for example, three “fingers” that recognize a total of nine nucleotides, a pair of ZFNs can recognize 18 base pairs. An 18-nucleotide sequence is sufficient to promote a unique binding event and, hence, a single cleavage within the human genome (3×10^9 bp) because there are 4^{18} (6.9×10^{10}) unique 18-mers.³

Although homologous recombination naturally occurs at a rate of 10^{-6} , a DSB is expected to increase the frequency of specific homologous recombination events by up to 1000-fold or more, thereby approaching recombination rates with therapeutic potential.^{4–8} This approach has been invoked at an endogenous locus in the human genome, resulting in permanent modification of up to 20% of chromatids in vitro.¹ Although mature neurons generally do not divide, there is evidence that double-strand break repair does occur by homologous recombination.^{9–11}

Retinitis pigmentosa (RP) is a group of retinal degenerative diseases that are characterized primarily by the loss of rod photoreceptor cells. RP is the most common cause of genetic blindness and affects approximately 1 in 3000 people in the United States.¹² Patients with RP experience night blindness and progressive loss of peripheral visual fields and eventually lose central vision as cone-cell viability is compromised by rod-cell death. There is no cure for RP. More than 200 different mutations in the human rhodopsin gene are known to be associated with RP.^{13–16} Although some of the mutations in the rhodopsin gene cause recessive RP and congenital stationary night blindness, most are implicated in autosomal-dominant RP.

In this study, a pair of ZFNs was designed to bind and cleave a sequence uniquely found in the human rhodopsin gene. We demonstrate that the engineered ZFNs induce a DSB at the rhodopsin locus and stimulate homologous recombination when delivered concurrently with an ectopic donor DNA fragment homologous to the human rhodopsin gene sequence. We go on to further quantify the relative frequency of homologous recombination based on several in vitro conditions. Delivery of these rhodopsin-targeting zinc finger nucleases and an appropriate donor fragment to photoreceptors, either separately in an AAV or concomitantly in a gutted adenovirus vector, would enable potential clinical application for RP patients with mutations in the human rhodopsin gene.

METHODS

Rhodopsin ZFN Design

Two ZFNs (RhoFA and RhoFB) were designed based on previously published guidelines.^{17–19} The human rhodopsin gene was scanned for a potential ZFN target of the form (NNC)₃N₆(GNN)₃. One such sequence, 5'-GGC CGC TGC CCA GCA GCA GGA GTC-3', is located at

FIGURE 1. ZFN design. (A) Schematic illustration of ZFN pair binding to DNA and *FokI* domains. The *FokI* cleavage domain is fused to the DNA-binding domain via (Gly₃Ser)₄ linker. An SV40 nuclear localization signal (SV40 NLS) located at 5' of ZFN sequence. (B) A pair of ZFNs was designed to target an 18-nucleotide sequence within the human rhodopsin gene. The targeted rhodopsin sequence contains nine nucleotides specific for each zinc finger binding domain (18 bp total), interspaced by six nonspecific nucleotides to permit *FokI* steric interaction and cleavage. (C) Schematic of ZFN modular design depicting amino acid sequences for NLS, H1, Sp1, and zinc fingers F1, F2, and F3 for rhodopsin ZFNs RhoFA and RhoFB. (D) Rhodopsin and control ZFN plasmid constructs. Sequences were cloned in front of a CMV enhancer and chicken β -actin promoter.

the 5' end of the fourth exon of the human rhodopsin gene (Fig. 1B). Each ZFN was engineered to constitute the sequence [NLS-Sp1-F1-H1-Sp1-F2-H1-Sp1-F3-H1-FokI], where NLS is the SV40 nuclear localization signal MPKKKRKV; Sp1 and H1 are the consensus Sp1 transcription factor backbone module TGEKPYKCPEGKSF5 and HQRTH, respectively; and F1, F2, and F3 are the specific zinc finger modules composed of DRSALAR (F1), QSGHLQR (F2), and QSGDLTR (F3) for RhoFA and ERGTLAR (F1), RSDDLQR (F2), and QSGDLTR (F3) for RhoFB, respectively (Fig. 1C). Each ZFN also contained a *FokI* nuclease domain.

