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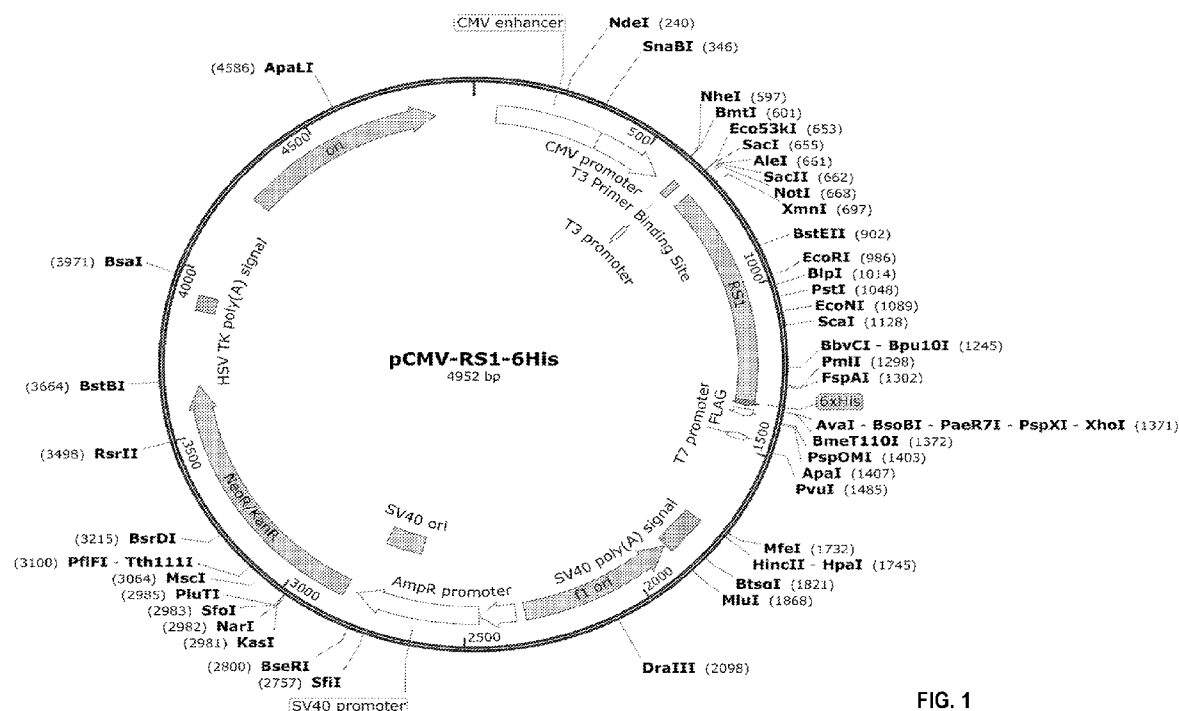


FIG. 1

(57) Abstract: Nucleic acid sequences encoding human retinoschisin 1 proteins, fragments thereof, and variants thereof, and human cell lines transformed with such nucleic acid sequences. Additionally, methods of producing and delivering these retinoschisin 1 proteins to model, study, and treat ophthalmic disorders in patients are provided.

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CELL LINES THAT PRODUCE HUMAN RETINOSCHISIN PROTEINS AND USES THEREOF

TECHNICAL FIELD

5 This invention relates generally to the fields of protein production and cell therapy.

BACKGROUND

Stable cell lines are used in a variety of research applications such as recombinant protein and antibody production, assay development, gene editing, functional studies, protein functions in unknown pathways, mechanism of action
10 elucidation, and more. Further, approximately 60–70% of all the recombinant pharmaceuticals currently on the market are produced in mammalian cells. A trend in the recombinant protein market is the use of human cells as an alternative to produce human therapeutic proteins. Human cells advantageously synthesize proteins that are similar to those naturally occurring in humans with respect to molecular structures and biochemical
15 properties, especially glycosylation patterns, which may reduce the possible immunogenic reactions caused by some recombinant proteins.

The human ARPE-19 cell line is a superior platform cell line for human protein production and cell-based protein delivery. The ARPE-19 cell line is hardy (i.e., the cell line is viable under stringent conditions, such as implantation into the intra-ocular
20 environment). ARPE-19 cells can be genetically modified to secrete proteins of therapeutic interest, and ARPE-19 cells have a relatively long life span. Furthermore, ARPE-19 cells elicit negligible host immune reaction and are non-tumorigenic.

Retinoschisin (RS1) is a 224-amino acid protein expressed and secreted from photoreceptor cells of the outer retina and bipolar cells of the inner retina as a multi-
25 subunit protein. The secreted protein associates with the surface of rod and cone photoreceptors at the level of the inner segment, outer nuclear, and outer plexiform layers and the surface of bipolar cells within the inner nuclear and inner plexiform layers of the retina. RS1 is generally believed to function as a retinal cell adhesion protein. Human retinoschisin is composed of a 23-amino acid signal sequence, a 39-amino acid Rs1
30 domain, a 157-amino acid discoidin domain, and a 5-amino acid C-terminal segment.

The dominant structural feature of the RS1 polypeptide is the 157-amino acid discoidin domain, which comprises over 75% of the processed polypeptide chain. Discoidin domains are present in a wide range of membrane and extracellular proteins. Some proteins that contain discoidin domains are Factors V and VIII involved in blood
35 coagulation, neuropilins 1 and 2, which mediate nervous system regeneration and degeneration, discoidin domain receptors implicated in cancer metastasis, and discoidin

I-like domain involved in cellular adhesion during slime mold differentiation and development. Discoidin domain containing proteins mediate a variety of functions, including cell adhesion, cell–extracellular matrix interactions, signal transduction, phagocytosis of apoptotic cells, axon guidance, angiogenesis, and blood clotting. Many of these proteins are involved in extracellular matrix or cell binding, although some bind ligands such as vascular endothelial growth factor and semaphorin. The RS1 leader sequence plays an essential role in the insertion of the nascent RS1 polypeptide chain into the endoplasmic reticulum (ER) membrane. It is subsequently cleaved in the lumen of the ER by a signal peptidase as a key step in the secretion of RS1 from cells. The Rs1 domain contains four cysteine residues. One of these residues, Cys59, forms an intermolecular disulfide bond with Cys223 of another subunit to form a disulfide-linked homo-oligomeric complex. Native RS1 has an oligomeric structure consisting of disulfide-linked dimers within a disulfide-linked homo-octameric complex.

X-linked juvenile retinoschisis (XLRS) is a neurodevelopmental retinal abnormality that manifests early in life and causes impaired acuity and a propensity for retinal detachment. The rate of retinal detachment in the XLRS population is considerably higher than in the general population (10 vs 0.01%, respectively), and the postoperative outcome is much worse. XLRS is caused by mutations in the gene encoding RS1. XLRS is characterized by structural abnormalities in normal lamination of the retinal neuronal and plexiform layers. Clinical examination shows microcysts within the macula, and schisis of the layers of the peripheral retina evident using ocular coherence tomography. The impaired retinal synaptic transmission of neural signals causes loss of dark-adapted absolute visual perception, evident on clinical electroretinogram (ERG) testing as a characteristic reduction of the b-wave response (from second-order retinal bipolar cells) relative to the photoreceptor a-wave, which frequently gives rise to an ‘electronegative’ ERG waveform. The fragile XLRS retina is also prone to disease related complications, such as vitreous hemorrhage and retinal detachment, and the condition worsens with age.

More than 150 mutations in the RS1 gene have been associated with XLRS. HEK 293 or COS-7 cells expressing RS1 mutants have been used to understand how selected mutations cause this disease. Most disease-linked missense mutations in the RS1 discoidin domain result in severe misfolding of the protein and retention in the ER. Mutations in the leader sequence prevent insertion of RS1 into the ER membrane resulting in mislocalization of these mutant polypeptides to the cytoplasm and rapid proteolytic degradation. Two disease-linked cysteine mutations (C59S and C223R) outside the discoidin domain do not significantly affect protein folding or secretion from

cells, but these mutants fail to assemble into a normal multisubunit complex suggesting that oligomerization is crucial for the functioning of RS1 as an extracellular adhesion protein.

It is important to obtain milligram amounts of RS1 protein to carry out further molecular and structural analyses. In all the expression systems tested (bacteria, yeast, baculoviral and mammalian), the RS1 protein yields are extremely low, making it difficult even to obtain microgram quantities of RS1 protein, indicating that there exists an 'expression limit'. Expression limit of secretory proteins, like RS1, is a standing challenge, not only in research labs but also in biopharmaceutical protein production. Recent studies have demonstrated that human protein secretory pathway genes are expressed in a tissue-specific pattern to match processing demands of the secretome and the functional difference between the host and parent secretion system limit protein yields (Felzi et. al., (2017) NPJ Syst Biol Appl. 3:22. doi: 10.1038/s41540-017-0021-4). For this reason, pure RS1 protein has been available only in relatively limited quantities and there is a need for a constant supply of enough RS1 protein material for a wider range of biochemical experiments.

In previous work, the inventors described a method for obtaining efficient AAV vector-mediated gene transfer to XLRs retinas without the use of subretinal injection. In that study, all layers of the retinoschisin knockout (Rs1-KO) mouse retina were efficiently transduced with AAV type 2 (AAV2) vectors when administered by simple vitreous injection (Zeng Y, et al., RS-1 Gene Delivery to an Adult Rs1h Knockout Mouse Model Restores ERG b-Wave with Reversal of the Electronegative Waveform of X-Linked Retinoschisis. *Invest Ophthalmol Vis Sci.* 2004; 45:3279-85; Kjellstrom S, et al., Retinoschisin gene therapy and natural history in the Rs1h-KO mouse: long-term rescue from retinal degeneration. *Invest Ophthalmol Vis Sci.* 2007; 48:3837-45). However, administration of AAV2 vector leads to a therapy-limiting immune response in the eye, because humans have a high preexisting immunity to AAV2.

Thus, there is a desire for methods of making native RS1 protein, and mutants and fragments thereof, for further research on RS1 function and its role in XLRs, and improved methods of delivering therapeutic human RS1 protein molecules to the eye of an individual.

SUMMARY

The present disclosure encompasses the recognition that it is desirable to engineer human cells to produce human retinoschisin proteins, fragments thereof, and variants thereof, for research and therapeutic purposes. Such proteins and the human cells that produce them can be used for the treatment and/or prevention of Retinoschisis

(e.g., X-linked Retinoschisis (XLRs)), and *in vivo* systems (or models) for eye-related diseases, disorders, and/or conditions.

Thus, this disclosure provides nucleic acid molecules comprising an open reading frame (ORF) encoding a human retinoschisin-1 (RS1) protein, or a portion thereof, or a variant thereof, that is efficiently expressed in human cells. When these nucleic acid molecules are administered to a patient having an XLRs-related disorder, the patient's visual function may be increased. The ORF may encode a fragment of the human RS1 protein, such as the N-terminal signal sequence, the Rs1 domain, the discoidin domain, and/or the C-terminal segment. The ORF may encode a protein comprising an amino acid sequence at least 85% identical to SEQ ID NO:2. The ORF may encode a protein consisting of the amino acid sequence of SEQ ID NO:2. The ORF may encode a protein comprising an amino acid sequence at least 85% identical to any one of SEQ ID NO:4, 7, 11, 15, 19, or 21. The ORF may encode a protein consisting of the amino acid sequence of any one of SEQ ID NO:4, 7, 11, 15, 19, or 21. The ORF may comprise a nucleotide sequence at least 85% identical to any one of SEQ ID NO:3, 6, 10, 14, 18, or 20. The ORF may consist of a nucleotide sequence of any one of SEQ ID NO:3, 6, 10, 14, 18, or 20. These nucleic acid molecules encoding the human RS1 protein may further comprise at least one intronic sequence from the human *RS1* gene, or a portion thereof, in particular, all or a portion of the first intron from the human *RS1* gene.

This disclosure also provides an expression cassette comprising any one or more of these nucleic acid molecules, operably linked to a promoter. The promoter in these expression cassettes may be a retinoschisin promoter or a functional fragment thereof, a rhodopsin promoter, a rhodopsin kinase promoter, a CRX promoter, or an interphotoreceptor retinoid binding protein (IRBP) promoter. The promoter in these expression cassettes may be a cytomegalovirus (CMV) promoter, a human elongation factor 1 α -subunit (EF-1 α), a chicken β -actin (CBA) or its derivative CAG, a β -glucuronidase (GUSB), a ubiquitin C (UBC), or an enhancer-less ubiquitously acting chromatin opening element (UCOE) promoter. In these expression cassettes, the nucleic acid molecule and the promoter may be operably linked to one or more viral post-transcriptional regulatory elements (PREs), such as the Hepatitis B Virus PRE or Woodchuck Hepatitis Virus PRE. These expression cassettes may further comprise a polyadenylation (polyA) signal sequence. These expression cassettes may further comprise an enhancer, such as a CMV enhancer, placed upstream or downstream (or both) of the promoter. These expression cassettes may further comprise an SV40 intron placed between the promoter and the nucleic acid molecule. These expression cassettes may further comprise one or more polynucleotide sequences encoding a 6x His tag, a c-

terminal Flag tag of SEQ ID NO:17, or a Kozak consensus sequence (GCCACC) upstream of the ATG start codon.

This disclosure also provides an expression vector comprising at least one of the nucleic acid molecules or expression cassettes described above. These expression
5 vectors may be a linear DNA molecule, a circular DNA molecule, a plasmid, a cosmid, a phage, and may comprise one or more nucleic acid sequences from a DNA or RNA virus, such as one or more nucleic acid sequences from an adeno-associated virus (an AAV vector), a cytomegalovirus (CMV), a retrovirus, an adenovirus, a herpes virus, a vaccinia virus, a poliovirus, or a Sindbis virus. These expression vectors may be a targeting vector
10 comprising a sequence that is identical or substantially identical to a sequence in a human cell directing integration of a polynucleotide sequence into a position within the genome of the cell via homologous recombination. These expression vectors may be an AAV vector comprising inverted terminal repeats (ITRs) from a virus selected from the group consisting of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9 and
15 AAV10, wherein the expression cassettes described above are flanked by the ITR sequences, and wherein at least one ITR comprises AAV sequences that allow packaging of the DNA molecule into an AAV particle. These expression vectors may be self-complementary AAV (scAAV). These expression vectors may be a Lentiviral vector.

This disclosure also provides a retinoschisin protein comprising or consisting of
20 the amino acid sequence of any one of SEQ ID NO: 4, 7, 11, 15, 19, or 21. These retinoschisin proteins may comprise an amino acid sequence at least 90% identical to any one of SEQ ID NO: 4, 7, 11, 15, 19, or 21.

This disclosure also provides compositions comprising a physiologically acceptable solution and a nucleic acid molecule, or an expression cassette or an
25 expression vector, or an RS1 protein of this disclosure. These compositions may further comprise at least one formulation component selected from the group consisting of salts, buffers, diluents, stabilizing agents, polymers, chelating agents.

This disclosure also provides human cells comprising a nucleic acid molecule, or an expression cassette, or an expression vector of this disclosure. These human cells
30 may be ARPE-19 cells. These human cells may be immortalized. A human cell line may be formed from these human cells.

This disclosure also provides implantable cell culture devices comprising a core comprising one or more human cells genetically engineered to express an RS1 protein of this disclosure and a semipermeable membrane surrounding the core. The
35 semipermeable membrane may permit diffusion of the RS1 protein therethrough. The human cells in these implantable cell culture devices may be ARPE-19 cells. The human

cells in these implantable cell culture devices may be transformed with a nucleic acid molecule, or an expression cassette, or an expression vector of this disclosure. In these implantable cell culture devices, the core may include a matrix comprising a hydrogel or extracellular matrix components disposed within the semipermeable membrane.

5 This disclosure also provides methods for treating an X-linked retinoschisis (XLRS)-related disorder by injecting an RS1 protein of this disclosure into the eye of a patient, thereby treating the disorder. This disclosure also provides related methods for treating an X-linked retinoschisis (XLRS)-related disorder by implanting a composition or a human cell or an implantable cell culture device of this disclosure into the eye of a
10 patient, thereby treating the disorder. In these treatment methods the injection or implantation may be made to the aqueous and vitreous humors of the eye, and/or the posterior and anterior chamber of the eye.

This disclosure also provides methods of making an RS1 protein by genetically engineering at least one human cell to express a retinoschisin protein comprising the
15 amino acid sequence of any one of SEQ ID NO: 4, 7, 11, 15, 19, or 21, and recovering the retinoschisin protein from the at least one human cell or a media in which the cells are suspended. In these methods, the genetically modified human cells may be ARPE-19 cells. In these methods, the genetically modified human cells may be transformed with a nucleic acid molecule, or an expression cassette, or an expression vector of this
20 disclosure.

Likewise, any of the nucleic acid molecules described herein can be used in the manufacture of one or more ARPE-19 cells that are genetically engineered to produce any of the RS1 polypeptides described herein.

This Summary is neither intended nor should it be construed as representative of
25 the full extent and scope of the present disclosure. Moreover, references made herein to "the present disclosure," or aspects thereof, should be understood to mean certain embodiments of the present invention and should not necessarily be construed as limiting all embodiments to a particular description. The present invention is set forth in various levels of detail in this Summary as well as in the attached drawings and the Detailed
30 Description and no limitation as to the scope of the present invention is intended by either the inclusion or non-inclusion of elements, components, etc. in this Summary. Additional aspects of the present invention will become more readily apparent from the Detailed Description, particularly when taken together with the figures.

BRIEF DESCRIPTION OF FIGURES

35 FIG. 1 shows a map of an expression vector of this disclosure.

FIGS. 2A-2C show ARPE-19 cells transfected in serum free conditions with a GFP-encoding reporter plasmid (pCMV EGFP) using various plasmid DNA to Lipofectamine ratios: 1:1 (FIG. 2A); 1:2 (FIG. 2B); and 1:3 (FIG. 2C). FIG. 2D is flow cytometry analysis of the transfected ARPE-19 cells.

FIG. 3A shows RS1 protein expressed in ARPE-19 cells 72 hours after transfection with an RS-1 encoding plasmid using various plasmid DNA to Lipofectamine ratios and incubated for 6 or 14 hours (last lane is molecular weight marker). FIG. 3B shows RS1 protein expression in 10% serum (FIG. 3B, Lane 1), 2% serum (FIG. 3B, Lane 2), and serum free conditions (FIG. 3B, Lane 3). (In FIG. 3B lane markers are Lanes 1 - 3; "M" is molecular weight marker; "PC" is positive control).

FIGS. 4A-4G show analysis of RS1 protein produced by ARPE cells stably transfected with a RS-1 encoding plasmid of this disclosure. FIG. 4A shows a Western blot of RS1 protein produced by polyclonal ARPE cells cultured in 1% serum ("P" indicates passage number of the transfected cells). FIG. 4B shows confirmation of the RS1 protein loading at passages 1 and 2 by silver staining. FIG. 4C shows amount of RS1 protein collected from serum free media from the RS1-transfected ARPE cells estimated by histidine pulldown. FIG. 4D is a photograph of the RS1-transfected ARPE polyclonal cells in culture (bottom), showing the cells are tightly adhered, larger in diameter than control ARPE-19 cells (top), and show highly flattened morphology. FIG. 4E shows immunocytochemical analysis of RS1 protein in the polyclonal RS1-transfected APRE cells. FIG. 4F shows a Western blot indicating the expression of RS1 protein in the RS1-transfected, polyclonal cells, analyzed by sampling the media collected from these cells at each of 10 passages (M = molecular weight markers). FIG. 4G shows staining of Na-ATPase enzyme subunits A1, A3, B1, and B2 in lysate of retinal cells (positive control), ARPE-19 cells cultured in vitro, and the RS-1 transfected cells.

FIG. 5 shows Western blot of RS1 protein produced by 27 single clones selected from the polyclonal cells and propagated and expanded with high, low, and medium secretion of RS1 protein in media.