The rhodopsin ZFNs were designed to exclusively bind the human rhodopsin gene, whose uniqueness in the human genome was confirmed by a BLAST search.²⁰ The rhodopsin ZFNs were designed to target the human rhodopsin genomic sequence and can also target the human rhodopsin cDNA. The zinc finger DNA-binding domain(s) were fused to the *FokI* restriction endonuclease domain through a (Gly₃Ser)₄ linker. As a negative control, a pair of ZFNs targeting the mouse tyrosinase gene (control ZFN) were generated based on previous studies.¹⁹ These control ZFNs were not expected to induce a DSB in the human genome, which was confirmed by a BLAST search.²⁰ All ZFN DNA sequences were synthesized by DNA2.0 (Menlo Park, CA) and were cloned into vector plasmid(s) regulated by a chicken β -actin promoter and a cytomegalovirus (CMV) enhancer (Fig. 1D).

ZFN Cutting qRT-PCR

The integrity of the rhodopsin gene was measured by quantitative real-time PCR (qRT-PCR) using primers flanking the hypothetical cut-site (forward primer, 5'-GCAGTGCTCGTGTGGAATCG-3'; reverse primer, 5'-ATGCGGGTGACCTCCTTCTC-3'; probe, 5'-TACCTCCTTGACGGTGAAGACG-3'). This qRT-PCR reaction was designed to produce a 326-nt amplicon and to discriminate between intact and disrupted human endogenous rhodopsin gene sequences. Six micrograms of rhodopsin or control ZFNs were transiently transfected into 2.1×10^6 human embryonic retinoblast (HER) cells²¹ at 90% confluence in media (Optimem; Lipofectamine; Invitrogen, Carlsbad, CA) and were

maintained in serum-free media. All experiments were performed in quadruplicate and were normalized to GSTT1 internal control (Hs00010004_cn; Applied Biosystems, Foster City, CA) using a real-time PCR detection system (iQ5 Multicolor; Bio-Rad, Hercules, CA).

P23H HER Stable Cell Line

The Pro23His mutation was introduced into human rhodopsin cDNA by PCR site-directed mutagenesis (CCC > CAC) using primers RhoMF (5'-CGGGACTTTCCAAATGTCGTAAC-3') and P23HR (5'-TACTGTGGGTACTCGAAGTGGCTGCGTACCACACCCGT-3'). Several P23H stable cell lines were generated by transfecting *EcoRV*-linearized (New England Biolabs, Ipswich, MA) plasmid containing the P23H rhodopsin cDNA regulated by a CMV promoter²² and selected using G418 (Geneticin; Gibco, Invitrogen). Western blot analyses from the resultant HER cell lines were used to confirm the expression of human rhodopsin cDNA using anti-rhodopsin 1D4 (courtesy of Robert S. Molday, University of British Columbia) and anti- α V integrin (catalog no. 611012; BD Biosciences).

Rhodopsin mRNA qRT-PCR

Six micrograms of rhodopsin or control ZFNs were transiently transfected into 2.1×10^6 HER cells at 90% confluence in media (Optimem; Lipofectamine, Invitrogen) and were maintained in serum-free media for 72 hours. Cells were treated with 1.0 mL RNA (STAT-60; Tel-Test, Inc., Friendswood, TX) and were incubated at room temperature for 5 minutes. Total mRNA was extracted and purified using standard phenol-chloroform and ethanol precipitation procedures. Six micrograms of RNA was treated with DNase (Turbo; Ambion, Foster City, CA) for 1 hour at 37°C and DNase inactivation reagent for 5 minutes before qRT-PCR reaction. mRNA (3.5 μ L) was used in a standard 1-step qRT-PCR protocol measuring human rhodopsin mRNA (Hs00173452_m1 Rho; Applied Biosystems) and was normalized to human GAPDH (Applied Biosystems).