FIGS. 6A-6D show the results of analysis of RS1 protein secretion efficiency from ARPE-19 cells. FIG. 6A shows a Western blot of growth media samples obtained from stably-transfected ARPE-19 cells expressing RS1 protein, grown in serum-free or serum conditions (first lane is molecular weight markers). FIG. 6B is a Western blot of growth media samples obtained from stably-transfected ARPE-19 cells expressing wild type (WT) RS1 protein, a secreted RS1 protein with a mutation (R141H), and a non-secreted RS1 protein with a mutation (R213W) (is molecular weight markers). FIG. 6C is a Blue Native PAGE gel run to resolve the expressed RS1 protein complex by molecular weight

and identify its native structure. Lanes 1-6 contain protein purified from six different cultures. FIG. 6D shows a Western blot of biotinylated RS1 proteins affinity-purified on agarose resin. M is molecular weight markers, lane 1 is lysate from mouse retina (positive control); lane 2 is cell lysate from ARPE-19 cells (negative control); lane 3 is cell lysate from RS1-transfected ARPE-19 cells; lane 4 is growth media sampled from RS1-transfected ARPE-19 cells; lane 5 is protein from biotinylated ARPE-19 cells; and lane 6 is protein from biotinylated, RS1-transfected ARPE-19 cells. FIG. 6E shows a Western blot of three RS1 proteins, both wild type and R141H and R213W mutants, expressed by ARPE-19 cells grown in serum-free conditions. Lanes for each of the three proteins are indicated, and lanes marked 1-4 indicate day of media sampling (1=Day 1; 2=Day 4; 3=Day 8; 4=Day 10); first lane is molecular weight markers; second lane is wild-type human RS1 protein.

FIGS. 7A and 7B are histology images of eyes from retinoschisin knockout mice injected with the RS1 protein secreting ARPE-19 cells intravitreally. FIG. 7A shows two representative histology images of treated and untreated eyes stained for RS1 protein. FIG. 7B shows three representative histology images of treated and untreated eyes stained for RS1 protein. FIG. 7C shows three Optical Coherence Tomography (OCT) images of eyes from the mice 6 weeks after injection of the transfected ARPE-19 cells.

FIGS. 8A-8C show results from the analysis of RS1 protein within the retina of the retinoschisin knockout mice injected with the RS1 protein secreting ARPE-19 cells. FIG. 8A shows Western blots of retinas from 7 mice. In each blot, the first three lanes are: molecular weight markers, lysate from ARPE-19 cells, and lysate from RS1-transfected ARPE-19 cells, respectively. RL represents retinal mouse lysates from wild-type mouse eyes. Numbers indicate individual animals and I=injected eye; C=control eye. FIG. 8B shows Western blots of Ezrin and CRALBP expression in these retina (normalized to beta-actin). FIG. 8C shows immunohistochemical staining of the retina cells with a bipolar cell marker, (PKC-a).

FIG. 9 shows a cryo-EM analysis of the purified human RS1 protein, demonstrating that RS1 is predominantly a 16-mer arranged as back-to-back octamer rings. Panel A in FIG. 9 is a silver-stained blue native gel showing an oligomer of ~400 kDa, much larger than an octamer (triangles indicate the expected band position). Panels B-E in FIG. 9 show selected 2D class averages derived from micrographs of frozen-hydrated RS1 particles. (Scale bars, 50 Å).

DETAILED DESCRIPTION

This disclosure provides nucleic acid molecules encoding human retinoschisin-1 (RS1) proteins, and variants thereof, methods of making and purifying human RS1 proteins, and methods of treating X-linked retinoschisis in a subject.

5 Throughout this disclosure, including the appended claims, conditional language, such as, among others, “can,” “could,” “might,” “may,” “e.g.,” and the like, unless specifically stated otherwise, or otherwise understood within the context as used, is generally intended to convey that certain embodiments include, while other embodiments do not include, certain features, elements, and/or steps. Thus, such conditional language
10 is not generally intended to imply that features, elements and/or steps are in any way required for one or more embodiments or that one or more embodiments necessarily include logic for deciding, with or without author input or prompting, whether these features, elements and/or steps are included or are to be performed in any particular embodiment. The terms “comprising,” “including,” “having,” and the like are synonymous
15 and are used inclusively, in an open-ended fashion, and do not exclude additional elements, features, acts, operations, and so forth. Also, the term “or” is used in its inclusive sense (and not in its exclusive sense) so that when used, for example, to connect a list of elements, the term “or” means one, some, or all of the elements in the list.

20 Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents,
25 and other references mentioned herein are incorporated by reference in their entirety. In the case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be limiting.

 Various aspects of this disclosure are described in detail in the following sections.
30 The use of such sections is not intended to be limiting. Each section can apply to one or more embodiments described herein. In this application, use of “or” means “and/or” unless stated otherwise.

 Proteins are a dominant class of therapeutics used in the treatment of eye diseases. However, large protein drugs are unable to bypass the blood-retinal barrier
35 and, thus, require repeated intraocular administration for treatment.

As used herein, and with particular regard to amino acid and nucleotide sequences, the term "about" refers to a variation of +/- 10%.

As used herein, the term "nucleic acid molecule" is intended to include DNA molecules (e.g., cDNA or genomic DNA), RNA molecules (e.g., mRNA), analogs of the DNA or RNA generated using nucleotide analogs, and derivatives, fragments, and
5 homologs thereof. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

As used herein, an "isolated" nucleic acid molecule is one that is separated from other nucleic acid molecules which are present in the source of the nucleic acid.
10 Preferably, an "isolated" nucleic acid is free of sequences which flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material or culture medium when produced by recombinant techniques, or of chemical precursors or
15 other chemicals when chemically synthesized. For example, those skilled in the art will recognize that the "isolated" nucleic acid may be a completely synthetic molecule that originally comes from phage display screening. Thus, in this context, an "isolated" nucleic acid could be a synthetic molecule substantially free of other cellular material, culture medium, chemical precursors, chemicals, etc.

As used herein, the term "oligonucleotide" or "polynucleotide" refers to a series of
20 linked nucleotide residues, which oligonucleotide has a sufficient number of nucleotide bases to be used in a PCR reaction. A short oligonucleotide sequence may be based on, or designed from, a genomic or cDNA sequence and is used to amplify, confirm, or reveal the presence of an identical, similar, or complementary DNA or RNA in a particular cell or
25 tissue.

Nucleic acid molecule of this disclosure can comprise only a portion of the nucleic acid sequences encoding a human RS1 protein, e.g., a fragment that can be used as a probe or primer or a fragment encoding a biologically active portion of any human RS1 protein. Fragments provided herein are defined as sequences of at least 6 (contiguous)
30 nucleic acids or at least 4 (contiguous) amino acids, a length sufficient to allow for specific hybridization in the case of nucleic acids or for specific recognition of an epitope in the case of amino acids, respectively, and are at most some portion less than a full-length sequence. Fragments may be derived from any contiguous portion of a nucleic acid or amino acid sequence of choice. Derivatives are nucleic acid sequences or amino acid
35 sequences formed from the native compounds either directly or by modification or partial substitution. Analogs are nucleic acid sequences or amino acid sequences that have a

structure similar to, but not identical to, the native compound but differs from it in respect to certain components or side chains. Analogs may be synthetic or from a different evolutionary origin and may have a similar or opposite metabolic activity compared to wild type. Homologs are nucleic acid sequences or amino acid sequences of a particular gene
5 that are derived from different species.

As used herein, the terms "protein" and "polypeptide" are intended to be interchangeable. The novel polypeptides of the invention include the RS1 having an amino acid sequence of SEQ ID NOs: 4, 7, 11, 15, 19, or 21. The invention also includes mutant or variant polypeptides any of whose residues may be changed from the
10 corresponding residue shown in SEQ ID NOs: 4, 7, 11, 15, 19, or 21. In the mutant or variant protein, up to 20% or more of the residues may be so changed. A variant polypeptide includes any variant in which residues at a particular position in the sequence have been substituted by other amino acids, and further includes the possibility of inserting an additional residue or residues between two residues of the parent protein as
15 well as the possibility of deleting one or more residues from the parent sequence. Any amino acid substitution(s), insertion(s), or deletion(s) is encompassed by the invention. In favorable circumstances, the substitution is a conservative substitution.

An "isolated" or "purified" polypeptide or biologically active portion thereof is substantially free of cellular material or other contaminating proteins or polypeptides from
20 the cell or tissue source from which the human RS1 polypeptide is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The phrase "substantially free of cellular material" includes preparations of human RS1 polypeptides in which the polypeptide is separated from cellular components of the cells from which it is isolated or recombinantly produced. For example, the phrase
25 "substantially free of cellular material" includes preparations of human RS1 polypeptides having less than about 30% (by dry weight), or less than about 20%, or less than about 10%, or less than about 5% non-RS1 protein. When the human RS1 polypeptides or fragments or variants thereof are recombinantly produced, they are also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%,
30 more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation.

To determine the percent homology of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal
35 alignment with a second amino or nucleic acid sequence). The amino acid residues or

nucleotides at corresponding amino acid positions or nucleotide positions are then compared.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses, adeno-associated viruses, and transposon-based recombination systems), which serve equivalent functions.

The expression vectors of this disclosure comprise any of the nucleic acids of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, that is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" is intended to mean that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner that allows for expression of the nucleotide sequence (e.g., in an in vitro transcription/translation system or in a host cell when the vector is introduced into the host cell).

The term "regulatory sequence" is intended to include promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; GENE EXPRESSION TECHNOLOGY: METHODS IN ENZYMOLOGY 185, Academic Press, San Diego, Calif. (1990). Regulatory sequences include those that direct constitutive expression of a nucleotide sequence in many types of host cell and those that direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector

can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein.

5 **Retinoschisin-1 and Retinoschisis**

Retinoschisin-1 is a small gene that is about 32.4 kb long containing six exons and five introns located on chromosome Xp22.2 and encodes a 3.1 kb mRNA that is translated into a 224-amino acid precursor polypeptide. Retinoschisin is expressed as a monomer containing four different domains: N-terminal signal sequence (23 amino acids) directing protein translocation to the exterior of the cell; a unique Rs1 domain (a long/highly conserved sequence motif 39 amino acids in length); a discoidin domain (157 amino acids), contributing to the adhesive function of RS1 to preserve the retinal cell architecture and to establish proper synaptic connectivity, and a small, C-terminal segment (5 amino acids).

Retinoschisin is assembled in the endoplasmic reticulum and secreted as functional disulfide-linked homo-octamer (eight subunits joined together by Cys59-Cys223 disulfide bonds. Subunits within the octamer are further organized into dimers mediated by Cys-Cys disulfide bonds. RS1 is bound by ionic forces to the outer leaflet of the photoreceptor inner segment plasma membrane, and function in cell-cell interactions and cell adhesion. RS1 is expressed in the retina, prominently by the rod and cone inner segments, and bipolar cells, and pineal gland. Immunostaining in the retina localizes Retinoschisin to the inner segments of photoreceptors, bipolar cells and the inner and outer plexiform layers. High sequence homology exists in human, mouse, rat, and rabbit.

Retinoschisis is a severe eye disease classified into degenerative, hereditary, tractional and exudative forms. In particular, X-linked juvenile Retinoschisis (XLRS), a hereditary form of Retinoschisis, is an early onset macular degeneration characterized by loss in visual acuity, abnormal splitting of the neurosensory layers of the retina and a reduction of the b-wave in an electroretinogram (ERG). XLRS is caused by mutations in the Retinoschisin-1 (RS1) gene and is transmitted in an X-linked recessive pattern that causes disease only in males. Mutations in the RS1 gene product result in the complete absence of an RS1 polypeptide, or the production of a defective RS1 polypeptide having reduced or no function. Almost 200 mutations of the RS1 gene have been reported to be associated with XLRS, and manifests in a phenotype that is highly variable across individuals. Of the almost 200 mutations reported, about 40% of the disease-causing mutations are nonsense or frame shift mutations, which are predicted to result in the absence of a full-length polypeptide. About 50% of the disease-causing mutations are

missense mutations that allow for production of full-length mutant polypeptide, and most of these mutations are found in the discoidin domain and result in a functionally incompetent misfolded polypeptide.

There is a spectrum of phenotypes for XLRS (referred to as XLRS-related diseases or disorders). Cystic Macular Lesions involving the fovea are characteristic clinical features of XLRS. In particular, foveal schisis with "cartwheel" or "spoke-wheel" pattern is a characteristic finding on fundus exam, presenting in nearly 100% of cases, and schisis may occur peripherally in up to 50% of patients or retinal detachments. Peripheral schisis can lead to holes and tears of the inner leaf with potential for hemorrhage from unsupported crossing vessels. Additional peripheral changes include pigmentation resembling retinitis pigmentosa, retinal fibrosis and white flecks, and vitreo-retinal dystrophy. ERGs show marked b-wave reduction, and abnormal a-wave in some patients, but in many, a-wave remains normal. XLRS-related disorders may also result in retinal detachment. Likewise, ocular injury may lead to separations within the retina and cause abrogated b-wave function. Thus, the supplementation of RS1 into XLRS eyes or injured eyes may improve ocular function, for example by restoring retinal structural integrity and/or visual function.

The clinical presentation of XLRS and course of disease is variable, presenting as early as at birth to later at school age with only mild visual symptoms. These variations and clinical severity do not appear to correlate with genotype, and female carriers are asymptomatic. Currently, spectral domain-OCT (SD-OCT) is the major diagnostic technique for this disease, while existing management options are limited to low vision aids. In small studies, carbonic anhydrase inhibitors have shown improvement in visual acuity in about 50% of eyes, however, definitive Phase III trials have yet to be executed.

Previous attempts at treating XLRS-related eye disorders through the use of gene therapy have had limited success partly attributable to the inflammation resulting from delivery of the gene therapy construct including the RS1 coding region into the eye of the patient.

Thus, one aspect of this disclosure is a nucleic acid molecule (e.g., deoxyribonucleic acid (DNA) molecule) comprising the RS1 coding region (open reading frame (ORF)), or a portion thereof, that is efficiently expressed in human cells to produce RS1 that is post-translationally modified to match wild type human RS1. This transcribed and translated RS1 can be purified and used to treat XLRS and related eye disorders by delivering this protein to the retina of the patient, with minimal inflammation, thereby increasing the visual function of the patient.

We have previously treated human XLRs patients using gene therapy by administration of an expression vector encoding the human RS1 protein (see, International Patent Application No. PCT/US2019/014418, which is incorporated herein by reference in its entirety). Alternative or additive therapeutic methods of treating XLRs include the administration of retinoschisin proteins to such patients and require a source of highly purified and correctly modified human retinoschisin proteins. Accordingly, we have invented methods of producing and administering human retinoschisin proteins that meet these requirements.

Thus, in one aspect, this disclosure provides polynucleotides encoding the human RS1 protein. These polynucleotides may, in whole or in part, be modified to include codons that are optimized for expression in human cells. Codon optimized sequences are synthetic sequences, and preferably encode the identical polypeptide (or a biologically active fragment of a full-length polypeptide which has substantially the same activity as the full-length polypeptide) encoded by the non-codon optimized parent polynucleotide. In some embodiments, the coding region of the genetic material encoding a reporter polypeptide (e.g., lacZ), in whole or in part, may include an altered sequence to optimize codon usage for a particular cell type (e.g., a human cell). Thus, in these polynucleotides, the coding region encoding a RS1 polypeptide, as described herein (e.g., a human RS1 polypeptide, and mutants or variants thereof), in whole or in part, may include an altered sequence to optimize codon usage for a particular cell type (e.g., a human cell). To give but one example, the codons of a mutant RS1 cDNA to be inserted into the genome of a human cell may be optimized for expression in a human cell. Such polynucleotide sequences may be described as a "codon-optimized sequence."

These polynucleotides encoding the human RS1 protein may have a deletion in the coding region, in whole or in part, of the RS1 protein, resulting in the production of an RS1 polypeptide with limited or no function, or with a gain of function. For example, these polynucleotides encoding the human RS1 protein may have a deletion in the coding region encoding any one or more of the N-terminal signal sequence, the Rs1 domain, the discoidin domain, and/or C-terminal segment of the human RS1 protein. Thus, these polynucleotides may encode individual polypeptide domains of the human RS1 protein, such as any one of: the N-terminal signal sequence, the Rs1 domain, the discoidin domain, and/or the C-terminal segment of the human RS1 protein. An exemplary polynucleotide of this disclosure encodes the discoidin domain of the human RS1 protein. Such polynucleotide sequences may be described herein as encoding RS1 "polypeptide fragments," or "fragments of" an RS1 polypeptide, or "truncated RS1 polypeptides."

These polynucleotides encoding the human RS1 protein may have one or more point mutations in the sequence encoding a human RS1 polypeptide that results in a variant RS1 polypeptide that includes one or more amino acid substitutions relative to the human, wild type RS1 polypeptide (encoded by the Rs1 gene without the point mutations). For example, a variant human RS1 polypeptide includes one or more amino acid substitutions relative to the wild type human RS1 polypeptide. The amino acid substitutions encoded by such polynucleotides may result in elimination or significant reduction in the level or activity of functional RS1 polypeptide produced from a RS1 mutant allele (i.e., a RS1 gene having a deletion or a point mutation). Amino acid substitutions can lead to elimination or significant reduction in the level of functional RS1 polypeptide produced as a result of, for example, misfolding of a polypeptide, defective subunit or oligomer assembly, and inability of a polypeptide to be inserted into the membrane of the ER as part of the protein secretion process.

An exemplary amino acid substitution encoded by these polynucleotides is in the signal sequence that results in an inability of a variant RS1 polypeptide having the amino acid substitution to be inserted into the membrane of the ER for secretion. For example, substitution of hydrophobic residues in the signal sequence with proline or with hydrophilic/charged residues may prevent the signal sequence from adopting an α -helix secondary structure required for insertion into the ER membrane.

Another exemplary amino acid substitution encoded by these polynucleotides is one or both regions flanking the discoidin domain, i.e., the Rs1 region composed of amino acids 24-62, and/or the C-terminal segment (composed of amino acids 220-224). These substitutions may include a substitution of a cysteine at any one of positions 38, 40, 42, 59, or 223, with an amino acid such as Ser, Arg, Trp, Tyr, or Gly.

Another exemplary amino acid substitution encoded by these polynucleotides is an amino acid substitution in the discoidin domain of the human RS1 polypeptide, which is composed of amino acids 63-219. For example, a substitution in the discoidin domain may be a substitution of one or more of the five Cys residues in the discoidin domain: C63, C83, C110, C142, and/or C219. Cys63 and Cys219, and Cys110-Cys142, form two intramolecular disulfide bonds that are important for RS1 protein folding. These cysteine residue(s) at any one of positions 63, 83, 110, 142, or 219 may be substituted with a Ser, Arg, Trp, Tyr or Gly. Additionally or alternatively, a substitution in the discoidin domain encoded by the polynucleotides of this disclosure may be a substitution of an amino acid residue not directly involved in formation of disulfide bonds but important for protein folding, formation or stability of the discoidin domain, and/or intermolecular interactions among adjacent subunits. Examples of such residues may include amino acid residues

such as E72, G109, E146, R182, and P203, R141, and/or D143. Additionally or alternatively, a substitution in the discoidin domain encoded by the polynucleotides of this disclosure replaces a non-cysteine residue with cysteine. Additionally or alternatively, a substitution in the discoidin domain encoded by the polynucleotides of this disclosure affects protein charge by eliminating or reversing the charge of amino acid residues or by replacing a non-charged residue with a charged residue. Additionally or alternatively, a substitution in the discoidin domain encoded by the polynucleotides of this disclosure affects conformation stability by insertion or removal of proline (Pro) residues. Additionally or alternatively, a substitution in the discoidin domain encoded by the polynucleotides of this disclosure may affect hydrophobicity by insertion or removal of polar residues (i.e., replacing a hydrophobic residue with a polar residue or replacing a polar residue with a hydrophobic residue).

Another exemplary amino acid substitution encoded by these polynucleotides is an amino acid substitution of an arginine (Arg;R) for the cysteine (Cys;C) at position 141 of the human RS1 protein (i.e., R141C). Another exemplary amino acid substitution encoded by these polynucleotides is an amino acid substitution of an arginine (Arg;R) for a tryptophan (Trp;W) at position 213 of the human RS1 protein (i.e., R213W).

Such polynucleotide sequences encoding one or more amino acid substitution(s) may be described herein as encoding RS1 “mutants”, or “variants”, or “variants of” a RS1 polypeptide, or “variant RS1 polypeptides.”

These polynucleotides encoding the human RS1 protein may include intron sequences from the human *RS1* gene (MIM# 312700). The human *RS1* gene contains six separate exons interspaced by five introns, and any one of these five introns, or portions thereof, may be present in the polynucleotide sequences of this disclosure that encode the human RS1 protein. An exemplary intronic sequence that may be included in the polynucleotide sequences of this disclosure is all, or a portion, of the first intron sequence of the human *RS1* gene. This first intron is 14351 nucleotides in length, and the entire intron, or a portion thereof, may be present in the polynucleotide sequences of this disclosure. An exemplary intronic sequence that may be included in the polynucleotide sequences of this disclosure comprises at least 200 nucleotides of the first intron sequence of the human *RS1* gene. An exemplary intronic sequence that may be included in the polynucleotide sequences of this disclosure comprises at least 300, or at least 400, or at least 500, or at least 600, or at least 700, or at least 800, or at least 900 nucleotides of the first intron sequence of the human *RS1* gene. Another exemplary intronic sequence that may be included in the polynucleotide sequences of this disclosure comprises at least 1000, or at least 2000, or at least 3000, or at least 4000, or at least 5000, or at least

6000, or at least 7000, or at least 8000, or at least 9000, or at least 10000 nucleotides of the first intron sequence of the human *RS1* gene. Another exemplary intronic sequence that may be included in the polynucleotide sequences of this disclosure comprises between 200 and 500, or between 100 and 400, or between 50 and 300 nucleotides of the first intron sequence of the human *RS1* gene. Another exemplary intronic sequence that may be included in the polynucleotide sequences of this disclosure comprises a 319 bp, truncated retinoschisin first intron. The truncated intron consists of base pairs +95 to +355 and +14396 to +14445 relative to the human *RS1* gene transcriptional start site. These sequences encode the splice donor and lariat/splice acceptor elements of the human *RS1* gene, respectively.

These polynucleotides encoding the human RS1 protein, or variants, or fragments thereof, may include a polynucleotide encoding a reporter molecule. The polynucleotide encoding a reporter molecule is fused in frame region encoding the human RS1 protein, or variant, or fragment thereof. As a result of such operable linkage to the RS1 polypeptide, the reporter molecule may be used to identify the location and expression characteristics of the RS1 polypeptide within cells or tissues. Exemplary reporter proteins that may be included in the polynucleotides of this disclosure include luciferase, green fluorescent protein (GFP), enhanced GFP (eGFP), cyan fluorescent protein (CFP), yellow fluorescent protein (YFP), enhanced yellow fluorescent protein (eYFP), blue fluorescent protein (BFP), enhanced blue fluorescent protein (eBFP), DsRed, and MmGFP.

These polynucleotides encoding the human RS1 protein, or variants, or fragments thereof, may further encode one or more fusion domains. Such fusion domain(s) or a fragment thereof may be selected to confer a desired property. For example, some fusion domains are particularly useful for isolation of the fusion proteins by affinity chromatography. For the purpose of affinity purification, relevant matrices for affinity chromatography, such as glutathione-, amylase-, and nickel- or cobalt-conjugated resins are used. Many of such matrices are available in "kit" form, such as the Pharmacia GST purification system and the QLAexpress™ system (Qiagen) useful with 6X-HIS fusion partners. As another example, a fusion domain may be selected to facilitate detection of the RS1 proteins. Examples of such detection domains include the various fluorescent proteins (e.g., GFP) as well as "epitope tags," which are usually short peptide sequences for which a specific antibody is available. Well known epitope tags for which specific monoclonal antibodies are readily available include FLAG, influenza virus haemagglutinin (HA), and c-myc tags. In some cases, the fusion domains have a protease cleavage site, such as for Factor Xa or Thrombin, which allows the relevant protease to partially digest the fusion proteins and thereby liberate the recombinant proteins therefrom. The liberated

proteins can then be isolated from the fusion domain by subsequent chromatographic separation. As another example, a fusion domain may be selected to stabilize the RS1 protein (a "stabilizer" domain), for example by increasing the serum half-life of the RS1 protein in vivo, regardless of whether this is because of decreased destruction, decreased clearance, or other pharmacokinetic effect. Fusions with the Fc portion of an immunoglobulin are known to confer desirable pharmacokinetic properties on a wide range of proteins. Likewise, fusions to human serum albumin can confer desirable properties. Fusions may be constructed such that the heterologous peptide is fused at the amino terminus of the RS1 polypeptides and/or at the carboxy terminus of the RS1 polypeptides.

Exemplary human RS1 sequences, variants thereof, and fragments thereof, are set forth in SEQ ID NOS: 1-21 and summarized in the following Table.

Name and description	Sequence	SEQ ID NO:
Homo sapiens retinoschisin 1 (RS1), mRNA, NCBI Reference Sequence: NM_000330.4	1 agttcagtaa ggtagagctt tggccgagga cgaggggaag atgtcacgca agatagaagg 61 ctttttgta ttacttctct ttggtatga agccacattg ggattatcgt ctaccgagga 121 tgaaggcgag gacctctggt accaaaaagc atgcaagtgc gattgccaag gaggacccaa 181 tgctctgtggt tctgcaaggtg ccacctctt ggactgtata ccagaatgcc catatcacaa 241 gacctctgggt ttcgaggtcag gggaggtcac accggaccag atcacctgct ctaacccggga 301 gcagtatgtg ggtcggtatt cttcgtggag tgcaaacaaag gcccggttca acagtcaaagg 361 ctttgggtgt gcttggctct ccaagttcca ggacagtagc cagtggttac agatagatct 421 gaaggagatc aaagtgattt cagggtatcct caccagggg cgctgtgaca tcgatgagtg 481 gatgaccaag tacagcgtgc agtacaggac cgatgagcgc ctgaactgga ttactacaa 541 ggaccagact ggaacaacc gggctcttcta tggcaactcg gaccgcacct ccacgggttca 601 gaacctgctg cggcccccca tcatctcccg cttcatccgc tgggctggca 661 cgtccgcatt gccatccgga tggagctgct ggagtgctgc agcaagtgtg cctgatgcct 721 gcctcagctc ggcgcctgcc agggggtgac tggcacagag cgggcccgtag gggacccct 781 cacacaccac cgagatggac agggctatat ttcgaaaagc aattgtaact gcaagtgtg 841 gtagataatt tttttttt taagatatag ctttctgatt tcaatgaaat aaaaatgaac 901 ttattcccca ctcaaggcca gagaaagtca gaacaaagaa aatgtccccg aaacgaattt 961 tcttacaaaa gcctaagtag caggggtaat ttctgtctca tttttgtct cagtgatact 1021 gtgaaagggt cagctctcagg ggaacacaaa gcagccctga taatttgaaa attcatttgc 1081 ttaccacat tcaagacaga aacatacagt ttctaaagc ctggcttga atgcagaagg 1141 gagcagctcc tcctagttaa gtttccacta aatcatcgcc aaagaggact tcagagccct 1201 ggggaggcag ctgaggggtc caagggtgac tgggtggcag ggatgagtgc ggtgggtgag 1261 aatccccggt cctgagagg ctatacgtga caaatgacca aaagcccaag gttaggggagt 1321 ttcctctgct cacagttctt accttcaagg cggatctggg cttccacct catgaacaca 1381 gggattgggg agggaccaga gcgcccaata cacacagctc cattatgcaa tccattccag 1441 caaattcccc gtgtctgtggt tcaccattta ggtgatcata caggacagggc tgcacatctc 1501 agtatatgta gggaccccaa atgaccacaa cacaglacaa ttgcccctta cctagggcta 1561 ccatttccta gcaaacccaaa catagttcga gaacagctgg cccagggagt accactggct 1621 actcagagga ggtcatttag ctggctacat gcttcgcaag aagtgggaaag gactcacatc 1681 ataaaaagga ccatgtagct ttttccctga aagcttctca cctccacct tctgccttgc 1741 aatacgcaaa ctgcccctgc tcctgaaaaa gctctggaag aggaatgggc ctggctttcc 1801 gttcctggag gcggcgctt agattgggag gcctcattgg ccacttagag cgcagcctga 1861 gtttccaggc cccttccctg gagaggctgt taacacgggg gaggggcagg agagggatat 1921 ggagagcagg tgggtggaatc agaggacgag gctgctctaa agactgttct ggccccagac 1981 acagggtagt ctttgctagc agtcatctt cgagttactt ttcattttca aatgccaaag 2041 caagtgacta gactcggct aatacagctc tggacaacac attcaccttt tctgtgaaca 2101 ggcagccttc taaaagcccc aaacatcctt cttgatgctt tgggggctca attattttat 2161 atccaaaccca gcatctttct agtccctatg ctgtatgctt gaacttcgga aaatgctttt 2221 ccccgcccaa tcttctctca aataaaaaa catcacacag ggtgttgggg gtggggggggg	1

	2281 ggggtggggg gacttatccc tggccttagg acacaggaca aatctatctt ggatagaaat 2341 gcctgaacag agacccttat ttggaaagggt gaattaaact ttggtcacgac atggactgtc 2401 agacaaaatg gcagtatcct aagagttaag gcacatcaaa cacaggagtc gagagagtgc 2461 agttcagggg aaaaggagag gaggaacacg tgaggcaggg agaaaggctt tccaaataag 2521 agttcatgtt ggaacttttt gtacgggctt tatlgagatt aagttcacat acaatttcta 2581 tccatttaaa gtgtacaatt tgatgacttt tggatatc tggatatc aaccattatc 2641 actagatcaa ttttagaaa tttatcacc ccaagagaaa tccgtgaccc atcagccaac 2701 actcccaaac ccacgggcca ccccaagccc tctgcaacca cgaatcgact gtctctgtag 2761 attggccttc tggacgttct acataaaatga aatcatatag tatgtggtat ttctgtactg 2821 gcttctttca cttagcatag tgttttaaa ttcatccacg ttataacatg tgtatcacta 2881 tgtcacttgt cactcctttt tattgctgaa catcatgttt cagtatcatg tcaagagcac 2941 attgttattt atccattcat ccattgatgg atatttgggt ttccactctt tagctattat 3001 gaataaatgct gctatgaaca ttgtgtata a	
Homo sapiens retinoschisin 1 (RS1), protein. Translation of SEQ ID NO:1	MSRKIEGFLLLLLFGYEATLGLSSTEDEGEDPWYQKACKKDCQGGPNALWSAGATSLDCIPECPYHKPLGF ESGEVTPDQITCSNPEQYVGWYSSWTANKARLNSQGFCAWLSKFQDSSQWLQIDLKEIKVISGILTQGRG DIDEMTKYSVQYRTDERLNIWYIKDQTGNNRVFYGNSDRTSTVQNLLRPPIISRFIRLIPLGWHVRIAIR MELLECVSKCA	2
Codon-optimized Homo sapiens retinoschisin 1 (RS1), mRNA.	atgtcacgcaagattgaaggctttctgctgctgctgtgttctgggtatgagggtacactg gggctgagttctactgaggacgagggcgaggacccctggtaccagaaggcctgcaagtgt gattgccaggaggacctaaccgactgtggagcgcggagccacccctccctggactgtatc ccagagtccccatcacaaagccactgggcttcgagtcgggcgaggtgacccctgatcag atcacatgttctaataccagagcagtagctgggctggtagctcctggacagccaaacag gcccggctgaattctcagggcttcggctgcgcctggctgagcaagtttcaggactctagc cagtggctgcagatcgatctgaaggagatcaaaagtgcagcggcatcctgacccagggc cgggtgcacatcgatgagtggtgaccaaactcctccgtgcagtaggacagacgagcgc ctgaactggatctactataaggatcagacaggcaacaactcgggtgttctacggcaactcc gacagaacctctacagtgcaagaatctgctgagggccccctatcatctcgggttatcaga ctgatccccctgggctggcacgtgcgcacatccgaatccgaactgctggaatgcgtg tctaagtggtgcc	3
Codon-optimized	MSRKIEGFLLLLLFGYEATLGLSSTEDEGEDPWYQKACKKDCQGGPNALWSAGATSLDCIPECPYH KPLGFESGEVTPDQITCSNPEQYVGWYSSWTANKARLNSQGFCAWLSKFQDSSQWLQIDLKEIKV	4

Homo sapiens retinoschisin 1 (RS1), protein. Translation of SEQ ID NO:3	ISGILTQGRCDIDEWMTKYSVQYRTDERLNWIYYKDQTGNNRVFYGNSDRTSTVQNLLRPPIISRF IRLIPLGWHVRIAIRMELLECVSKCA	
6xHis tag	CACCACCACCACCAC	5
nucleotide construct encoding the codon-optimized Rs1-discoïdin domain-C-terminal domain	atgtcacgcaagattgaaggcttctgctgctgctgctgttcgggtatgaggctacactg gggtgagtgaactgtatccagagtgccctatcacaaagccactgggcttcgagtcgggc gagtgacccctgatcagatcacatgttctaataccagagcagtaactgggtggtatagc tcctggacagccaaagggccggctgaattctcagggttcggctgcgctggctgagc aagtttcaggactctagccagtggtgcagatcgatctgaaggagatcaaagtgcagc ggcatcctgacccagggccgggtgtgacatcgatgagtgagtgacaaactccgtgcag tataggacagacgagcgcctgaactggatctactataaggatcagacagggcaacaatcgg gtgttctacggcaactccgacagaaacctctacagtgagaaatctgctgagggccctatc atctctcggtttatcagactgatccccctgggctggcacgtgcgcacatcgcaatccgaatg gaactgctggaatgcgtgtctaagtgtgcc	6
Protein sequence of the Rs1-discoïdin domain-C-terminal domain construct. Translation of SEQ ID NO:6.	MSRKIEGFLLLLLFGYEATLGLSDCIPECPYHKPLGFESGEVTPDQITCSNPEQYVGVYSSWTANKA RLNSQGFGCWLSKFQDSSQWLQIDLKEIKVISGILTQGRCDIDEWMTKYSVQYRTDERLNWIYYKD QTGNNRVFYGNSDRTSTVQNLLRPPIISRFIRLIPLGWHVRIAIRMELLECVSKCA	7

Synthetic primer used for R141H mutagenesis	gaccaggggccactgtgacatcgatg	8
Synthetic primer used for R141H mutagenesis	aggatgccgctgatcact	9
nucleotide construct encoding the mutated (R141H) codon-optimized human RS1 protein	atgtcacgcaagattgaaggctttctgctgctgctgctgtgttcgggtatgaggctacactg gggctgagttctactgaggacgagggcgagggacccctggtaccagaaggcctgcaagtgt gattgccaggaggacctaacgcactgtggagcgcggagaccacctccctggactgtatc ccagagtccccctatcacaaagccactgggcttcgagtcgggcgaggtgacccctgatcag atcacatgttctaatccagagcagtagctgggctggtagctcctggacagcccaacaag gccccgctgaattctcagggcttcggctgcgcctggctgagcaagtctcaggactctagc cagtggctgcagatcgatctgaaggagatcaaaagtgcacgggcacacctgacccagggc cactgtgacatcgatgagtggaatgaccaaagtactcctgcagtataggacagacgagcgc ctgaactggatctactataaggatcagacagggcaacaatcgggtgttctacggcaactcc gacagaacctctacagtcagaaatctgctgagggccccctatcatctcgggttatcaga ctgatccccctgggctggcacgtgcgcacatccgaatccgaatggaactgctggaatgctg tctaagtgtgcc	10
Protein sequence of the mutated (R141H) codon-optimized human RS1 protein	MSRKIEGFLLLLLFGYEATLGLSSTEDGEDPWYQKACKKDCQGGPNALWSAGATSLDCIPECPYHK PLGFESGEVTPDQITCSNPEQYVGWYSSWTANKARLNSQGFGCWLKSFQDSSQWLQIDLKEIKVIS GILTQGHCDIDEWMTKYSVQYRTDERLNLWIIYKDQGTGNRRVIFYGNSDRSTSTVQNLLRPPIISRFIRL IPLGWHVRIAIRMELLECVSKCA	11

Synthetic primer used for R213W mutagenesis	ctgggctggcacgtccgcattgccatctggatggagctgctggagtgcgctc	12
Synthetic primer used for R213W mutagenesis	gacgcactccagcagctccatccagatggcaatgcggacgtgccagcccag	13
nucleotide construct encoding the mutated (R213W) codon-optimized human RS1 protein	atgtcacgcaagatagaaggcttttgttattacttctcttggctatgaagccacattg ggattatcgtctaccgagatgaaggcaggaccctggtacaaaaagcatgcaagtgc gattgccaaaggaggacccaatgctctgtggtctgcaggtgccacctccttggaactgtata ccagaaatgcccatatcacaaagcctctgggttccagtcaggggaggtcacacccggaccag atcacctgctctaacccggagcagatgtgggctggtattcttcgtggactgcaaaacag gccggctcaacagtcaggctttgggtgtgctggcttcccaagttccaggacagtagc cagtggttacagatagatctgaaggagatcaaaagtgatttcagggtatcctcaccagggg cgctgtgacatcgatgagtggtggtgaccaaagtacagcgtgcagtcaggaccgatgagcgc ctgaactggatttactacaaggaccagactggaaacaaacccgggtcttctatggcaactcg gaccgcacctccacgggttcagaaacctgctgcggcccccatcatctcccgcttcacccgc ctcatcccgctgggctggcacgtccgcattgccatctggatggagctgctggagtgcgctc agcaagtgtgcc	14
Protein sequence of the mutated (R213W) codon-optimized human RS1 protein	MSRKIEGFLLLLLFGYEATLGLSSTEDGEDPWYQKACKDCQGGPNALWSAGATSLDCIPECPY HKPLGFESGEVTPDQITCSNPEQYVGWYSSWTANKARLNSQFGCAWLSKFQDSSQWLQIDLKEI KVISGILTQGRCDIDEWMTKYSVQYRTDERLNLWIIYYKDQGTGNRRVFFGNSDRITSTVQNLLRPPII SRFIRLLIPLGWHVRIAIWMELLECVSKCA	15
Nucleotide sequence encoding Flag Tag	gattacaaggatgacgacgataag	16

Peptide sequence of Flag Tag	DYKDDDDK	17
nucleotide construct encoding the Flag-tagged codon-optimized human RS1 protein	atgtcacgcaagatagaaggcttttggttattacttctcttttggtatgaagccacattg ggattatcgtctaccgagatgaagcgaggacccttggtacaaaaagcatgcaagtgc gattgccaaaggaggaccctctctgtggtctgcaggtgccacctccttgactgtata ccagaatgcccatatcacaagcctctgggttccagtcagggagggtcacaccggaccag atcacctgctctaaaccggagcagtagtggtggtggtatctctcgtggactgcaaaacag gccggctcaacagtcaggcttgggtgtgctggtctccaagttccaggacagtagc cagtggtaacagatagatctgaaggagatcaaaagtattcagggtcctcaccagggg cgtgtgacatcgatgagtggtgaccaaagtcagcgtgcagtcaggtacagaccgatgagcgc ctgaactggatttactacaaggaccagactggaaacaaaccccatctctatggcaactcg gaccgcacctccacgggtcagaacctgctgcggccccccatcatctccgcttcacccgc ctcatcccgctgggtggcagctccgcatcgccatccggatggagctgctggagtgcgctc agcaagtgtgccgattacaaggatgacgacgataagtga	18
Protein sequence of the Flag-tagged codon-optimized human RS1 protein. Translation of SEQ ID NO:18	MSRKIEGFLLLLFGEATLGLSSTEDEGEDPWYQKACKDCQGGPNALWSAGATSLDCIPEC PYHKPLGFESGEVTPDQITCSNPEQYVGWYSSWTANKARLNSQFGCAWLSKFQDSSQWLQID LKEIKVISGILTQGRCDIDEMWTKYSVQYRTDERLNWIYYKDQTGNNRVFYGNSDRTSTVQNL LRPPIISRFIPLGLWHVRIAIRMELLECVSKCADYKDDDDK	19
Nucleotide sequence encoding the mutated (R213W), codon-optimized,	atgtcacgcaagatagaaggcttttggttattacttctcttttggtatgaagccacattg ggattatcgtctaccgagatgaagcgaggacccttggtacaaaaagcatgcaagtgc gattgccaaaggaggaccctctctgtggtctgcaggtgccacctccttgactgtata ccagaatgcccatatcacaagcctctgggttccagtcagggagggtcacaccggaccag atcacctgctctaaaccggagcagtagtggtggtggtatctctcgtggactgcaaaacag gccggctcaacagtcaggcttgggtgtgctggtctccaagttccaggacagtagc cagtggtaacagatagatctgaaggagatcaaaagtattcagggtcctcaccagggg cgctgtgacatcgatgagtggtgaccaaagtcagcgtgcagtcaggtacagaccgatgagcgc	20

Flag-tagged human RS1 protein	ctgaactggatttactacaaggaccagactggaacaacccgggtcttctatggcaactcg gaccgcacctccacggttcagaaacctgctgcgggcccccatcatctcccgcttcacccgc ctcatcccgctgggctggcacgtccgcattgccatctggatggagctgctggagtgcgctc agcaagtgtgccgattacaaggatgacgacgataagtga	
Protein sequence of the mutated (R213W), codon-optimized, Flag-tagged human RS1 protein. Translation of SEQ ID NO:20.	MSRKIEGFLLLLLFGYEATLGLSTEDGEDPWYQKACKDCQGGPNALWSAGATSLDCIPEC PYHKPLGFESGEVTPDQITCSNPEQYVGWYSSWTANKARLNSQGFCAWLSKFQDSSQWLQID LKEIKVISGILTQGRCDIDEWMTKYSVQYRTDERLNWIYYKDQGTGNRRVIFYGNSDRTSTVQNL LRPPIISRFIRLIPLGWHVRIAIIWMELLECVSKCADIKDDDDDK	21

As used herein, "a portion of a RS1 ORF" refers to at least 500 contiguous nucleotides from a RS1 ORF, wherein the at least 500 contiguous nucleotides encode a protein having at least one activity of the human RS1 protein specified herein, and wherein the portion of a RS1 ORF does not comprise a full-length RS1 ORF (i.e., the portion of a RS1 ORF is less than a full-length RS1 ORF). The portion of the RS1 ORF may comprise at least about 1,000, at least about 1,500, at least about 2,000, at least about 2,500, or at least about 3,000, contiguous nucleotides from a RS1 ORF. Similarly, the portion of a RS1 ORF may be less than about 3,000 nucleotides, less than about 2,900 nucleotides, less than about 2,800 nucleotides, less than about 2,700, less than about 2,600 nucleotides, less than about 2,500 nucleotides, less than about 2,400 nucleotides, less than about 2,300 nucleotides, less than about 2,200 nucleotides, less than about 2,100, less than about 2,000 nucleotides, less than about 1,900 nucleotides, about 1,800 nucleotides, less than about 1,700 nucleotides, less than 1,600 nucleotides, or less than about 1,500 nucleotides in length.