Rhodopsin Immunocytochemistry

Six micrograms of rhodopsin or control ZFNs were transiently transfected into 2.1×10^6 P23H#5 (HER) cells at 90% confluence in media (Optimem; Lipofectamine, Invitrogen) and were maintained in serum-free media for 72 hours. Cells were stained for rhodopsin using rho 1D4 antibody, and the rhodopsin-associated fluorescence signal was quantified using Image J software.

Cell Viability

Six micrograms of rhodopsin ZFNs, control ZFNs, or GFP-encoding plasmid (pCAGGFP; courtesy of Connie Cepko, Harvard University School of Medicine) were transiently transfected into 2.1×10^6 P23H#5 (HER) cells at 90% confluence in media (Optimem; Lipofectamine, Invitrogen) and were maintained in serum-free media for 72 hours. Cells were suspended in 400 μ L PBS by pipetting. One microliter of propidium iodide (Fluka; Sigma-Aldrich, St. Louis, MO) was added immediately before measurements were taken on a flowmeter (FACSCalibur; BD Biosciences).

Donor DNA Fragment

The rhodopsin donor DNA fragment was generated by digesting a plasmid containing the human genomic rhodopsin sequence (courtesy of Tiansen Li, National Eye Institute) with the *Eco*RI and *Xba*I restriction enzymes (New England Biolabs). Oligonucleotides containing the sequences 5'-GGCCGAGC TCAACAACAAGAAAGTGC-CACCACACAGAAGGCAGAGAAGGAG3' and 5'-GTGACCTCCTTC-TCTGCCTTCTGTGTGGTGGCACTTCTTGTGTGAGCTGCGGCC-TGCA3' were annealed and ligated using *Sbf*I and *Bst*EII to create pRhoXE4.4. The resultant plasmid was linearized with *Xba*I and *Eco*RI and further digested with *Eco*47III to create the "short" donor fragment where indicated.

Long-Range PCR

The forward primer (5'-AAACTTCATGGGGTGGTGAGC-3') is specific to the endogenous rhodopsin gene and is not theoretically capable of binding the donor fragment. The reverse primer (5'-TCTGTGTGGTG-GCACTTCTTG-3') is specific to the donor fragment, producing a 2.3-kb PCR amplicon only if the donor fragment is incorporated into the endogenous human rhodopsin gene. The resultant PCR product was sequenced using the reverse primer and a primer spanning the junction between the donor fragment and the endogenous rhodopsin gene (5'-CACACACACAAAACTCC-3') by the Tufts University Core DNA Facility (Boston, MA).

Allele-Specific qRT-PCR

The allele-specific probe was designed to bind exclusively to the donor DNA fragment (5'-6-FAM TGGTGGCACTTCTTGTGTGA-3' BHQ-1) (IDT, Coralville, IA). The forward primer (5'-ATGCGGGTGACCTCT-TCTC-3') binds outside the region of homology between the donor fragment and the endogenous rhodopsin sequence, and the reverse primer (5'-TGGGTGGTGTCATCTGGTAACG-3') binds within the region of homology between the donor and the endogenous rhodopsin sequence. The donor DNA fragment was digested with *Eco*47III (Promega, Madison, WI) and *Xba*I (New England Biolabs) for qRT-PCR design constraints. The resultant PCR product was sequenced using the reverse primer by the Tufts University Core DNA Facility.

Homologous Recombination Standard Curve

Donor DNA was digested with *Xba*I and *Eco*47III restriction enzymes, and DNA concentration was determined by optical density and comparison (High DNA Mass Ladder; Invitrogen). Serial dilutions from 10^{10} to 10^2 were assayed by allele-specific qRT-PCR, and Ct values were normalized to GSTT1 internal control. Rhodopsin and control ZFN sample Ct values were plotted against the homologous recombination standard curve, and the absolute percentage of homologous recombination

was calculated as homologous recombination events per total rhodopsin genes tested.