As used in this disclosure, a RS1 open reading frame (ORF) refers to a series of contiguous nucleotides that does not contain any intron sequences or stop codons, and which encode a human RS1 protein, or variant thereof, or fragment thereof. Exemplary human RS1 proteins encoded by the ORF of this disclosure function to associate with the surface of rod and cone photoreceptors at the level of the inner segment, outer nuclear, and outer plexiform layers, and the surface of bipolar cells within the inner nuclear and inner plexiform layers of the retina, function as retinal cell adhesion proteins, and/or improving the visual function of a patient suffering from XLRS-related disorders when the encoded protein is expressed in the cells of an eye of the patient. A human RS1 ORF is represented by SEQ ID NO:1, which encodes a RS1 protein of SEQ ID NO:2.

Those skilled in the art recognize that genes found in nature often contain polymorphisms. A polymorphism, or variant, or mutant, refers to a nucleic acid molecule (or its encoded protein), the sequence of which is similar, but not identical, to a reference sequence, often referred to as the wild-type sequence. While some sequence variations result in the reduction or elimination of the activity of the encoded protein, many have minimal or no effect on the activity of the encoded protein. According to the present invention, the portion of a RS1 ORF can be obtained from any polymorphic variant of a RS1 ORF, so long as the portion encodes a protein that is capable of increasing the visual function of a patient suffering from XLRS-related disorders.

Variations in the sequence of a human RS1 ORF, or portions thereof, used in the present invention may be made through the use of genetic engineering techniques known to

those skilled in the art. Examples of such techniques may be found in Sambrook J, Fritsch E F, Maniatis T et al., in *Molecular Cloning-A Laboratory Manual*, 2nd Edition, Cold Spring Harbor Laboratory Press, 1989, pp. 9.31-9.57, or in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6. With regard to such variants, any type of alteration in the nucleic acid sequence is permissible so long as the resulting variant protein retains the ability to increase the visual function of a patient suffering from XLRS-related disorders. Examples of such variations include, but are not limited to, deletions, insertions, substitutions and combinations thereof. For example, with regard to proteins, it is well understood by those skilled in the art that one or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9 or 10), amino acids can often be removed from the amino and/or carboxyl terminal ends of a protein without significantly affecting the activity of that protein. Similarly, one or more (e.g., 2, 3, 4, 5, 6, 7, 8, 9 or 10) amino acids can often be inserted into a protein without significantly affecting the activity of the protein.

Any variation in the sequence of these proteins is permissible so long as the ability of the variant protein to increase visual function in the XLRS patient is not significantly affected. In this regard, it is appreciated in the art that amino acids can be classified into groups based on their physical properties. Examples of such groups include, but are not limited to, charged amino acids, uncharged amino acids, polar uncharged amino acids, and hydrophobic amino acids. Preferred variants that contain substitutions are those in which an amino acid is substituted with an amino acid from the same group. Such substitutions are referred to as conservative substitutions. Naturally occurring residues may be divided into classes based on common side chain properties, as follows:

- 1) hydrophobic: Met, Ala, Val, Leu, Ile;
- 2) neutral hydrophilic: Cys, Ser, Thr;
- 3) acidic: Asp, Glu;
- 4) basic: Asn, Gln, His, Lys, Arg;
- 5) residues that influence chain orientation: Gly, Pro; and
- 6) aromatic: Trp, Tyr, Phe.

Non-conservative substitutions may involve the exchange of a member of one of these classes for a member from another class.

In making amino acid changes in the protein encoded by the portion of a RS1 ORF, the hydropathic index of amino acids may be considered. Each amino acid has been assigned a hydropathic index on the basis of its hydrophobicity and charge characteristics. The hydropathic indices are: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine

(-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate (-3.5); glutamine (-3.5); aspartate (-3.5); asparagine (-3.5); lysine (-3.9); and arginine (-4.5). The importance of the hydropathic amino acid index in conferring interactive biological function on a protein is generally understood in the art (Kyte et al., 1982, J. Mol. Biol. 157:105-31). It is known that certain amino acids may be substituted for other amino acids having a similar hydropathic index or score and still retain a similar biological activity. In making changes based upon the hydropathic index, the substitution of amino acids whose hydropathic indices are within ± 2 is preferred, those within ± 1 are preferred, and those within ± 0.5 are even more preferred.

It is also understood in the art that the substitution of like amino acids can be made effectively on the basis of hydrophilicity, particularly where the biologically functional equivalent protein or peptide thereby created is intended for therapeutic uses, as in the present case. The greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with its immunogenicity and antigenicity, i.e., with one biological property of the protein. The following hydrophilicity values have been assigned to these amino acid residues: arginine (+3.0); lysine (+3.0); aspartate (+3.0 $\pm$ 1); glutamate (+3.0 $\pm$ 1); serine (+0.3); asparagine (+0.2); glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5 $\pm$ 1); alanine (-0.5); histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine (-1.8); isoleucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); and tryptophan (-3.4). In making changes based upon similar hydrophilicity values, the substitution of amino acids whose hydrophilicity values are within ± 2 is preferred, those within ± 1 are particularly preferred, and those within ± 0.5 are even more particularly preferred.

Desired amino acid substitutions (whether conservative or non-conservative) can be determined by those skilled in the art at the time such substitutions are desired. For example, amino acid substitutions can be used to identify important residues of the therapeutic protein, or to increase or decrease the immunogenicity, solubility or stability of the therapeutic proteins described herein. Exemplary amino acid substitutions are shown in the following table:

Amino Acid Substitutions

Original Amino Acid Exemplary Substitutions

Ala	Val, Leu, Ile
Arg	Lys, Gln, Asn
Asn	Gln
Asp	Glu
Cys	Ser, Ala
Gln	Asn

	Glu	Asp
	Gly	Pro, Ala
	His	Asn, Gln, Lys, Arg
	Ile	Leu, Val, Met, Ala
5	Leu	Ile, Val, Met, Ala
	Lys	Arg, Gln, Asn
	Met	Leu, Phe, Ile
	Phe	Leu, Val, Ile, Ala, Tyr
	Pro	Ala
10	Ser	Thr, Ala, Cys
	Thr	Ser
	Trp	Tyr, Phe
	Tyr	Trp, Phe, Thr, Ser
	Val	Ile, Met, Leu, Phe, Ala

15 As used herein, the phrase “significantly affect a protein’s activity” refers to a decrease in the activity of a protein by at least 20%, at least 30%, at least 40% or at least 50%. Methods of measuring such activities are known to those skilled in the art.

The portion of a RS1 ORF may include a nucleotide sequence at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or identical to at least 500 contiguous
20 nucleotides from a RS1 ORF, wherein the portion encodes a protein that is capable of increasing visual function in a patient suffering from XLRS-related disorders. The portion of a RS1 ORF may include a nucleotide sequence at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or identical to at least 600 contiguous nucleotides from a RS1 ORF, wherein the portion encodes a protein that is capable of increasing visual function in a patient suffering
25 from XLRS-related disorders. The portion of a RS1 ORF may include a nucleotide sequence at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or identical to at least 650 contiguous nucleotides from a RS1 ORF, wherein the portion encodes a protein that is capable of increasing visual function in a patient suffering from XLRS-related disorders. The portion of a RS1 ORF may include a nucleotide sequence at least 85%, at least 90%, at least 95%, at least
30 98%, at least 99%, or identical to SEQ ID NO:3. In specific embodiments, the portion of a RS1 ORF consists of SEQ ID NO:3.

While any portion of a RS1 ORF having one or more of the desired activities described above can be used in the methods of the present disclosure, the inventors have discovered that particular portions of a RS1 ORF may be more suitable than others in studying RS1 proteins

and their interactions, and in increasing visual function in a patient suffering from XLRS-related disorders. Thus, the portion of a RS1 ORF may include a nucleotide sequence at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or identical to any one of SEQ ID NO:3, 6, 10, 14, 18, or 20. Portions of a RS1 ORF region represented by SEQ ID NO:6 is an example of particularly suitable portions. In specific embodiments, the portion of a RS1 ORF consists of SEQ ID NO:6.

RS1 Proteins

This disclosure includes all human RS1 proteins, variants thereof, and fragments thereof, encoded by the RS1 polynucleotides of this disclosure.

As noted above, a polynucleotide of this disclosure encodes at least a portion of a RS1 protein that is capable of increasing the visual function in a patient suffering from XLRS-related disorders. The portion of a RS1 ORF may encode a protein comprising an amino acid sequence at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or identical to at least 200 contiguous amino acid residues from SEQ ID NO:2, wherein the portion of the RS1 protein is capable of increasing visual function in a patient suffering from XLRS-related disorders. The portion of a RS1 ORF may encode a protein comprising an amino acid sequence at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or identical to at least 210 contiguous amino acid residues from SEQ ID NO:2, wherein the portion of the RS1 protein is capable of increasing visual function in a patient suffering from XLRS-related disorders. The portion of a RS1 ORF may encode a protein comprising an amino acid sequence at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or identical to at least 220 contiguous amino acid residues from SEQ ID NO:2, wherein the portion of the RS1 protein is capable of increasing visual function in a patient suffering from XLRS-related disorders.

The RS1 ORF may encode a protein comprising an amino acid sequence at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or identical to SEQ ID NO:2. The RS1 ORF may encode a protein comprising an amino acid sequence at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or identical to any one of SEQ ID NO:4, 7, 11, 15, 19, or 21. The RS1 ORF may encode a protein consisting of an amino acid sequence at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or identical to any one of SEQ ID NO:2, 4, 7, 11, 15, 19, or 21. Thus, this disclosure also provides a RS1 protein, or fragment thereof, or variant thereof, comprising an amino acid sequence at least 85%, at least 90%, at least 95%, at least 97%, at least 99%, or identical to any one of SEQ ID NO:2, 4, 7, 11, 15, 19, or 21. This disclosure also provides a RS1 protein, or fragment thereof, or variant thereof, comprising an amino acid sequence consisting of any one of SEQ ID NO:2, 4, 7, 11, 15, 19, or 21.

These RS1 proteins, or fragments or variants thereof, may have many therapeutic uses (as described in greater detail, below), as well as invaluable uses in research on the RS1 protein chemistry, interactions, and biochemical effects to better understand the many complexities of RS1 and the diseases and disorders affected or caused by RS1. For example, during protein clinical development or manufacturing, biochemical standards are a necessity. These standards may act as a positive control or in establishing a dosage range over which an assay is accurate. In one example, to measure RS1 concentrations in biological samples (such as peripheral blood, aqueous humor, or vitreous humor in the eye) obtained from individuals injected with one or more therapies selected from a gene therapy containing the RS1 cDNA, cells producing RS1 protein, or ocular injections of RS1 protein, a positive control is needed to demonstrate that the assay is working and/or to establish a standard curve of known quantities of RS1 protein in a sample. A source of purified RS1 protein is also needed for use in cell-based in vitro/ex-vivo assays designed to determine the therapeutic, safe, and/or toxic threshold amounts of RS1 protein. The RS1 proteins of this disclosure that contain mutations are also needed for in vitro assays developed to study the role of particular RS1 mutations on protein function, and the biochemical pathways affected by these mutations. The RS1 proteins of this disclosure, in particular those containing a His tag, may be used in protein interaction studies (like pull down assays) and proteomics to better understand RS1 protein-protein interactions. The RS1 proteins of this disclosure in studies designed to better understand the growth of retinal cells. It is difficult to grow primary retinal cells in vitro and such studies can be used to analyze whether RS1 protein helps in faster or better growth and morphological expansion of retinal cells in vitro. If these RS1 proteins increase the rate or extent of growth, attachment, expansion, and/or differentiation of retinal cells in vitro, the RS1 protein could be used as a matrix protein for primary retinal cell culture.

Another example is the development of an ELISA assay for use in monitoring the level of antibodies that are generated in an individual administered an RS1 gene therapy. To assess whether such individuals produce antibodies to RS1 protein post administration of the gene therapy, an ELISA assay would be created in which purified RS1 protein is coated on plates, patient serum is applied to the plates, and the plates are washed. If the individual's serum contains anti-RS1 protein antibodies, those antibodies are retained on the plates and may be detected, typically with a fluorescent tag or other colorimetric signal. Thus, a source of purified RS1 protein is required for such ELISA assays. Another example is the application of the recombinant RS1 protein and RS1-transfected ARPE-19 cells to elucidate the molecular mechanisms, such as RS1-membrane interactions, underlying RS1 function in the retina. RS1 is

an extracellular protein that plays a crucial role in the cellular organization of the retina. In X-linked retinoschisis (XLRs), caused by loss of function mutations in X-linked RS1 gene, the retina splits into two layers, leading to progressive decline in vision in young males. This schisis pathology, has been attributed to the loss of RS1 function as a cell-cell adhesion protein. The double octameric structure of RS1 revealed by cryo-EM agrees with the predicted function. However, there is no direct *in vivo* evidence that RS1 is a cell-cell adhesion protein. Thus, the purified RS1 protein, the human cells expressing the recombinant RS1 protein, and animal models comprising these cells and RS1 proteins, or fragments or variants thereof, will provide *in vitro* and *in vivo* models of the molecular and structural functions of the RS1 protein.

Expression cassette

An expression cassette is a distinct component of vector DNA consisting of a gene and regulatory sequence to be expressed by a transfected cell. In successful transformants, the expression cassettes of this disclosure direct the human cell to make RS1 mRNA and RS1 proteins. Thus, within the retinoschisin expression cassettes of this disclosure, the protein coding portion of these expression cassettes is composed of a polynucleotide sequence encoding human retinoschisin protein, or variants thereof, or fragments thereof. In these expression cassettes, the protein coding portion may include a polynucleotide sequence at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or identical to any one of SEQ ID NO:3, 6, 10, 14, 18, or 20. The protein coding portion represented by SEQ ID NO:6 is an example of particularly suitable RS1 coding portion. Within these expression cassettes, the protein coding portion may consist of the polynucleotide sequences SEQ ID NO:3 or 6. Within these expression cassettes, the protein coding portion may encode a human RS1 protein, or fragment thereof, or variant thereof, at least 85%, at least 90%, at least 95%, at least 98%, at least 99%, or identical to the amino acid sequence of any one of SEQ ID NO:2, 4, 7, 11, 15, 19, or 21. Within these expression cassettes, the protein coding portion may encode a human RS1 protein, or fragment thereof, or variant thereof, consisting of the amino acid sequence of any one of SEQ ID NO:2, 4, 7, 11, 15, 19, or 21.

Lentivirus and adeno-associated virus (AAV) expression cassettes encoding human proteins can be designed for target specificity and protein expression levels, which can be enhanced using cell-specific promoters or enhancers. For example, transgene expression levels can be enhanced by including the CMV promoter and enhancers (that includes transcription factor binding sites) in the expression cassette, or mRNA stability/nuclear export cis-acting elements, such as introns, polyA signals, or post-transcriptional regulatory elements (PREs). Additionally, a Kozak consensus sequence may be included as the optimum protein translation

initiation site for the human RS1 ORF. Expression cassettes designed for AAV and lentivirus vectors require thoughtful design due to foreign DNA packaging size constraints of AAV and lentivirus.

In the expression cassettes of this disclosure, the choice of promoter is an important cis-acting element that can dictate the overall strength of the human RS1 expression. In these expression cassettes, the promoter is chosen to drive high-level expression of the human RS1 protein in a human cell. The promoter may be an eye-specific promoter. For example, the promoter may be a retinoschisin promoter, or a functional fragment thereof that has retinoschisin gene promoter activity, a rhodopsin promoter, a rhodopsin kinase promoter, a CRX promoter, or an interphotoreceptor retinoid binding protein (IRBP) promoter. Additionally or alternatively, the promoter may be a non-tissue specific promoter or constitutive promoter, such as a cytomegalovirus (CMV) promoter, such as a CMV intermediate-early (IE) promoter. Additionally or alternatively, the promoter may be a human elongation factor 1 α -subunit (EF-1 α), a chicken β -actin (CBA) and its derivative CAG, a β -glucuronidase (GUSB), or ubiquitin C (UBC), or enhancer-less ubiquitously acting chromatin opening element (UCOE) promoter. In these expression cassettes, the promoter sequence is functionally linked to the RS1 ORF. As used herein, the term functionally linked refers to the fact that the promoter is connected to a polynucleotide sequence containing an open-reading frame such that when the construct is placed into the appropriate conditions, the promoter causes transcription (expression) of the codon-optimized human RS1 open reading frame.

In these expression cassettes, one or more viral post-transcriptional regulatory elements (PREs) may be included to enhance the human RS1 ORF expression. When functionally linked to the promoters and RS1 ORF, these cis-acting elements may enhance nuclear export of intronless RNA. For example, HPRE (Hepatitis B Virus PRE, 533 bps) or WPRE (Woodchuck Hepatitis Virus PRE, 600 bps) may be included in these cassettes to increase the expression of the human RS1 ORF and/or prevent long-term silencing of expression that may be seen in cultured human cells, particularly when using lentiviral and AAV vectors.

In these cassettes, a polyadenylation (polyA) signal sequence may be useful for nuclear export, translation, and RS1 mRNA stability, thereby boosting expression of the RS1 ORF. PolyA signal strength has been shown to be generally independent of cell type. Thus, the expression cassettes of this disclosure may include a functionally linked polyA signal sequence selected from the late SV40, the bGHpA, or the minimal SPA polyA signal sequences.

In these expression cassettes, the efficiency of polyadenylation may be increased by the addition of a polyA signal upstream enhancer (USE) placed upstream of the polyA signals.

Thus, the expression cassettes of this disclosure may include one or more polyA signal upstream enhancer (USE) sequence(s), such as the SV40 late polyA signal upstream enhancer.

In these expression cassettes, an enhancer may be included and functionally linked to increase promoter-driven RS1 ORF expression. For example, the CMV enhancer, which contains transcription binding sites, has been shown to increase transgene expression under different cell-specific promoters and different cell types making it a broadly applicable tool to increase transgene expression levels. Thus, in the expression cassettes of this disclosure, a CMV enhancer may be placed upstream or downstream (or both) of the promoter to drive increased expression of the RS1 ORF.

In these expression cassettes, an intron may be included and functionally linked for mRNA processing and increased RS1 ORF expression. For example, an SV40 intron may be placed between the promoter and the RS1 ORF, in the expression cassette to increase RS1 ORF expression under the CMV promoter and enhancer. Exemplary introns that may be placed between the promoter and the RS1 ORF in the expression cassettes of this disclosure include an MVM (minute virus of mice) intron, a F.IX truncated intron 1, or a hybrid or chimeric intron, such as a human β -globin SD / immunoglobulin heavy chain acceptor, or Adenovirus splice donor / immunoglobulin splice acceptor intron, or SV40 late splice donor / splice acceptor (19S/16S) intron, or Hybrid adenovirus splice donor / IgG splice acceptor.