RESULTS

Rhodopsin Locus Cleavage by Zinc Finger Nucleases

To measure the integrity of the endogenous rhodopsin gene after transient transfection of the rhodopsin ZFNs into human embryonic retinoblasts, we used quantitative real-time PCR (qRT-PCR) using primers flanking the hypothetical cut-site (Fig. 2A). We confirmed the specificity of the qRT-PCR on a 1% agarose gel (Fig. 2B). At 24 hours after ZFN transfection, there was no significant ($P = 0.177$) difference at the rhodopsin locus between rhodopsin-specific and control ZFNs (Fig. 2C). However, at 48 and 72 hours after transfection, we noted a significant ($P < 0.001$) reduction in the qRT-PCR signal at the rhodopsin locus when the rhodopsin-targeting ZFNs were used compared with control ZFNs (Fig. 2C). A representative qRT-PCR at the rhodopsin locus at 72 hours after transfection is shown (Fig. 2D). This led us to conclude that a significant level of DSB induction by rhodopsin ZFNs occurred by 48 hours after transfection in vitro.

ZFN-Related Cytotoxicity

To assay for ZFN-related cytotoxicity, we examined the uptake of propidium iodide (PI) by HER cells transiently transfected with rhodopsin ZFNs, control ZFNs, or GFP-expressing plasmid DNA. Although 7.70% of untransfected HER cells were PI-positive after 72 hours, there was no significant ($P = 0.244$) difference between the HER cells transiently transfected with GFP plasmid (25.59%) and control ZFNs (21.57%) (Fig. 2E). Notably, there was a significant ($P < 0.05$) decrease in PI uptake between the GFP plasmid (25.59%) and rhodopsin ZFN (18.90%)-transfected HER cells (Fig. 2E). This led us to conclude that the rhodopsin ZFNs were not causing cytotoxicity beyond that typically observed in cells transiently transfected with control ZFNs or similar amounts of DNA.

Rhodopsin ZFN Influence on Gene Expression

To measure the ZFN-mediated impact on rhodopsin gene expression, rhodopsin mRNA levels in human embryonic retinoblast cell lines stably expressing P23H rhodopsin cDNA were measured by qRT-PCR. A higher rhodopsin expressing cell line (P23H#2) and a lower-expressing stable cell line (P23H#5) were assayed (Fig. 3A). Although there was no reduction in rhodopsin mRNA expression in the higher-expressing rhodopsin HER stable cell line (P23H#2), there was a 40% reduction in rhodopsin mRNA in the lower-expressing HER stable cell line (P23H#5) transiently transfected with the rhodopsin-specific ZFNs compared with control ZFNs (Fig. 3B).

We found a significant ($P < 0.05$) 38% reduction in rhodopsin-associated fluorescence signal when P23H#5 stable HER cells were transiently transfected with rhodopsin-specific ZFNs relative to control ZFNs (Fig. 3C). Pixel intensity was measured with Image J software (developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at <http://rsb.info.nih.gov/ij/index.html>), and efficiency of transient transfection was determined using a GFP-expressing plasmid (Fig. 3D). These observations, taken with the decrease in rhodopsin gene integrity and rhodopsin mRNA, strongly suggest that the rhodopsin ZFNs were targeting the rhodopsin gene.

Homologous Recombination of Donor DNA

To determine the quantity of donor DNA fragment necessary to detect ZFN-mediated homologous recombination, a long-range

FIGURE 2. Rhodopsin locus cleavage by ZFNs. (A) Schematic of qRT-PCR. Primers flanking the ZFN binding site were used to quantitate cleavage at the rhodopsin gene locus. (B) Agarose gel electrophoresis of the resultant 326-nt qRT-PCR amplicon. (C) Fold difference of intact rhodopsin gene at 24, 48, and 72 hours after transfection ($*P < 0.001$; $n = 8$). (D) qRT-PCR 72 hours after transient transfection of control and rhodopsin ZFN transfections. (E) PI uptake by HERs 72 hours after transient transfection of indicated plasmid DNA ($**P < 0.0001$, untransfected vs. GFP; $*P < 0.05$, GFP vs. Rho; $P = 0.2444$, control vs. Rho; $n = 12$, 25,000 cell counts per n).

PCR assay was developed in which one primer binds outside the region of homology between the donor fragment and the endogenous rhodopsin sequence and the second primer binds exclusively to the donor fragment (Fig. 4A). This assay design theoretically ensures that only homologous recombination events were capable of producing a PCR product. We used the degeneracy of the genetic code to design the donor fragment to encode the wild-type rhodopsin amino acid sequence while introducing nine novel nucleotides (Fig. 4B). As a negative control for the assay, rhodopsin ZFNs were cotransfected into HERs with a GFP-encoding plasmid in place of the donor DNA fragment; no amplicon was generated when GFP was used (Fig. 4C).

To determine the minimum amount of linear donor DNA required for detectable rhodopsin ZFN-mediated homologous recombination, we performed a dilution curve using 6 μ g ZFN plasmids with decreasing amounts of donor fragment. The minimum amount of linear donor DNA required to detect homologous recombination with rhodopsin ZFNs was 500 ng (Fig. 4C; relevant concentrations shown). No homologous recombination was observed with the same amount of donor fragment in the presence of control ZFNs (Fig. 4C). We did, however, observe a PCR product indicating homologous recombination when 5 μ g donor DNA fragment was transiently transfected in the presence of either rhodopsin ZFN or control ZFN (Fig. 4C).

Sequencing of the 2.3-kb PCR amplicon confirmed that the product generated was specific to the donor DNA fragment

and, therefore, accurately represented homologous recombination at this locus (Fig. 4D).

Quantification of ZFN-Mediated Homologous Recombination

To confirm rhodopsin ZFN-mediated homologous recombination, we designed an allele-specific qRT-PCR. Similar to the previously described long-range PCR, the forward primer was designed to bind outside the region of homology between the donor fragment and the endogenous rhodopsin sequence, and the reverse primer was designed to bind within the region of homology (Fig. 5A). For this assay to provide an efficient qRT-PCR reaction, the donor DNA fragment was shortened to 2.9 kb to address the size limitations on PCR amplicon imposed by qRT-PCR. The probe used in this assay was designed to be specific for the donor fragment (Fig. 5B), and this specificity was confirmed using plasmid rhodopsin DNA (pRhoXE4.4) as a template. Rhodopsin ZFNs transiently transfected into HER cells in the absence of donor fragment yielded no qRT-PCR amplification or fluorescence signal (Fig. 5C). When 5 μ g donor DNA fragment was transiently transfected into HER cells, a positive PCR signal was detected, reconfirming that this amount of donor fragment alone was capable of inducing homologous recombination (Fig. 5C). When the same amount of donor fragment was transiently transfected in the presence of the rhodopsin ZFNs, an increase in qRT-PCR fluorescence signal was observed, leading us to conclude that the rhodopsin ZFNs were increasing the frequency of homologous recombination.

FIGURE 3. ZFN-mediated knockdown of rhodopsin in stable P23H rhodopsin cells. (A) Western blot analysis of HER stable cell lines expressing human rhodopsin cDNA containing Pro23His mutation. α V Integrin was used as a loading control. (B) qRT-PCR of rhodopsin mRNA from P23H stable cell lines transiently transfected with rhodopsin-targeting compared with control ZFNs. (C) Quantification of rhodopsin-associated fluorescence signal (* $P < 0.05$). (D) Immunocytochemistry of GFP, control ZFN, and rhodopsin ZFN transient transfections in P23H#5 HER stable cell line.

nation (Fig. 5C). By using standard qRT-PCR calculations, this difference in PCR amplification resulted in a 7.67-fold increase in homologous recombination relative to endogenous levels of homologous recombination (Fig. 5D). Compared with a standard curve of positive control plasmid serially diluted into HER genomic DNA, this corresponded to an absolute value of 16.98% homologous recombination.