To construct an exemplary expression cassette of this disclosure, we synthesized a codon-optimized human RS1 cDNA having the polynucleotide sequence of SEQ ID NO:3 in frame with 6xHis tag (as a method of tagging proteins for purification) with a NotI restriction site at the 5' end and XhoI restriction site at the 3' end, and subcloned into a pUC57 vector. This construct was further subcloned into pCMV Tag 4A vector. The resulting expression cassette encodes the cytomegalovirus (CMV) promoter for constitutive expression of the cloned RS1 ORF in a wide variety of mammalian cell lines with a c-terminal Flag tag. But we inserted the 6X His tag followed by stop codon immediately downstream of the RS1 ORF to silence the Flag epitope (SEQ ID NO:17). We also introduced a Kozak consensus sequence (GCCACC) upstream of ATG start codon to provide optimal expression of the human RS1-His fusion protein.

Expression Vectors

Polynucleotide molecules of this disclosure are useful within expression vectors of this disclosure that are used to transfect and transduce human cells, which express the human RS1 proteins, and fragments thereof, and variants thereof. The transfected human cells may be cultured *in vitro* or may be a cell in the eye of a human patient suffering from XLRs-related

disorders, such that the human RS1 protein is delivered to cells of the eye. An expression vector of the present disclosure is produced by human intervention and can be DNA, RNA, or variants thereof. The expression vector may be a linear molecule (e.g., a linear nucleic acid molecule, a linear viral genome, etc.) or it may be a circular molecule such as a plasmid. In one embodiment, an expression vector may comprise one or more nucleic acid sequences from an adeno-associated virus (an AAV vector), a cytomegalovirus (CMV) (a CMV vector), a retrovirus, an adenovirus, a herpes virus, a vaccinia virus (a vaccinia vector), a poliovirus, a Sindbis virus, or any other DNA or RNA virus. The expression vector may be a DNA molecule, either linear or circular, comprising nucleic acid sequences from a plasmid and nucleic acid sequences from a viral genome to enable nucleic acid molecule delivery and high-level expression of the encoded therapeutic molecule.

As explained above, polynucleotide molecules of this disclosure include a promoter sequence that is functionally linked to the RS1 ORF. Thus, preferred polynucleotides used in the expression vectors of this disclosure may be any one or more of the expression cassettes of this disclosure, described above. Therefore, an aspect of this disclosure is an expression vector comprising at least one polynucleotide molecule of this disclosure, as described above. As used herein, a vector is any agent comprising a polynucleotide molecule of this disclosure that can be used to deliver an RS1 ORF into a human cell. Examples of suitable vectors include, but are not limited to, plasmids, cosmids, phage, and viruses. The vector may be a virus (i.e., a “viral vector”). The vector may be a targeting vector (i.e., a “targeting construct”). As used herein, a targeting vector refers to a polynucleotide molecule that comprises a targeting region. A targeting region comprises a sequence that is identical or substantially identical to a sequence in a target cell or tissue and provides for integration of the targeting construct (and/or a polynucleotide sequence contained therein) into a position within the genome of the cell or tissue via homologous recombination. Targeting regions that target into a position of the cell or tissue via recombinase-mediated cassette exchange using site-specific recombinase recognition sites (e.g., loxP or Frt sites) are also included. These targeting vectors may further comprise a polynucleotide sequence or gene (e.g., a reporter gene, homologous gene, heterologous gene, or mutant gene) of particular interest, such as a selectable marker, control and/or regulatory sequences, and other nucleic acid sequences that encode a recombinase or recombinogenic polypeptide. In some embodiments, a targeting construct may comprise a gene of interest in whole or in part, wherein the gene of interest encodes a polypeptide, in whole or in part, that has a similar function as a protein encoded by an endogenous sequence. In some embodiments, a targeting construct may comprise a mutant gene of interest, in whole or in part,

wherein the mutant gene of interest encodes a variant polypeptide, in whole or in part, that has a similar function as a polypeptide encoded by an endogenous sequence. In some embodiments, a targeting construct may comprise a reporter gene, in whole or in part, wherein the reporter gene encodes a polypeptide that is easily identified and/or measured using techniques known in the art.

Those skilled in the art appreciate that in some instances, packaging of heterologous DNA into a virus requires specific sequences from the DNA of the virus into which the heterologous DNA is being packaged. Thus, a polynucleotide molecule of this disclosure may include viral DNA that allows for packaging of the isolated DNA molecule into a virus. Any viral vector can be used to package RS1 polynucleotides of this disclosure, so long as the virus is capable of containing the DNA and delivering it to a human cell.

One example of a suitable viral vector for use in the present invention is adeno-associated virus. Adeno-associated viruses are small, replication-defective, non-enveloped viruses that belong to the family Parvoviridae. The Parvoviridae family is characterized by having a single-stranded linear DNA genome of about 4,800 nucleotides and a small icosahedral shaped capsid measuring about 20 nm in diameter. The AAV genome contains two open reading frames called 'rep' and 'cap.' The rep ORF encodes all of the non-structural proteins that are necessary for replication and packaging of the viral genome, while the cap ORF encodes the viral capsid proteins. The viral capsid proteins are the structural proteins of the virus and assemble into the viral particle. The AAV genome is terminated at each end by an inverted terminal repeat (ITR) of approximately 150 nucleotides in length. The sequences of the ITRs are palindromes that fold back on themselves to form T-shaped hairpin structures. Each ITR contains a Rep binding site (RBS) and a sequence referred to as the terminal resolution site (trs), which is cleaved by the viral Rep protein. These sequences in the ITR are important for replication and packaging of the viral genome. Thus, the ITRs can be combined with DNA molecules of the present invention to produce nucleic acid molecules that can be packaged into AAV particles and/or virus-like particles. Similar use of ITRs is described in U.S. Patent No. 8,927,269, the entirety of which is incorporated herein by reference. Thus, a polynucleotide molecule of this disclosure may be flanked by ITR sequences, wherein at least one ITR comprises AAV sequences that allow packaging of the DNA molecule into an AAV particle. The AAV sequences may be from an AAV ITR. At least one ITR may include an AAV RBS and a trs. The ITRs can contain sequence from any AAV, so long as the virus strain from which the ITRs are obtained is capable of delivering the packaged DNA into a human cell. A polynucleotide molecule of this disclosure may be flanked by ITRs from a virus selected from the group

consisting of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9 and AAV10.

Accordingly, one aspect of this disclosure is a polynucleotide molecule of this disclosure flanked by ITRs. Another aspect of this disclosure is a nucleic acid molecule comprising: a) a pair of inverted terminal repeats (ITRs), each of which is capable of forming a T-shaped hairpin

structure, wherein at least one inverted terminal repeat comprises an AAV RBS and an AAV trs; and, b) a polynucleotide sequence between the terminal repeats, wherein the polynucleotide sequence encodes at least a human RS1 protein, a fragment thereof, or a variant thereof.

Examples of suitable portions of a RS1 protein have been described above, and include, without limitation, any one of SEQ ID NO: 4, 7, 11, 15, 19, or 21.

Another example of a suitable viral vector for use in the present invention is lentivirus. Lentiviral vectors are another promising new tool for the establishment of transgenic cells and the manipulation of the mammalian genome. Lentiviral vectors are useful vectors for transgenesis in part because of their ability to incorporate into genomic DNA with high efficiency, especially in cells that are not actively dividing. Lentiviral vector-mediated transgene expression can also be maintained for long periods of time. High efficiencies for lentiviral transgenesis, even in animal species and strains that are very difficult to manipulate using the standard transgenic techniques, have been demonstrated. These advantages of the lentiviral vector system have broadened its use as a gene therapy vector to additional applications that include transgenesis and functional genetics.

It is well established in the art that the efficiency of delivery of nucleic acid molecules into cells can be increased using a delivery means such as a viral particle. Moreover, polynucleotide molecules of this disclosure that comprise viral packaging sequences may be packaged into viral particles for use in delivering the RS1 ORF to a human cell, which cell may be in the eye of a patient in need of treatment for XLRS-related disorders. As used herein, a viral particle refers to a particle comprising capsid proteins from one or more viruses, and which can encapsulate, or contain, isolated DNA containing the appropriate packaging sequences. Thus, this disclosure provides viral particles, such as AAV particles or lentivirus particles, comprising a polynucleotide molecule of this disclosure.

As described in greater detail below, any of the nucleic acid molecules described herein can be used in the manufacture of one or more human cells that are genetically engineered to produce the RS1 polypeptides of this disclosure for treating ophthalmic disorders by implantation into the eye of a patient as an isolated cell or within an implantable cell culture device, thereby allowing the RS1 protein to diffuse from the genetically engineered cells into the eye.

Compositions

Another aspect of this disclosure provides compositions including nucleic acid molecules, or RS1 proteins, or viral particles of this disclosure. Such compositions include physiologically acceptable solutions that comprise, for example, water, saline, salts, buffers, diluents, stabilizing agents, polymers, chelating agents, and the like. One example of a physiologically acceptable solution is a solution comprising about 10 mM Tris-HCl (pH 7.4) and about 180 mM NaCl. It will be appreciated by those skilled in the art that such concentrations are approximate and may vary by as much as 10% or more, without significant effect on the efficacy or stability of the composition.

Purified RS1 protein and methods of making/purifying

This disclosure also provides the use of one or more human cells (in particular, ARPE-19 cells) that are genetically engineered to produce any of the RS1 polypeptides of this disclosure (e.g., any one of SEQ ID NO:4, 7, 11, 15, 19, or 21). These cells may also be used in the manufacture of any of the implantable cell culture devices of this disclosure for treating XLRs-related disorders, for example, by implantation of the device into the eye of the patient.

Human cell lines for production of RS1

This disclosure also provides host cells or cell lines containing the polynucleotides, expression cassettes, and/or expression vectors of this disclosure. Human cells useful in the methods of producing and delivering the RS1 proteins of this disclosure preferably: are hardy under stringent conditions (the cells should be functional in the eye, especially in the intra-ocular environment), able to be genetically modified (the desired therapeutic factors needed to be engineered into the cells), have a relatively long life span (the cells should produce sufficient progenies to be banked, characterized, engineered, safety tested and clinical lot manufactured), exhibit greater than 80% viability for a period of more than one month *in vivo* (which ensures long-term delivery), produce and deliver an efficacious quantity of RS1 protein(s) (which ensures effectiveness of the treatment), induce a low level of host immune reaction, and are nontumorigenic (to provide added safety to the host). An exemplary human cell line for use in the methods and devices of this disclosure are the ARPE-19 cell line (described in Finnemann et al., 94 Proc. Natl. Acad. Sci. USA 12932-7 (1997), and U.S. Pat. No. 6,361,771). ARPE-19 cells are normal retinal pigmented epithelial (RPE) cells and express the retinal pigmented epithelial cell-specific markers CRALBP and RPE-65, and form stable monolayers, which exhibit morphological and functional polarity, demonstrate all of these preferable characteristics of a

useful human cell line, and are available from the American Type Culture Collection (ATCC Number CRL-2302).

As used herein, "host cell" and "recombinant host cell" are used interchangeably to refer not only to the particular subject cell but to the progeny or potential progeny of such cells.

5 Because certain modifications may occur in succeeding generations of these host cells, due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used in this disclosure.

A host cell can be any eukaryotic cell that can effectively support the production of human RS1 proteins, or fragments thereof, or variants thereof. Any eukaryotic cell type can be
10 used as a host cell, as long as the cell is capable of supporting transcription and translation of a polynucleotide molecule of this disclosure to produce a human RS1 protein, or fragment thereof, or variant thereof. Non-limiting examples of suitable mammalian host cells include cell lines such as ARPE-19, HEK-293 (293), Vero, RD, BHK-21, HT-1080, A549, Cos-7, and MRC-5 cells. A preferred cell line for production of human RS1 proteins of this disclosure is the ARPE-
15 19 cell line.

Vector DNA can be introduced into eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation,
20 DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (Molecular Cloning: A Laboratory Manual. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the
25 expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., resistance to antibiotics) is generally introduced into the host cells along with the human RS1 protein-encoding polynucleotides of this disclosure. Various selectable markers include those that confer resistance to drugs, such as G418, hygromycin, or
30 methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding the human RS1 protein, or can be introduced on a separate vector. Cells stably transfected with the RS1 polynucleotide can then be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the remaining cells are killed). Additionally or alternatively, cells transfected human cells that

express a variant or fragment RS1 protein that reduces or eliminates the activity of RS1 may be selected using methods that include, but are not limited to, Southern blot analysis, DNA sequencing, PCR analysis, or phenotypic analysis. Such cells may then be employed in various methods and compositions described in this disclosure.

5 A host cell of this disclosure, such as a eukaryotic host cell in culture, can be used to produce (i.e., express) the human RS1 protein, or fragments thereof, or variants thereof. Accordingly, this disclosure also provides methods for producing these human RS1 proteins using the host cells of this disclosure. These methods comprise culturing a host cell of this disclosure (into which a recombinant expression vector encoding human RS1 proteins have
10 been introduced) in a suitable medium such that the human RS1 proteins of this disclosure are expressed. The medium may include serum or may be serum-free liquid cell culture media. These methods may further include isolating the human RS1 proteins of this disclosure from the medium or the host cell.

Similarly, this disclosure also provides cell lines of ARPE-19 cells genetically engineered
15 to produce the human RS1 proteins of this disclosure, wherein the human RS1 proteins of this disclosure are encoded by a polynucleotide molecule of this disclosure. In these ARPE-19 cells, the polynucleotide molecule may have the nucleotide sequence of any one of SEQ ID NO:3, 6, 10, 14, 18, or 20.

These recombinant human cells can be isolated and used on an ad hoc basis or can be
20 maintained in culture for many generations. These human cells described herein may be immortalized (e.g., via use of a virus, cell fusion, etc.) and maintained in culture indefinitely (e.g., in serial cultures).

These human cells provide an improved *in vivo* system and source of biological materials (e.g., RS1 proteins, fragments thereof, variants thereof) that are useful for a variety of
25 assays. In various embodiments, the recombinant human cells described herein are used to develop therapeutics that treat, prevent, and/or inhibit one or more symptoms associated with a lack of RS1 expression and/or activity and/or variant RS1 polypeptide expression or activity (e.g., gene therapy/replacement). These variant RS1 polypeptides may be used in various assays to determine the functional consequences on retinal structure and development.

30 These recombinant human cells may also provide an *in vivo* system for identifying a therapeutic agent for treating, preventing, and/or inhibiting progressive vision loss resulting from XLRS-related disorders. In these methods, an effect of a therapeutic agent is determined *in vitro* by contacting a potential therapeutic agent with a recombinant human cell whose genome comprises an RS1 polynucleotide molecule, as described herein. Similarly, these recombinant

human cells may provide *in vitro* models that translate to conditions characterized by a breakdown of cell-to-cell adhesion in the neurosensory layers of one or both retinas. Similarly, these recombinant human cells may be used to identify, screen, and/or develop candidate therapeutics that affect (e.g., enhance) photoreceptor function. Similarly, these recombinant human cells may be used to determine the efficiency or efficacy of retinoschisin gene delivery to retinal cells, which may include determination and/or optimization of vectors designed to deliver retinal gene therapies that encode an RS1 polypeptide. The recombinant human cells of this disclosure that express mutant RS1 proteins may also be used *in vitro* to screen drug compounds, immune suppressants, and/or other target drugs to determine or anticipate their therapeutic or toxic effects *in vivo* in certain XLRN-related diseases or disorders in which these mutations are present. The recombinant human cells of this disclosure that express mutant RS1 proteins may also be used in gene expression analysis to study biochemical pathways and the differential expression of genes using the wild type and mutant RS1 protein-expressing cells, for example using RNASeq analysis.

As described below, these recombinant human cells may be used to produce RS1 polypeptides, which are in turn used for therapeutic purposes in methods of administering RS1 polypeptides to a subject. Additionally or alternatively, these recombinant human cells may be used for therapeutic purposes in which the cells themselves are administered to a subject. Thus, this disclosure also provides one or more human cells (preferably ARPE-19 cells) that are genetically engineered to produce any of the RS1 polypeptides of this disclosure for treating ophthalmic disorders (such as XLRN-related disorders) by implantation into the eye of a patient and by allowing the encoded RS1 protein(s) to diffuse into the eye.

In these methods, the cells may be administered to a subject in the form of an implantable cell culture device having a core containing any of the recombinant human cell lines of this disclosure genetically engineered to express the RS1 polypeptides of this disclosure, and a semipermeable membrane surrounding the core, wherein the membrane permits diffusion of the RS1 polypeptides therethrough. The therapeutic use of cells encapsulated in a semipermeable membrane is based on the concept of isolating cells from the recipient host's immune system by surrounding the cells with a semipermeable biocompatible material before implantation within the host. For example, this disclosure provides a device in which the recombinant human cells of this disclosure are encapsulated in an immunoisulatory capsule, which, upon implantation into a recipient host, minimizes the deleterious effects of the host's immune system on the cells in the core of the device. The cells are therefore immunoisolated from the host by enclosing them within an implantable polymeric capsule formed by a

microporous membrane. This approach prevents the cell-to-cell contact between the host and implanted tissues, thereby eliminating antigen recognition through direct presentation. The advantage of this mode of RS1 protein delivery includes the ability to deliver a low, continuous, and therapeutic dose of the protein. Moreover, because it is administered directly to the eye, this delivery device can potentially deliver an effective, yet much lower, dose than bolus dosing of drops, thereby potentially avoiding or reducing untoward side effects. Additionally, these devices may provide constant and continuous even dosing, which can potentially eliminate dosing fluctuations.

In these devices, the preferred cell is a recombinant ARPE-19 cell line that expresses one or more of the RS1 polypeptide encoding polynucleotides of this disclosure. When ARPE-19 cells are used in these implantable cell culture devices, typically between 100,000 and 100 million of these recombinant ARPE-19 cells are encapsulated in each device, and more typically, between 200,000 and 400,000 of these recombinant ARPE-19 cells are encapsulated in each device. Thus, methods of modifying the dosage of the dose of the RS1 proteins of this disclosure delivered to the eye of a subject include implanting fewer or greater number of devices, or fewer or greater numbers of cells in each device. Preferably, between 1 and 50 devices are implanted per eye.

A variety of biocompatible capsules are suitable for delivery of RS1 proteins of this disclosure. Useful biocompatible polymer capsules comprise a core which contains a recombinant human cell (i.e. a human cell transformed with one or more of the polynucleotides of this disclosure) or cells, either suspended in a liquid medium or immobilized within a biocompatible matrix, and (b) a surrounding jacket comprising a membrane which does not contain isolated cells, which is biocompatible, and permits diffusion of the cell-produced biologically active molecule into the eye. The cells are advantageously isolated within a capsule having a liquid core that may further optionally include a nutrient medium, and/or a source of additional factors to sustain cell viability and function. For example, the core of the devices may function as a reservoir for growth factors, growth regulatory substances, and/or nutrient-transport enhancers. Certain of these substances are also appropriate for inclusion in liquid media.

These devices can also be used as a reservoir for the controlled delivery of needed drugs or biotherapeutics, such that the core contains a high concentration of the selected drug or biotherapeutic (alone or in combination with cells or tissues).

In these devices, the core may comprise a biocompatible matrix of a hydrogel or other biocompatible material (e.g., extracellular matrix components) which stabilizes the position of

the cells. The term "hydrogel" herein refers to a three-dimensional network of cross-linked hydrophilic polymers. Any suitable matrix or spacer may be employed within the core, including precipitated chitosan, synthetic polymers and polymer blends, microcarriers and the like, depending upon the growth characteristics of the human cells to be encapsulated. Alternatively or additionally, the capsule may have an internal scaffold to prevent cells from aggregating and improve cellular distribution within the device.

All components of these devices may be composed of biocompatible materials. Thus, a surrounding semipermeable membrane and an internal cell-supporting scaffolding may be formed from biocompatible materials. In such device, the recombinant human cells may be seeded onto the scaffolding, which is encapsulated by the permselective membrane. Exemplary biocompatible and/or biodegradable polymers useful in forming all or part of the encapsulation delivery devices of this disclosure include those comprised of poly(lactic acid) PLA, poly(lactic-coglycolic acid) PLGA, and poly(glycolic acid) PGA and their equivalents, polyacrylates (including acrylic copolymers), polyvinylidenes, polyvinyl chloride copolymers, polyurethanes, polystyrenes, polyamides, cellulose acetates, cellulose nitrates, polysulfones (including polyether sulfones), polyphosphazenes, polyacrylonitriles, poly(acrylonitrile/covinyl chloride), as well as derivatives, copolymers and mixtures thereof. Preferably, the surrounding semipermeable membrane is a biocompatible semipermeable hollow fiber membrane. Methods of making such implantable delivery devices are disclosed by U.S. Pat. Nos. 6,361,771; 5,639,275; 5,653,975; 4,892,538; 5,156,844; 5,283,138; and 5,550,050, each of which is incorporated herein by reference.