Sequencing of the 578-bp PCR amplicon confirmed that the product generated was specific to the donor DNA fragment and, therefore, accurately represented homologous recombination at this locus (data not shown).

Because we demonstrated that 5 μ g donor fragment transiently transfected without rhodopsin ZFNs was capable of inducing homologous recombination, we next examined the fold difference in homologous recombination events when transiently transfecting the minimal amount of donor fragment required to observe homologous recombination (500 ng). Under these conditions, an 11.7-fold increase in homologous recombination was observed in the cells treated with the rhodopsin versus control ZFNs (Fig. 5E).

DISCUSSION

In this study we designed a pair of ZFNs that target the human rhodopsin gene and induce DSB. To our knowledge, this is the first application of zinc finger nuclease technology to target a

retinal disease gene and to establish directed homologous recombination. We did not observe any significant increase in toxicity compared with control ZFNs or plasmid transfections and determined that 72 hours was sufficient for homologous recombination to occur in vitro. Other groups have reported some levels of ZFN-related cytotoxicity and have begun to devise methods to inhibit such effects.²³

Given that HER cells do not normally express rhodopsin (Fig. 3A), we generated several stable HER cell lines that use a CMV promoter to stably express human rhodopsin from a cDNA containing the P23H mutation. The P23H mutation is located in the first exon of the human rhodopsin gene. The

FIGURE 4. Homologous recombination of donor DNA fragment at rhodopsin locus. (A) Schematic depicting 4.4-kb homology between linear donor DNA and the human rhodopsin gene and location of primers used for long-range PCR specific to homologous recombination. The forward primer is not theoretically capable of binding the donor fragment, and the reverse primer is specific to the donor fragment, producing a 2.3-kb PCR amplicon only if the donor fragment is incorporated into the endogenous human rhodopsin gene. (B) Design of key nucleotide sequences in donor DNA fragment to which reverse primer binds. Arrows: specific nucleotides modified between donor fragment and endogenous rhodopsin sequence. (C) Long-range PCR illustrating that 5 μ g donor DNA is sufficient to induce HR without ZFNs, and 0.5 μ g donor DNA is sufficient to distinguish HR in the presence of targeted ZFNs. (D) Sequencing confirms that a 2.3-kb PCR product is generated from donor fragment (FinchTV, Geospiza). Asterisks: novel nucleotides attributed to ZFN-mediated homologous recombination.

FIGURE 5. Quantitative real-time PCR using an allele-specific probe to homologous recombination. **(A)** Schematic of allele-specific qRT-PCR. The probe binds exclusively to the donor fragment, the forward primer binds outside the region of homology between the donor fragment and endogenous rhodopsin sequence, and the reverse primer binds within the region of homology between the donor and endogenous rhodopsin sequence. **(B)** Design of allele-specific probe for homologous recombination based on novel nucleotides in donor DNA fragment. **(C)** qRT-PCR results indicate that homologous recombination is enhanced when donor fragment is transfected with ZFNs. **(D)** Rhodopsin-targeted ZFNs have a 7.67-fold increase in homologous recombination over control ZFNs when transfected with 5 μ g donor fragment. **(E)** Rhodopsin-targeted ZFNs have a 11.7-fold increase in homologous recombination over control ZFNs when transfected with 500 ng donor fragment.

design of the ZFNs allows for recognition of the human rhodopsin cDNA and the endogenous human genomic rhodopsin sequence. P23H has been documented as the most common pathologic rhodopsin mutation in the United States.²² The differences in expression between P23H#2 and P23H#5 are likely responsible for the observed differences in the effect of rhodopsin ZFNs on rhodopsin mRNA and protein levels. The decrease in rhodopsin mRNA and protein signal was observed only when cells were maintained in serum-free media (data not shown). This observation may not be unexpected given that serum starvation of cells inhibits cell cycle progression, a period during which the cell may be less likely to repair a ZFN-induced DSB.²⁴