These implantable delivery devices capsule can be any configuration appropriate for maintaining biological activity and providing access for delivery of the product or function, including for example, cylindrical, rectangular, disk-shaped, patch-shaped, ovoid, stellate, or spherical. Moreover, the capsule can be coiled or wrapped into a mesh-like or nested structure. Certain shapes, such as rectangles, patches, disks, cylinders, and flat sheets offer greater structural integrity and are preferable where retrieval is desired. These devices may have a tether that aids in maintaining device placement during implant, and aids in retrieval. Such a tether may have any suitable shape that is adapted to secure the capsule in place. For example, the suture may be a loop, a disk, or a suture.

The surrounding or peripheral membrane, which surrounds the core of these delivery devices may be permselective, biocompatible, and/or immunoisulatory, and is produced free of isolated cells, and completely surrounds (i.e., isolates) the core, thereby preventing contact between any cells in the core and the recipient's body. Biocompatible semi-permeable hollow

fiber membranes, and methods of making them are disclosed in U.S. Pat. Nos. 5,284,761 and 5,158,881 (See also, WO 95/05452), each of which incorporated herein by reference in its entirety.

To be permselective, the outer, surrounding membrane of these devices is formed in such a manner that it has a molecular weight cut off ("MWCO") range appropriate both to the type and extent of immunological reaction anticipated to be encountered after the device is implanted and to the molecular size of the largest RS1 protein for which passage into and out of the device and into the eye is desirable. Thus, the outer membrane of these devices allows passage into the eye of RS1 proteins up to a predetermined size, but prevents the passage of larger substances. More specifically, the surrounding or peripheral region is produced in such a manner that it has pores or voids of a predetermined range of sizes, and, as a result, the device is permselective. The MWCO of the surrounding jacket must be sufficiently low to prevent access of the substances required to carry out immunological attacks to the core, yet sufficiently high to allow delivery of RS1 proteins of this disclosure to the recipient's eye. Preferably, the MWCO of the biocompatible outer membrane of these devices is from about 1 kD to about 150 kD. In some embodiments, the outer membrane of these devices may be either an ultrafiltration membrane or a microporous membrane. Those skilled in the art will recognize that ultrafiltration membranes are those having a pore size range of from about 1 to about 100 nanometers while a microporous membrane has a range of between about 0.05 to about 10 microns. The thickness of this outer membrane can vary, but it will always be sufficiently thick to prevent direct contact between the cells and/or substances on either side of the barrier. The thickness of this region generally ranges between 5 and 200 microns; thicknesses of 10 to 100 microns are preferred, and thickness of 20 to 50 or 20 to 75 microns are particularly preferred. Those skilled in the art will recognize that outer membranes with permselective, immunoisulatory membranes are preferable for sites that are not immunologically privileged. In contrast, microporous membranes or permselective membranes may be suitable for immunologically privileged sites. For implantation into immunologically privileged sites, capsules made from polyether sulfone or polysulfone membranes.

The external surface of the outer membrane of these devices can be selected or designed in such a manner that it is particularly suitable for implantation at a selected site. For example, the external surface can be smooth, stippled, or rough, depending on whether attachment by cells of the surrounding tissue is desirable. The shape or configuration can also be selected or designed to be particularly appropriate for the implantation site chosen.

Any suitable method of sealing these delivery devices known in the art may be used, including the employment of polymer adhesives and/or crimping, knotting, and heat sealing. In addition, any suitable "dry" sealing method can also be used. In such methods, a substantially non-porous fitting is provided through which the cell-containing solution is introduced, and subsequent to filling, the delivery device is sealed. Such methods are described in, e.g., U.S. Pat. Nos. 5,653,688; 5,713,887; 5,738,673; 6,653,687; 5,932,460; and 6,123,700, each of which are herein incorporated by reference.

Methods of Treatment

One aspect of this disclosure provides methods of treating a patient having a XLRN-related disorder. One major problem in treatment of such diseases is the inability to deliver adequate amounts of therapeutic agents into the eye, due to the presence of the blood-retinal barrier, and to maintain them there at therapeutically effective concentrations. The treatment methods of this disclosure include administering to the patient's eye a RS1 protein of this disclosure. As used herein, the terms "patient," "individual" and "subject" are used interchangeably to refer to any human in need of treatment of a disease of the eye, in particular XLRN-related disorders. The terms patient, individual, and subject by themselves do not denote a particular age, sex, race, and the like. Thus, individuals of any age, whether male or female, are intended to be covered by the present disclosure and include, but are not limited to the elderly, adults, children, babies, infants, and toddlers. Likewise, the methods of the present invention can be applied to any race, including, for example, Caucasian (white), African-American (black), Native American, Native Hawaiian, Hispanic, Latino, Asian, and European.

A related aspect of this disclosure provides methods of improving the visual function of a patient having an XLRN-related disorder comprising administering a RS1 protein of this disclosure to a patient in need thereof. As used in this disclosure, improvements in visual function may include increased retinal synaptic transmission of neural signals that may increase dark-adapted absolute visual perception, increased b-wave response (from second-order retinal bipolar cells) relative to the photoreceptor a-wave measured on clinical electroretinogram (ERG) testing, lower absolute or relative rate of retinal detachment in the XLRN patient population, improvement of the disease phenotype, including restoration of the normal positive ERG b-wave and a reduction of the cyst-like structures that are characteristic of XLRN disease, improvement in retinal function, visual acuity or reduction in nystagmus, statistically-significant improvement in ERG a-wave amplitudes, and/or statistically significant improvement in ERG b-wave amplitudes compared to untreated eyes, significant improvement in ERG a- and b-wave amplitudes compared to untreated eyes, and statistically significant improvement in schisis

cavity scoring relative to untreated eyes (typically when evaluated following days or weeks post-treatment).

Any method that delivers an RS1 polypeptide of this disclosure into the eye of the patient can be employed in these treatment methods. For example, in one embodiment, the RS1 polypeptide is delivered as “naked” protein (i.e., the RS1 polypeptide may be injected into the eye such that it is taken up by the appropriate cells of the eye). Alternatively, the RS1 polypeptide may first be mixed with a suitable carrier to form a composition of this disclosure. Following injection of the composition into the eye, the RS1 protein enters the cells of the eye. Methods of injecting therapeutic molecules, including proteins, into the eye are known to those skilled in the art.

Alternatively or additionally, RS1 polypeptides of this disclosure may be administered as a recombinant human cell of this disclosure (i.e., a human cell transformed with an RS1-encoding polynucleotide of this disclosure). Thus, these treatment methods comprise a method of treating a patient having an XLRs-related disorder by administering to the eye of a patient an RS1 protein, or fragment thereof, or variant thereof, and/or recombinant human cell of this disclosure. Such administration may result in delivery of the RS1 protein and subsequent improvement in visual function.

In these treatment methods, the administration of the RS1 protein may be made by implanting the implantable cell culture devices of this disclosure into the eye of a patient and allowing the RS1 proteins of this disclosure to diffuse from the device into the eye, thereby treating the disorder. Treatment of XLRs-related disorders according to these methods may require only one or at most less than 50 implanted devices per eye to supply an appropriate therapeutic dose of RS1 protein.

In these treatment methods, the RS1 proteins either alone or in a human cell or in an encapsulated form, may be injected into the eye. This administration may include subconjunctival, sub-Tenon's, intravitreal, subretinal, intracameral, or alternative mode of injection. Such injections can deliver an RS1 protein to the intraocular fluid or to a location within the retina.

In these treatment methods, the RS1 proteins, either alone or in a human cell or in an encapsulated form, may be administered to the eye of a patient receiving gene therapy for an XLRs-related disorder. In these methods, the gene therapy may include the administration of an expression vector encoding the human RS1 protein (see, for example, International Patent Application No. PCT/US2019/014418, which is incorporated herein by reference in its entirety).

Another aspect of this disclosure provides methods of treating XLRs-related disorders in a human including administering to a human subject diagnosed with, or suspected of having, or being at risk of developing XLRs-related disorders, a therapeutically-effective amount of an RS1 protein, wherein administration of the protein reduces at least one symptom of the disorder. The RS1 protein may be administered as naked protein, or within a composition of this disclosure, or within a recombinant human cell of this disclosure, or encapsulated within a delivery device of this disclosure. The vector may be administered as a viral particle. In these methods, the administration may be made using intravitreal, subretinal or subtenon or alternative injection techniques.

The present invention also provides kits for practicing the disclosed methods. Kits of the present invention may include polynucleotides of this disclosure (such as RS1 protein-encoding polynucleotides, and/or expression cassettes, and/or expression vectors of this disclosure), or human cells of this disclosure, or encapsulated cells of this disclosure, or encapsulation delivery devices of this disclosure. Such kits may also include reagents and tools necessary for practicing these treatment methods, such as buffers, diluents, syringes, needles, and instructions for administering such reagents.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

EXAMPLES

Example 1

Construction of high expression Retinoschisin vector

An expression vector was designed to express the gene of interest: Retinoschisin comprising mutated Rs1 and discoidin domains.

We chose the heterologous ARPE19 cells as a host organism to overexpress the human RS1 protein for therapeutic use and for understanding protein-protein interactions. ARPE-19 cells do not naturally express the *RS1* gene. Normally, the RS1 protein is selectively expressed by the retinal photoreceptor and bipolar cells and in the pineal gland. Human RS1 protein likely expresses poorly in ARPE-19 cells because proteins are often difficult to express outside their original host due to codon usage bias. Codon usage bias refers to differences in the frequency of occurrence of synonymous codons in coding DNA. Most amino acids can be encoded by multiple synonymous codons, but these codons occur with different frequencies in different organisms (see, [genscript.com/tools/codon-frequency-table](https://www.genscript.com/tools/codon-frequency-table)). For example, there are six codons for the amino acid serine, and in mammalian expression systems these are used at different frequencies as shown in the following table:

Serine Codons	Mammalian usage: Serine codon frequency/1000 codons	hRS1 gene: Serine codon frequency/19 codons*	Codon Optimized RS1 cDNA: Serine codon frequency/ 19 codons*
AGU	11.9	2	1
AGC	19.4	3	5
UCG	4.5	3	0
UCA	11.7	3	1
UCU	14.6	4	7
UCC	17.4	4	5
CAI ϕ		0.79	0.93

* Total number of Serine residues in hRS1=19; ϕ Codon Adaptation Index

Because the choice of codons could affect RS1 protein expression, we optimized codon usage for increased protein expression using OptimumGene® (GenScript). Based on the mammalian cell codon usage table ([genscript.com/tools/codon-frequency-table](https://www.genscript.com/tools/codon-frequency-table)), we selected the two or three most used codons for each amino acid. Most of these optimal codons have abundant matching tRNAs. To optimize the codon usage in the RS1 gene (cDNA), we replaced the lesser used codons with the most used codons. The effectiveness of codon optimization on gene (cDNA) expression was confirmed by the Codon Adaptation Index (CAI), which is a quantitative method of predicting the level of expression of a gene based on its codon sequence. Optimizing the codon usage for the human RS1 cDNA increased the CAI value from 0.79 to 0.93 (CAI values range between 0.0 and 1.0; with higher values predicting high levels of gene expression).

A codon-optimized human RS1 cDNA (a truncated Homo sapiens Retinoschisin 1 (RS1), comprising codons 36 to 710 of NCBI Reference Sequence: NM_000330.30; SEQ ID NO:3), was created, as follows (shown with translation using the single-letter amino acid code, which is SEQ ID NO:4):

```

atg tca cgc aag att gaa ggc ttt ctg ctg ctg ctg ctg ttc ggg tat gag gct aca ctg
M  S  R  K  I  E  G  F  L  L  L  L  L  F  G  Y  E  A  T  L
ggg ctg agt tct act gag gac gag ggc gag gac ccc tgg tac cag aag gcc tgc aag tgt
G  L  S  S  T  E  D  E  G  E  D  P  W  Y  Q  K  A  C  K  C
gat tgc cag gga gga cct aac gca ctg tgg agc gcc gga gcc acc tcc ctg gac tgt atc
D  C  Q  G  G  P  N  A  L  W  S  A  G  A  T  S  L  D  C  I
cca gag tgc ccc tat cac aag cca ctg ggc ttc gag tcc ggc gag gtg acc cct gat cag
P  E  C  P  Y  H  K  P  L  G  F  E  S  G  E  V  T  P  D  Q
atc aca tgt tct aat cca gag cag tac gtg ggc tgg tat agc tcc tgg aca gcc aac aag
I  T  C  S  N  P  E  Q  Y  V  G  W  Y  S  S  W  T  A  N  K
gcc cgg ctg aat tct cag ggc ttc ggc tgc gcc tgg ctg agc aag ttt cag gac tct agc

```

```

A R L N S Q G F G C A W L S K F Q D S S
cag tgg ctg cag atc gat ctg aag gag atc aaa gtg atc agc ggc atc ctg acc cag ggc
Q W L Q I D L K E I K V I S G I L T Q G
cgg tgt gac atc gat gag tgg atg acc aag tac tcc gtg cag tat agg aca gac gag cgc
5 R C D I D E W M T K Y S V Q Y R T D E R
ctg aac tgg atc tac tat aag gat cag aca ggc aac aat cgg gtg ttc tac ggc aac tcc
L N W I Y Y K D Q T G N N R V F Y G N S
gac aga acc tct aca gtg cag aat ctg ctg agg ccc cct atc atc tct cgg ttt atc aga
D R T S T V Q N L L R P P I I S R F I R
10 ctg atc ccc ctg ggc tgg cac gtg cgc atc gca atc cga atg gaa ctg ctg gaa tgc gtg
L I P L G W H V R I A I R M E L L E C V
tct aag tgt gcc (SEQ ID NO:3)
S K C A (SEQ ID NO:4)

```

In this codon-optimized cDNA construct, nucleotides 1-69 are a leader signal sequence to allow secretion of the protein product into the growth medium; nucleotides 70-186 are an RS1 domain of unknown function; nucleotides 187-657 are the Discoidin domain implicated in cell-cell adhesion; nucleotides 658-672 are the C-terminal flank. The codon-optimized nucleotides are nucleotides 1-69 and 172-699.

This truncated, codon-optimized RS1 construct was fused in frame with 6x His tag (5'-CACCACCACCACCACCAC -3'; SEQ ID NO:5) and synthesized with a NotI restriction site at the 5' end and XhoI restriction site at 3' end, and subcloned into pUC57 vector (GenScript, Piscataway, NJ). This construct was further subcloned into pCMV Tag 4A vector (Agilent Technologies, Santa Clara, CA). This mammalian expression vector encodes the cytomegalovirus (CMV) promoter for constitutive expression of the cloned DNA in a wide variety of mammalian cell lines with a c-terminal Flag tag. But the 6X His tag in our construct is followed immediately by a stop codon downstream of the RS1 coding sequence to silence the Flag epitope in the pCMV Tag 4A vector. We also introduced a Kozak consensus sequence (GCCACC) upstream of the ATG start codon to provide optimal expression of the RS1-His fusion protein.

All constructs were sequenced to verify the absence of random mutations (Integrated DNA Technologies, Skokie, IL). The plasmid DNA was isolated from bacterial cell cultures using maxiprep plasmid purification kit (Qiagen Sciences Inc, Germantown, MD). A map of the expression vector (pCMV-RS1-6His) is shown in FIG. 1.

Example 2

Translation of pCMV-RS1-6His Coding nucleotide sequence to RS1 protein sequence

The RS1 cDNA expressed from the pCMV-RS1-6His expression vector described in Example 1 is translated into a 224 amino-acid (aa) protein (corresponding to NCBI Reference Sequence:NP_000321.1). The functional domains of RS1 are:

- (1) a signal peptide: aa 1-23,
- (2) Rs1 domain: aa 24-62,
- (3) the discoidin domain (aa 63-219), and
- (4) a C-terminal domain: aa 220-224.

The signal sequence guides the translocation of nascent RS1 from the endoplasmic reticulum (the site of synthesis) to external leaflet of the plasma membrane, during which signal sequence is cleaved by signal peptidase to generate mature protein with characteristics of native, human RS1 and a highly-conserved discoidin domain.

Example 3

Construction of high expression discoidin domain vector

A similar expression vector was designed to express the retinoschisin discoidin domain, flanked by the Rs1 and C-terminal domains. A sequence encoding a codon-optimized Rs1-Discoidin Domain (DD) (SEQ ID NO:6) was cloned in the pCMV Tag 4A Vector at NotI and XhoI sites, including an upstream Kozak sequence, and c-terminal 6X His Tag, as described in Example 1.

The nucleotide construct encoding the Rs1-discoidin-C-terminal domains included:

- (1) the Rs1 Signal Sequence (nucleotides 1-69),
- (2) the discoidin domain sequence (nucleotides 70-558), and
- (3) an additional C-terminal sequence (nucleotides 559-570). The translation of the Discoidin Domain, using the single letter amino acid code, is SEQ ID NO:7).

Example 4

Site Directed Mutagenesis of RS1 (secreted and non-secreted forms)

A commercial Q5 site-directed mutagenesis kit (New England Biolabs, Ipswich, MA) was used to introduce point mutations in the pCMV-RS1-6His expression vector described in Example 1.

A. R141H RS1 mutant

In codon 141, nucleotides c422 (G) and c423 (G) were mutated to A and C, respectively (CGG>CAC), resulting in the substitution of Histidine (CAC) for the native Arginine (CGG) when the mutated construct is translated. The sequence of the primers used for introducing R141H Mutation in RS1-6His CDNA were:

Forward Primer: R141H-F 5'-GACCCAGGGCcacTGTGACATCGATG-3' (SEQ ID NO:8)

Reverse Primer: R141H-R 5'-AGGATGCCGCTGATCACT-3' (SEQ ID NO:9)

The nucleotide sequence encoding the mutated R141H human RS1 protein (SEQ ID NO:10) is as follows (shown with translation using the single-letter amino acid code, which is SEQ ID NO:11) (mutated codon and resulting Histidine residue are underlined):

```

5  atg tca cgc aag att gaa ggc ttt ctg ctg ctg ctg ctg ttc ggg tat gag gct aca ctg
   M  S  R  K  I  E  G  F  L  L  L  L  L  F  G  Y  E  A  T  L
   ggg ctg agt tct act gag gac gag ggc gag gac ccc tgg tac cag aag gcc tgc aag tgt
   G  L  S  S  T  E  D  E  G  E  D  P  W  Y  Q  K  A  C  K  C
10 gat tgc cag gga gga cct aac gca ctg tgg agc gcc gga gcc acc tcc ctg gac tgt atc
   D  C  Q  G  G  P  N  A  L  W  S  A  G  A  T  S  L  D  C  I
   cca gag tgc ccc tat cac aag cca ctg ggc ttc gag tcc ggc gag gtg acc cct gat cag
   P  E  C  P  Y  H  K  P  L  G  F  E  S  G  E  V  T  P  D  Q
   atc aca tgt tct aat cca gag cag tac gtg ggc tgg tat agc tcc tgg aca gcc aac aag
   I  T  C  S  N  P  E  Q  Y  V  G  W  Y  S  S  W  T  A  N  K
15 gcc cgg ctg aat tct cag ggc ttc ggc tgc gcc tgg ctg agc aag ttt cag gac tct agc
   A  R  L  N  S  Q  G  F  G  C  A  W  L  S  K  F  Q  D  S  S
   cag tgg ctg cag atc gat ctg aag gag atc aaa gtg atc agc ggc atc ctg acc cag ggc
   Q  W  L  Q  I  D  L  K  E  I  K  V  I  S  G  I  L  T  Q  G
   cac tgt gac atc gat gag tgg atg acc aag tac tcc gtg cag tat agg aca gac gag cgc
20 H  C  D  I  D  E  W  M  T  K  Y  S  V  Q  Y  R  T  D  E  R
   ctg aac tgg atc tac tat aag gat cag aca ggc aac aat cgg gtg ttc tac ggc aac tcc
   L  N  W  I  Y  Y  K  D  Q  T  G  N  N  R  V  F  Y  G  N  S
   gac aga acc tct aca gtg cag aat ctg ctg agg ccc cct atc atc tct cgg ttt atc aga
   D  R  T  S  T  V  Q  N  L  L  R  P  P  I  I  S  R  F  I  R
25 ctg atc ccc ctg ggc tgg cac gtg cgc atc gca atc cga atg gaa ctg ctg gaa tgc gtg
   L  I  P  L  G  W  H  V  R  I  A  I  R  M  E  L  L  E  C  V
   tct aag tgt gcc (SEQ ID NO:10)
   S  K  C  A  (SEQ ID NO:11)

```

B. R213W RS1 mutant

30 A R213W mutation was introduced into the human RS1 sequence using the Quick Change site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Nucleotide c673 (C) was mutated to T (CGG>TGG), resulting in the substitution of Tryptophan (CAC) for the native Arginine (CGG) when the mutated construct is translated. The nucleotide sequence of the primers used to introduce the R213W mutation were:

35 Forward: 5'-ctgggctggcagctccgcatcTggatggagctgctggagtgcgtc-3' (SEQ ID NO:12)
 Reverse: 5'- gacgcactccagcagctccatccAgatggcaatgcggacgtgccagcccag-3' (SEQ ID NO:13)

The nucleotide sequence encoding the mutated R213W human RS1 protein (SEQ ID NO:14) is as follows (shown with translation using the single-letter amino acid code, which is SEQ ID NO:15) (mutated codon and resulting Tryptophan residue are underlined):

```

atg tca cgc aag ata gaa ggc ttt ttg tta tta ctt ctc ttt ggc tat gaa gcc aca ttg
M  S  R  K  I  E  G  F  L  L  L  L  L  F  G  Y  E  A  T  L
gga tta tcg tct acc gag gat gaa ggc gag gac ccc tgg tac caa aaa gca tgc aag tgc
5  G  L  S  S  T  E  D  E  G  E  D  P  W  Y  Q  K  A  C  K  C
gat tgc caa gga gga ccc aat gct ctg tgg tct gca ggt gcc acc tcc ttg gac tgt ata
D  C  Q  G  G  P  N  A  L  W  S  A  G  A  T  S  L  D  C  I
cca gaa tgc cca tat cac aag cct ctg ggt ttc gag tca ggg gag gtc aca ccg gac cag
P  E  C  P  Y  H  K  P  L  G  F  E  S  G  E  V  T  P  D  Q
10 atc acc tgc tct aac ccg gag cag tat gtg ggc tgg tat tct tcg tgg act gca aac aag
I  T  C  S  N  P  E  Q  Y  V  G  W  Y  S  S  W  T  A  N  K
gcc cgg ctc aac agt caa ggc ttt ggg tgt gcc tgg ctc tcc aag ttc cag gac agt agc
A  R  L  N  S  Q  G  F  G  C  A  W  L  S  K  F  Q  D  S  S
cag tgg tta cag ata gat ctg aag gag atc aaa gtg att tca ggg atc ctc acc cag ggg
15 Q  W  L  Q  I  D  L  K  E  I  K  V  I  S  G  I  L  T  Q  G
cgc tgt gac atc gat gag tgg atg acc aag tac agc gtg cag tac agg acc gat gag cgc
R  C  D  I  D  E  W  M  T  K  Y  S  V  Q  Y  R  T  D  E  R
ctg aac tgg att tac tac aag gac cag act gga aac aac cgg gtc ttc tat ggc aac tcg
L  N  W  I  Y  Y  K  D  Q  T  G  N  N  R  V  F  Y  G  N  S
20 gac cgc acc tcc acg gtt cag aac ctg ctg cgg ccc ccc atc atc tcc cgc ttc atc cgc
D  R  T  S  T  V  Q  N  L  L  R  P  P  I  I  S  R  F  I  R
ctc atc ccg ctg ggc tgg cac gtc cgc att gcc atc tgg atg gag ctg ctg gag tgc gtc
L  I  P  L  G  W  H  V  R  I  A  I  W  M  E  L  L  E  C  V
agc aag tgt gcc (SEQ ID NO: 14)
25 S  K  C  A  (SEQ ID NO: 15)

```

Example 5

Cloning human Retinoschisin-1 cDNA into pCMVTag4A Vector

30 for RS1-Flag Protein Expression

To obtain proteins of sufficient purity and quality for mechanistic and therapeutic studies, human RS1 cDNA was cloned in frame with a nucleotide sequence:

(GATTACAAGGATGACGACGATAAG; SEQ ID NO:16)

encoding a Flag Tag (DYKDDDDK; SEQ ID NO:17) was synthesized with a NotI restriction site
 35 at the 5' end and XhoI restriction site at the 3' end (GenScript, Piscataway, NJ) and subcloned into the pCMV Tag 4A vector.

The nucleotide sequence encoding the Flag-tagged human RS1 protein (SEQ ID NO:18) is as follows (shown with translation using the single-letter amino acid code, which is SEQ ID NO:19) (the Flag Tag inserted before the stop codon is underlined):

atg tca cgc aag ata gaa ggc ttt ttg tta tta ctt ctc ttt ggc tat gaa gcc aca ttg
 M S R K I E G F L L L L L F G Y E A T L
 gga tta tcg tct acc gag gat gaa ggc gag gac ccc tgg tac caa aaa gca tgc aag tgc
 G L S S T E D E G E D P W Y Q K A C K C
 5 gat tgc caa gga gga ccc aat gct ctg tgg tct gca ggt gcc acc tcc ttg gac tgt ata
 D C Q G G P N A L W S A G A T S L D C I
 cca gaa tgc cca tat cac aag cct ctg ggt ttc gag tca ggg gag gtc aca ccg gac cag
 P E C P Y H K P L G F E S G E V T P D Q
 atc acc tgc tct aac ccg gag cag tat gtg ggc tgg tat tct tcg tgg act gca aac aag
 10 I T C S N P E Q Y V G W Y S S W T A N K
 gcc cgg ctc aac agt caa ggc ttt ggg tgt gcc tgg ctc tcc aag ttc cag gac agt agc
 A R L N S Q G F G C A W L S K F Q D S S
 cag tgg tta cag ata gat ctg aag gag atc aaa gtg att tca ggg atc ctc acc cag ggg
 Q W L Q I D L K E I K V I S G I L T Q G
 15 cgc tgt gac atc gat gag tgg atg acc aag tac agc gtg cag tac agg acc gat gag cgc
 R C D I D E W M T K Y S V Q Y R T D E R
 ctg aac tgg att tac tac aag gac cag act gga aac aac cgg gtc ttc tat ggc aac tcg
 L N W I Y Y K D Q T G N N R V F Y G N S
 gac cgc acc tcc acg gtt cag aac ctg ctg cgg ccc ccc atc atc tcc cgc ttc atc cgc
 20 D R T S T V Q N L L R P P I I S R F I R
 ctc atc ccg ctg ggc tgg cac gtc cgc att gcc atc cgg atg gag ctg ctg gag tgc gtc
 L I P L G W H V R I A I R M E L L E C V
 agc aag tgt gcc gat tac aag gat gac gac gat aag tga (SEQ ID NO:18)
 S K C A D Y K D D D D K - (SEQ ID NO:19)

25

Similarly, to obtain mutated proteins for study, mutated human RS1 cDNAs were cloned in frame with the Flag Tag (DYKDDDDK; SEQ ID NO:17) and subcloned into the pCMV Tag 4A vector.

The nucleotide sequence encoding the mutated, R213W Flag-tagged human RS1
 30 protein (SEQ ID NO:20) is as follows (shown with translation using the single-letter amino acid
 code, which is SEQ ID NO:21) (the R213W and the Flag Tag inserted before the stop codon are
 underlined):

atg tca cgc aag ata gaa ggc ttt ttg tta tta ctt ctc ttt ggc tat gaa gcc aca ttg
 M S R K I E G F L L L L L F G Y E A T L
 35 gga tta tcg tct acc gag gat gaa ggc gag gac ccc tgg tac caa aaa gca tgc aag tgc
 G L S S T E D E G E D P W Y Q K A C K C
 gat tgc caa gga gga ccc aat gct ctg tgg tct gca ggt gcc acc tcc ttg gac tgt ata
 D C Q G G P N A L W S A G A T S L D C I
 cca gaa tgc cca tat cac aag cct ctg ggt ttc gag tca ggg gag gtc aca ccg gac cag
 40 P E C P Y H K P L G F E S G E V T P D Q

```

atc acc tgc tct aac ccg gag cag tat gtg ggc tgg tat tct tcg tgg act gca aac aag
I   T   C   S   N   P   E   Q   Y   V   G   W   Y   S   S   W   T   A   N   K
gcc cgg ctc aac agt caa ggc ttt ggg tgt gcc tgg ctc tcc aag ttc cag gac agt agc
A   R   L   N   S   Q   G   F   G   C   A   W   L   S   K   F   Q   D   S   S
5  cag tgg tta cag ata gat ctg aag gag atc aaa gtg att tca ggg atc ctc acc cag ggg
Q   W   L   Q   I   D   L   K   E   I   K   V   I   S   G   I   L   T   Q   G
cgc tgt gac atc gat gag tgg atg acc aag tac agc gtg cag tac agg acc gat gag cgc
R   C   D   I   D   E   W   M   T   K   Y   S   V   Q   Y   R   T   D   E   R
ctg aac tgg att tac tac aag gac cag act gga aac aac cgg gtc ttc tat ggc aac tcg
10 L   N   W   I   Y   Y   K   D   Q   T   G   N   N   R   V   F   Y   G   N   S
gac cgc acc tcc acg gtt cag aac ctg ctg cgg ccc ccc atc atc tcc cgc ttc atc cgc
D   R   T   S   T   V   Q   N   L   L   R   P   P   I   I   S   R   F   I   R
ctc atc ccg ctg ggc tgg cac gtc cgc att gcc atc tgg atg gag ctg ctg gag tgc gtc
L   I   P   L   G   W   H   V   R   I   A   I   W   M   E   L   L   E   C   V
15 agc aag tgt gcc gat tac aag gat gac gac gat aag tga (SEQ ID NO:20)
S   K   C   A   D   Y   K   D   D   D   D   K   -   (SEQ ID NO:21)

```

Example 6

Culturing ARPE-19 cells

ARPE-19 cells were obtained from the American Type Culture Collection (ATCC Number CRL-2302). ARPE-19 cells are human retinal pigmented epithelial (RPE) cells which form stable monolayers and show morphological and functional polarity. The ARPE-19 cells were cultured in DMEM/F12 (1:1 mixture with HEPES buffer containing 10% fetal bovine serum, 56 mM final concentration sodium bicarbonate and 2 mM L-glutamine) and incubated at 37°C in 5% CO₂. The cells were typically grown in Corning tissue culture-treated 6 well plates or T25/T75 flasks. To subculture, spent medium was removed and the cells were incubated at 37°C for 9 minutes 0.25% trypsin solution. 5-10 ml complete media was added to the trypsinized cells and spun at 1400 rpm for 10 minutes. The obtained pellet was washed with phosphate buffered saline (PBS) once under similar conditions and then subcultivated at a recommended ratio of 1:3 to 1:5.

1. Generation of a kill curve to determine the optimal selection antibiotic concentration

0.1X 10⁶ cells were plated in 0.5 ml complete growth medium per well in a 24-well tissue culture plate one day prior to introducing antibiotic selection. Ideally, cells should have reached high confluence (~60-80%) prior to adding the selection antibiotic. Increasing amounts of G418 were added to duplicate wells of cells plated in complete media. A no-antibiotic control was added. Concentration range for the selection antibiotic was taken between the range of 0 to

1000 ug/ml (0, 50, 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 µg/ml). The selection media with selection antibiotic was replaced every 2-3 days for up to a week. The culture was examined every day for signs of visual toxicity. The optimum antibiotic dosage was determined to be the lowest antibiotic concentration at which all cells are dead after one week of antibiotic selection.

2. Transfection

While performing the kill curve (1 week), the transfection conditions were optimized in a T75 flask by transfecting a GFP encoding reporter plasmid into cells at high confluence. The appropriate dose of plasmid (5-30 µg) and transfection reagent (15-45 µl) was determined.

Reporter gene expression (GFP) and toxicity was observed at regular time points over at least a 48-hour period, optimally for 72 hours. The optimal DNA and transfection reagent dosage used for generating stable transfectants was DNA:Lipofectamine = 1:1 (30ug pDNA and 30 ul Lipofectamine for transfection of a T75 flask).

3. Characterization of cell lines

The transfected mammalian cells were characterized as outlined in the worldwide regulatory guidance documents, using the following methods.

Authenticity & Characteristics	Methods
Growth / Morphology	Doubling time and growth analysis in different serum/serum free conditions
Genetic Stability	By protein expression analysis every passage till passage 30
Tumorigenicity	Analysis by in vivo studies for at least 6-8 weeks after intravitreal injection
Mycoplasma	Tested negative
Sterility	Tested sterile
General Virus Screens	Tested negative
Adventitious Agents from Raw Material	Yes; the media is sterile filtered twice and so is Fetal bovine serum (triple filtered)

4. Polyclones to monoclones selection

The selection of monoclones was carried out at higher cell concentrations and repeated a few times to ensure monoclonality using cloning rings. The selected cells were seeded sparsely but not at limiting dilution in 10 cm dishes and allowed to expand and form discernible colonies for 2-3 weeks. The individual colonies were then trypsinized and transferred to another smaller culture vessel using cloning rings for monoclonal expansion.

5. Clone transfer and expression analysis

Selected single-colonies were expanded to high confluence and transferred to a 12-well tissue culture plate. Once the 12-well tissue culture plate clones were confluent, they were passaged to a 6-well tissue culture plate and further to a T25 flask. The media and cells were used to analyze the expression of the protein. A small portion of selected cells was propagated for 50-90 doublings to confirm stability of expression by verifying expression of the target gene at multiple time points.

6. Expansion and cryopreservation of high expressing clones

Once expression was verified, clones of interest were scaled up to larger volumes. Once expanded, the cell stocks were frozen down using appropriate freezing medium lacking the selection antibiotic. The stable cell lines were critically examined for their expression till passage 20 because some clones may lose expression after several passages. The samples were frozen down from early passage to prolong their use after thawing.

7. Choosing the cell line for stable cell line generation

We established this *in vitro* human RS1 production system for therapeutic and research-based studies of the protein. ARPE-19 is a well-established human epithelial cell line that has been extensively evaluated as a platform cell line for cell-based protein delivery technology. ARPE-19 cells are highly viable under stringent, hypoxic, and nutrition-deficient conditions and can be genetically modified to secrete the protein of choice. They have a relatively a long-life span and are efficiently transfected to produce useful amounts of a desired protein. Because they are human cells, the recombinant human protein is correctly glycosylated, and these cells trigger no or low-level host immune reactions and are non-tumorigenic.

8. Characterization of the expression vectors

The expression vectors were characterized for protein expression by transient transfection in ARPE-19 cells. Once that data was established, we made a stable cell line using each expression vector.

9. Analysis and optimization of transfection efficiency using a GFP reporter plasmid in ARPE-19 cells

Because it is easier to analyze the transfection efficiency with a fluorescent reporter, ARPE-19 cells were transfected with a green fluorescent protein (GFP)- encoding reporter plasmid (pCMV EGFP)

Various plasmid DNA to Lipofectamine ratios [1:1, 1:2, and 1:3] were used to transfect ARPE-19 cells in serum-free conditions. The GFP expression was monitored from 6 hours to 72 hours and checked for toxicity and cell death (FIGS. 2A-2C).

Flow-cytometric analysis was used to evaluate transfection efficiency at various pDNA and Lipofectamine concentrations (FIG. 2D). Average transfection efficiency is shown in the following table:

DNA (pCMV EGFP) : Lipo	Average % transfection
1:1	50.7%
1:2	42.3%
1:3	30.4%

The conditions for optimal transfection in ARPE-19 cells in serum-free conditions were established as follows:

Highest transfection efficiency was observed at a DNA:Lipofectamine ratio of 1:1.

The optimal pDNA concentration was determined to be 7.5 µg/ 0.5 million cells for widespread and efficient transfection with no or minimum cell death.

The time points when we observed the brightest and highest expression were at 6 and 14 hours.

10. Optimization of media for various culture conditions

We optimized the culture conditions of these cells in high serum (10%), low serum (2% and 1%), and serum free conditions. The doubling time in high serum is 36-48 hours while that in low serum or serum free conditions is 72-90 hours. Specific growth rate was calculated by: (maximum cell density – cell density plated) / days from last passage.

We used 7.5µg pDNA /0.5 million cells and incubated for 6 hours or 14 hours after transfection and added fresh media. RS1 expression was checked at 72 hours after media change. We found that expression of RS1 at 14 hours post transfection (DNA: Lipofectamine 1:1) was highest and most consistent (FIG. 3A). RS1 expression was found to be robust upon

transfection and culture in 10% serum (FIG. 3B, Lane 1); 2% serum (FIG. 3B, Lane 2); and serum-free conditions (FIG. 3B, Lane 3).

Even though the doubling time increased considerably, the viability and protein expression was not affected.

11. Analysis of expression vector in ARPE-19 for protein expression by transient transfection

The expression vector was transiently transfected in ARPE-19 cells and analyzed for protein expression. Once the conditions were optimized for transfection yielding the highest protein expression, we proceeded to generate the stable cell line. The conditions for transfection were: DNA: LIPO = 1:1, Transfection time: 14-16 hours, Analysis time: 72 hours.

The culture supernatant was collected, concentrated 0.6 ml media to 200 ul, and RS1 expression was analyzed. RS1 expression was consistent with passages and we could find protein expression without loss until passage 10. Further analysis was also performed at passage 20 and 30 to confirm the stability of cell line. The cells were stable and secreted RS1 until passage 30.

12. Stable cell line generation and polyclone expansion and characterization

A polyclonal ARPE-RS1 stable cell line was generated by transfecting with the pCMV-RS1-6x-his plasmid and selecting the stably transfected cells for 10 generations in antibiotic selection media containing 700µg/ml G418 solution. The polyclonal ARPE-RS1 cells expanded and cultured in 1% serum showed robust expression of the RS1 protein over the 10 passages of these stably transfected cells (FIG. 4A; "P" indicates Passage number; FIG. 4B confirmation of RS1 protein expression by silver stain). After confirmation of stable protein expression, the cells were further cultured in media containing no selection antibiotic and continued for at least 20 generations with frequent freeze downs.

Cell culture media from five different polyclonal RS1-transfected ARPE cell cultured in serum free was analyzed for RS1 protein by histidine pulldown. The average concentration of RS1 secreted from the polyclonal cells was 771 ng per 0.1 million cells (FIG. 4C). The ARPE-RS1 polyclonal cells were tightly adhered, larger in diameter than the control ARPE-19 cells, and displayed highly flattened morphology (FIG. 4D). Immunocytochemical analysis of RS1 in the polyclonal ARPE-RS1 cells also demonstrated the RS1 protein membrane-bound expression (FIG. 4E). These polyclonal cells were grown and passed for at least 10 passages in serum-free media. The expression of RS1 protein in these cells was analyzed by sampling the serum-free media collected from these cells at each passage, and the cells from passages 3 and 5 (P3 and P5) demonstrated higher RS1 protein concentrations (FIG. 4F).

It has previously been reported that ATPase subunit B2 plays a major role in RS1 binding and downstream action. To further study ATPase-independent actions of RS1 protein, the polyclonal RS1-transfected ARPE-19 cell line, as well as the control ARPE-19 cells were analyzed for Na-ATPase subunits. Both the ARPE-19 and ARPE-RS1 cells did not express ATPase subunits A3 and B2 (FIG. 4G).

13. Monoclonal selection for high/mid/low protein expressing clones

Monoclones were selected by clonal ring and 27 colonies were propagated and expanded with high, low, and medium secretion of RS1. Protein estimation was performed by Western blot using an anti-RS1 antibody (FIG. 5) and all 27 monoclonal clones were cryopreserved.

14. Characterization for cell line stability

The RS1 protein secretion efficiency was compared qualitatively by Western blot in both serum (10% FBS) and serum-free conditions. ARPE-19 cells were used as the negative control. FIG. 6A is a Western blot of growth media samples obtained from each cell line; lanes 1 and 2 are media from stably-transfected ARPE-19 cells expressing RS1 protein, grown in serum-free or serum conditions, respectively. Lanes 3 and 4 are the control ARPE-19 cells grown in serum-free or serum conditions, respectively.