Our results demonstrate that transfection with our rhodopsin-targeting ZFNs results in an increase in homologous recombination when supplied in concert with a donor DNA fragment with 2.9 kb of homology to the endogenous human rhodopsin sequence. The degeneracy of the genetic code was used to engineer the donor DNA fragment to contain nine nucleotide changes directly adjacent to the zinc finger DNA-binding site that would prevent repetitive cutting by the rhodopsin ZFNs once the donor fragment was incorporated into the endogenous rhodopsin locus and that could distinguish the donor from the endogenous sequence while still encoding the wild-type rhodopsin amino acid sequence (Fig. 4B).

Based on previous studies, we estimated that 5 μ g donor DNA fragment would be required to detect homologous recombination induced by ZFNs in 2.1×10^6 cells.¹ Interestingly, though we observed a PCR product indicating homologous recombination when 5 μ g donor fragment was supplied concomitantly with rhodopsin ZFNs, we also observed homologous recombination when 5 μ g donor DNA fragment was transiently transfected with control ZFNs (Fig. 4C), leading us to conclude that 5 μ g donor fragment is capable of inducing homologous recombination regardless of targeted ZFNs in HER cells. This may be a result of supersaturation of the system with ectopic donor fragment, leading to an increase in inherent rates of homologous recombination. To determine the minimum amount of linear donor DNA required for detectable ZFN-mediated homologous recombination, we performed a dilution curve with decreasing amounts of donor fragment. The minimum amount of linear donor DNA required to distinguish rhodopsin ZFN-mediated homologous recombination in our system was 500 ng. No homologous recombination was observed with the same amount of donor fragment with control ZFNs (Fig. 4C). Therefore, though 5 μ g donor fragment is sufficient to induce homologous recombination without ZFNs, 500 ng donor DNA is sufficient to distinguish HR in the presence of targeted ZFNs.

Notably, we observed homologous recombination only when a linear, not a circular, donor DNA fragment was used (data not shown). This may be attributed to single-strand annealing between the resected ends of the linear fragment and the resected ends of the DSB on the chromosome.

We designed our ZFNs to target the human rhodopsin gene with the long-term goal of repairing mutations that lead to retinitis pigmentosa. Although the amount of homologous recombination observed is modest, RP may be an ideal candidate disease for potential ZFN-mediated treatment because a relatively modest amount of photoreceptor rescue may improve vision in these patients or may delay photoreceptor degeneration.

An additional point to take into consideration is that there are more than 200 known mutations in the human rhodopsin gene that lead to various forms of RP. The approach described herein may be used for a multitude of RP patients as long as the specific mutation does not lie in the ZFN-binding site and does not significantly disrupt the homology of the donor DNA fragment. One distinct advantage of ZFNs is obviating the need to deliver episomal heterologous DNA sequences with inadequate regulatory signals.

In theory, only one donor DNA molecule will be used as a template for HR with a target allele, and the remaining donor molecules could potentially integrate into other naturally occurring or ZFN-induced off-target DSBs. Therefore, an additional critical parameter for therapeutic gene targeting approaches will be to assess the ratio of targeted versus untargeted donor integration events. Delivery of these rhodopsin-targeting zinc finger nucleases and an appropriate donor fragment to photoreceptors, either separately in an adeno-associated virus or concomitantly in a gutted adenovirus vec-

tor, would enable potential clinical application for RP patients with mutations in the human rhodopsin gene.

Although we have elucidated some of the parameters that have led to ZFN-mediated homologous recombination, others remain. How much homology between donor fragment and endogenous genomic sequence is required for ZFN-directed HR? What is the relative frequency of homologous recombination as the distance from the DSB increases? These questions and many others will have to be addressed before ZFN treatment can be used in the clinic.

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