Similarly, Western blot analysis of growth media from polyclonal ARPE-19 cell lines stably transfected with: wild type (WT) RS1 protein, a secreted RS1 protein with a mutation (R141H), and a non-secreted RS1 protein with a mutation (R213W) is shown in FIG. 6B. The R141H-mutated RS1 protein-transfected cells showed slightly reduced RS1 expression, and, as expected, the R213W-mutated RS1 protein-transfected cells showed no, or significantly reduced, RS1 protein expression.

Blue Native Page gel was run to resolve the expressed protein complex by molecular weight and identify its native structure (FIG. 6C). Lanes 1-6 contain protein purified from six different cultures, showing high-molecular weight bands conforming to RS1 protein forming double octamers.

A cell surface biotinylation assay was run to reveal 6-His-tagged RS1 protein stably expressed in polyclonal ARPE-19 cells. ARPE-19 (control) and RS1-expressing ARPE-19 cells were cultured and surface biotinylated. The biotinylated proteins were affinity-purified using Thermo Scientific NeutrAvidin® agarose resin and analyzed by Western blot (FIG. 6D), demonstrating that the expressed RS1 protein was membrane-associated with sufficient extracellular exposure. Cell line stability and viability was evaluated in serum-free conditions. Secretion of RS1 protein, both wild type and mutated (R141H), from the transfected ARPE-19

cells was stable for at least 8-10 days (FIG. 6E). As noted above, the R213W mutant is a non-secreted mutant protein, hence no R213W RS1 mutant expression was detected.

15. Characterization for *in vivo* survival and function

The RS1-transfected cells that stably express RS-1 protein were transplanted into one eye of 6-8-week-old retinoschisin knockout (RS1 KO) mice by intravitreal injection of 0.1 million polyclonal cells/1 microliter/eye. Large histology images (FIG. 7A) show the RS1 protein secreted by the transfected and transplanted ARPE-19 cells translocates to retinal layers and binds to the ganglion cell layer (GCL), Inner Nuclear Layer (INL), Inner Plexiform Layer (IPL) and Outer Plexiform Layer (OPL). The images also showed RS1 protein binding to Inner segments at times. The schisis in RS1 KO retina treated with the transfected ARPE-19 cells, which showed robust RS1 protein binding to retinal cells in the Inner Nuclear Layer, were observed to have lesser and smaller schisis compared to the untreated eye.

Additional images (FIG. 7B) show the cluster of RS1-transfected ARPE-19 cells were present in the VC while the secreted RS1 protein translocated through ILM to GCL, IPL, and ILM. The RS1 protein was also bound to Inner segments when there was robust concentrations of RS1 protein secreted from the transfected ARPE-19 cells.

The RS1 KO mice were also analyzed by Optical Coherence Tomography (OCT) to observe morphological changes 6 weeks after injection of the transfected ARPE-19 cells (FIG. 7C). Mice with robust RS1 cell injection showed decreases in the number and size of cavities. Some animals had aggregated cells in the vitreous, which caused difficulty in acquiring a clearer OCT image of the injected eye. For example, plate 3 shows an aggregation of ARPE-19 cells near the optic nerve head, which casts shadows onto the retina, making the OCT image difficult to interpret.

Example 7

Characterization of recombinant RS1 protein

1. Purification of recombinant RS1 protein

Human RS1 protein recovered from serum-free culture media from RS1-transfected ARPE-19 cells, described above, was purified to near-homogeneity by previously published methods of affinity chromatography in combination with size exclusion chromatography (Tolun G, et. al., (2016) Paired octamer rings of retinoschisin suggest a junctional model for cell-cell adhesion in the retina. Proc Natl Acad Sci U S A. 113:5287-92; Bush M, et. al., (2016). Cog-Wheel Octameric Structure of RS1, the Discoidin Domain Containing Retinal Protein Associated with X-Linked Retinoschisis. PLoS One. 11:e0147653. doi: 10.1371; and Ramsay, E.P., et al.,

(2016) Structural analysis of X-linked retinoschisis mutations reveals distinct classes which differentially effect retinoschisin function, Human Molecular Genetics, 25(24):5311–20).

2. Cryo-electron microscopy analysis of purified RS1 protein

During the last decade, single-particle cryo-electron microscopy (cryo-EM) has emerged
5 as a leading method for elucidating macromolecular structures at near-atomic resolution,
rivaling even the established technique of X-ray crystallography. A large, multi-subunit protein
complex, like RS1 (16 RS1 molecules in a double octamer) are not amenable to crystallization
and therefore X-ray crystallography analysis. In contrast, cryo-EM overcomes these problems
with crystallization, and quickly creates high-resolution models of even multi-subunit protein
10 complexes. We recently showed that double octamer rings of RS1 assemble into higher order
complexes forming extensive branched networks (Heymann, et. al., (2019) Cryo-EM of
retinoschisin branched networks suggests an intercellular adhesive scaffold in the retina. J Cell
Biol. 218:1027-38).

Using the RS1 purified by these chromatography methods, we determined a 4.1-Å-
15 resolution structure of RS1 by cryo-EM (FIG. 9). In FIG. 9, panel B is a top view average; panel
C is an oblique view average of double octamer rings; panel D is a side view average of double
octamer rings; and panel E is a side view average of single octamer rings. We showed that the
assembled state of the mature RS1 is a double octamer, back-to-back double octameric rings.
The ability to resolve the RS1 protein structure to this level validates the protein production and
20 purification procedures and the purity level of RS1 protein resulting from these procedures.

3. Fate of RS1 protein expressed *in vivo* following injection of ARPE-19 cells into RS1 KO mice

The injected and un-injected retina from the RS1 KO mice, described above, were
obtained 6 weeks post injection of the RS1-transfected ARPE-19 cells, and RS1 protein was
25 extracted. Western blots (FIG. 8A) were used to analyze the presence of RS1 protein, and 6 of
7 retina analyzed showed RS1 protein expression, suggesting that the RS1-transfected ARPE-
19 cells secreted RS1 protein in the vitreous chamber, and the secreted RS1 protein bound to,
and was non-toxic to the retinal cells. The un-injected and injected retina were also analyzed for
human Ezrin and CRALBP to determine whether RS1-transfected ARPE-19 cells injected into
30 the vitreous chamber migrated to the retina. Ezrin and CRALBP expression remained the same
for both injected and un-injected retina (FIG. 8B), suggesting no or minimal migration/infiltration
of RS1-transfected ARPE-19 cells into the retina.

The concentration of RS1 protein present in the injected retina was estimated using a
RS1 standard. 500 pg of the RS1 standard was loaded and the intensity units (IU) of RS1

protein bands in the Western blots obtained from the retina of injected eyes were compared to the IU of the RS1 standard. The results are provided in the following table:

	Intensity Units	Picograms	pg/Retina
RS1 Standard	328500	500	
54I*	210000	320	2666
76I	74600	114	950
77I	116000	177	1475

* Values /30ug protein

Total Retina Protein 250 µg

5 Immunohistochemical staining of the retina cells with a bipolar cell marker (PKC-a) was conducted to determine whether the RS1 protein secreted by the RS1-transfected ARPE-19 cells binds to bipolar cells only (FIG. 8C). The staining showed a set of PKC-a cells stained for RS1 protein but another set of RS1 stained cells were PKC-a negative, suggesting that RS1 protein also binds to retinal cell types other than Bipolar cells.

10 Genomic analysis of two sets of the polyclonal RS1-transfected ARPE-19 and wild type ARPE-19 cells was conducted and compared. The differentially-regulated genes were shortlisted and preliminary analysis using the PANTHER platform (Protein Analysis THrough Evolutionary Relationships software: pantherdb.org) was performed to assess molecular function, biological process, cellular components, protein class altered, and pathway hits.

15 The various features and processes described above may be used independently of one another, or may be combined in various ways. All possible combinations and subcombinations are intended to fall within the scope of this disclosure. In addition, certain method or process blocks may be omitted in some implementations. The methods and processes described herein are also not limited to any particular sequence, and the blocks or states relating thereto can be performed in other sequences that are appropriate. For example, described blocks or states
20 may be performed in an order other than that specifically disclosed, or multiple blocks or states may be combined in a single block or state. The example blocks or states may be performed in serial, in parallel, or in some other manner. Blocks or states may be added to or removed from the disclosed example embodiments. The example systems and components described herein

may be configured differently than described. For example, elements may be added to, removed from, or rearranged compared to the disclosed example embodiments.

While certain example embodiments have been described, these embodiments have been presented by way of example only, and are not intended to limit the scope of the inventions disclosed herein. Thus, nothing in the foregoing description is intended to imply that any particular feature, characteristic, step, module, or block is necessary or indispensable. Indeed, the novel methods and materials described herein may be embodied in a variety of other forms; furthermore, various omissions, substitutions and changes in the form of the methods and materials described herein may be made without departing from the spirit of the inventions disclosed herein. The accompanying claims and their equivalents are intended to cover such forms or modifications as would fall within the scope and spirit of certain of the inventions disclosed herein.

What is claimed is:

1. A nucleic acid molecule comprising an open reading frame (ORF) encoding a human retinoschisin-1 (RS1) protein, or a portion thereof, or a variant thereof, that is efficiently expressed in human cells.
2. The nucleic acid molecule of claim 1, wherein the nucleotide sequence encodes a protein that when administered to a patient having an XLRS-related disorder, increases visual function in the patient.
3. The nucleic acid molecule of claim 1, wherein the ORF comprises codons encoding the human RS1 protein that are optimized for expression in human cells.
4. The nucleic acid molecule of claim 1, wherein the ORF encodes a fragment of the human RS1 protein selected from the N-terminal signal sequence, the Rs1 domain, the discoidin domain, and/or the C-terminal segment.
5. The nucleic acid molecule of claim 1, wherein the ORF encodes a protein comprising an amino acid sequence at least 85% identical to SEQ ID NO:2.
6. The nucleic acid molecule of any one of claims 1-5, wherein the ORF encodes one or more point mutations in the sequence encoding a human RS1 polypeptide that encodes a variant RS1 polypeptide that includes one or more amino acid substitutions relative to the human, wild type RS1 polypeptide.
7. The nucleic acid molecule of claim 6, wherein the point mutation results in an inability of a variant RS1 polypeptide having the amino acid substitution to be inserted into the membrane of the endoplasmic reticulum for secretion.
8. The nucleic acid molecule of claim 6, wherein the point mutation results in an amino acid substitution in one or both regions flanking the discoidin domain comprising amino acids 24-62 and amino acids 220-224 of SEQ ID NO:2.
9. The nucleic acid molecule of claim 6, wherein the point mutation results in an amino acid substitution of a cysteine at any one of positions 38, 40, 42, 59, or 223 of SEQ ID NO:2 with an amino acid selected from the group consisting of Ser, Arg, Trp, Tyr, and Gly.
10. The nucleic acid molecule of claim 6, wherein the point mutation results in an amino acid substitution in the discoidin domain of the human RS1 polypeptide comprising amino acids 63-219 of SEQ ID NO:2.
11. The nucleic acid molecule of claim 10, wherein the point mutation results in an amino acid substitution at a position of SEQ ID NO:2 selected from the group consisting of E72, G109, E146, R182, P203, R141, and D143.

12. The nucleic acid molecule of claim 10, wherein the point mutation results in an amino acid substitution in SEQ ID NO:2 selected from the group consisting of R141C and R213W.
13. The nucleic acid molecule of claim 1, wherein the ORF encodes a protein consisting of the amino acid sequence of SEQ ID NO:2.
14. The nucleic acid molecule of claim 1, wherein the ORF encodes a protein comprising an amino acid sequence at least 85% identical to any one of SEQ ID NOs:4, 7, 11, 15, 19, or 21.
15. The nucleic acid molecule of claim 1, wherein the ORF encodes a protein consisting of the amino acid sequence of any one of SEQ ID NOs:4, 7, 11, 15, 19, or 21.
16. The nucleic acid molecule of claim 1, comprising a nucleotide sequence at least 85% identical to any one of SEQ ID NOs:3, 6, 10, 14, 18, or 20.
17. The nucleic acid molecule of claim 1, consisting of a nucleotide sequence of any one of SEQ ID NOs:3, 6, 10, 14, 18, or 20.
18. The nucleic acid molecule of claim 1, further comprising at least one intronic sequence from the human *RS1* gene, or a portion thereof.
19. The nucleic acid molecule of claim 18, wherein the at least one intronic sequence is at least a portion of the first intron from the human *RS1* gene.
20. The nucleic acid molecule of claim 18, wherein the at least one intronic sequence comprises at least 300 nucleotides of the first intron sequence of the human *RS1* gene.
21. The nucleic acid molecule of claim 18, wherein the at least one intronic sequence comprises at least 1000 nucleotides of the first intron sequence of the human *RS1* gene.
22. The nucleic acid molecule of claim 18, wherein the at least one intronic sequence comprises between 200 and 500 nucleotides of the first intron sequence of the human *RS1* gene.
23. An expression cassette comprising a nucleic acid molecule of any one of claims 1-22, operably linked to a promoter.
24. The expression cassette of claim 23, wherein the promoter is a tissue-specific promoter.
25. The expression cassette of claim 24, wherein the promoter is selected from the group consisting of a retinoschisin promoter or a functional fragment thereof, a rhodopsin promoter, a rhodopsin kinase promoter, a CRX promoter, and an interphotoreceptor retinoid binding protein (IRBP) promoter.
26. The expression cassette of claim 23, wherein the promoter is a non-tissue-specific promoter.
27. The expression cassette of claim 26, wherein the promoter is selected from the group consisting of a cytomegalovirus (CMV) promoter, a human elongation factor 1 α -subunit (EF-1 α), a chicken β -actin (CBA) and its derivative CAG, a β -glucuronidase (GUSB), ubiquitin C

- (UBC), and an enhancer-less ubiquitously acting chromatin opening element (UCOE) promoter.
28. The expression cassette of any one of claims 23-27, wherein the nucleic acid molecule and the promoter are operably linked to one or more viral post-transcriptional regulatory elements (PREs).
 29. The expression cassette of claim 28, wherein the PRE is selected from the group consisting of Hepatitis B Virus PRE and Woodchuck Hepatitis Virus PRE.
 30. The expression cassette of any one of claims 23-29, further comprising a polyadenylation (polyA) signal sequence.
 31. The expression cassette of any one of claims 23-30, further comprising an enhancer placed upstream or downstream of the promoter.
 32. The expression cassette of claim 31, wherein the enhancer is a CMV enhancer.
 33. The expression cassette of claims 23-32, further comprising an SV40 intron placed between the promoter and the nucleic acid molecule.
 34. The expression cassette of any one of claims 23-33, further comprising a polynucleotide sequence encoding at least one of a His tag, a c-terminal Flag tag of SEQ ID NO:17, and a Kozak consensus sequence (GCCACC) upstream of an ATG start codon.
 35. An expression vector comprising a nucleic acid molecule of any one of claims 1-14 or an expression cassette of any one of claims 23-34.
 36. The expression vector of claim 35, that is a linear DNA molecule,
 37. The expression vector of claim 35, that is a circular DNA molecule.
 38. The expression vector of claim 35, that is a plasmid, cosmid, or phage.
 39. The expression vector of claim 35, wherein the vector comprises one or more nucleic acid sequences from a DNA or RNA virus.
 40. The expression vector of claim 35, wherein the vector comprises one or more nucleic acid sequences from an adeno-associated virus (an AAV vector), a cytomegalovirus (CMV), a retrovirus, an adenovirus, a herpes virus, a vaccinia virus (a vaccinia vector), a poliovirus, or a Sindbis virus.
 41. The expression vector of claim 35, wherein the vector is a targeting vector comprising a sequence that is identical or substantially identical to a sequence in a human cell directing integration of a polynucleotide sequence into a position within the genome of the cell via homologous recombination.

42. The expression vector of claim 35, wherein the vector is an AAV vector comprising inverted terminal repeats (ITRs) selected from the group consisting of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, and AAV10.
43. The expression vector of claim 42, wherein the expression cassette of any one of claims 15-26 is flanked by the ITR sequences, and wherein at least one ITR comprises AAV sequences that allow packaging of the DNA molecule into an AAV particle.
44. The expression vector of claim 42, wherein the vector is a self-complementary AAV (scAAV) vector.
45. The expression vector of claim 35, wherein the vector is a Lentiviral vector.
46. A retinoschisin protein comprising the amino acid sequence of any one of SEQ ID NO: 4, 7, 11, 15, 19, or 21.
47. A retinoschisin protein comprising an amino acid sequence at least 90% identical to any one of SEQ ID NO: 4, 7, 11, 15, 19, or 21.
48. A retinoschisin protein consisting of an amino acid sequence of any one of SEQ ID NO: 4, 7, 11, 15, 19, or 21.
49. A composition comprising a physiologically acceptable solution and a nucleic acid molecule of any one of claims 1-22.
50. A composition comprising a physiologically acceptable solution and an expression cassette of any one of claims 23-34.
51. A composition comprising a physiologically acceptable solution and an expression vector of any one of claims 35-45.
52. A composition comprising a physiologically acceptable solution and an RS1 protein of any one of claims 46-48.
53. The composition of any one of claims 49-52, wherein the physiologically acceptable solution is a solution comprising about 10 mM Tris-HCl (pH 7.4) and about 180 mM NaCl.
54. The composition of any one of claims 49-52, further comprising at least one formulation component selected from the group consisting of salts, buffers, diluents, stabilizing agents, polymers, chelating agents.
55. A human cell comprising a nucleic acid molecule of any one of claims 1-22.
56. A human cell comprising expression cassette of any one of claims 23-34.
57. A human cell comprising an expression vector of any one of claims 35-45.
58. The human cells of any one of claims 55-57, wherein the human cell is an ARPE-19 cell.
59. An immortalized cell made from human cells of any one of claims 55-58.
60. A cell line comprising the human cells of any one of claims 55-59.

61. A cell line comprising an ARPE-19 cell genetically engineered to express an RS1 protein of any one of claims 46-48.
62. An implantable cell culture device comprising:
 - a) a core comprising one or more human cells genetically engineered to express an RS1 protein of any one of claims 46-48; and,
 - b) a semipermeable membrane surrounding the core, wherein the membrane permits diffusion of the RS1 protein therethrough.
63. The implantable cell culture device of claim 62, wherein the human cells are ARPE-19 cells.
64. The implantable cell culture device of claims 62 or 63, wherein the human cells have been transformed with a nucleic acid molecule of any one of claims 1-22, or an expression cassette of any one of claims 23-34, or an expression vector of any one of claims 35-45.
65. The implantable cell culture device of any one of claims 62-64, wherein the core further comprises a matrix comprising a hydrogel or extracellular matrix components disposed within the semipermeable membrane.
66. A method for treating an ocular disorder, comprising injecting an expression vector of any one of claims 35-45 into the eye of a patient, thereby treating the disorder.
67. A method for treating an ocular disorder, comprising implanting a RS1 protein of any one of claims 46-48 into the eye of a patient, thereby treating the disorder.
68. A method for treating an ocular disorder, comprising implanting a composition of any one of claims 49-54 into the eye of a patient, thereby treating the disorder.
69. A method for treating an ocular disorder, comprising implanting a human cell of any one of claims 55-59 into the eye of a patient, thereby treating the disorder.
70. A method for treating an ocular disorder, comprising implanting an implantable cell culture device of any one of claims 62-65 into the eye of a patient, thereby treating the disorder.
71. The methods of any one of claims 66-70, wherein the injection or implantation is made to the aqueous and vitreous humors of the eye, and/or the posterior and anterior chamber of the eye.
72. The methods of any one of claims 66-71, wherein the injection or implantation is made to an eye of a patient receiving gene therapy for an XLRs-related disorder.
73. The methods of any one of claims 66-72, wherein the ocular disorder is one of an ocular injury, retinal detachment, or an XLRs-related disorder.
74. A method for making an RS1 protein, comprising:
 - a) genetically engineering at least one human cell to express a retinoschisin protein comprising the amino acid sequence of any one of SEQ ID NO: 4, 7, 11, 15, 19, or 21; and,

- b) recovering the retinoschisin protein from the at least one human cell or a media in contact with the cells.
75. The method of claim 74, wherein the genetically modified human cells are ARPE-19 cells.
76. The method of claims 74 or 75, wherein the genetically modified human cells have been transformed with a nucleic acid molecule of any one of claims 1-22, or an expression cassette of any one of claims 23-34, or an expression vector of any one of claims 35-45.
77. The method of any one of claims 74-76, wherein the media in contact with the cells is serum-free liquid cell culture media.
78. The method of any one of claims 74-77, wherein the genetically engineering comprises transfecting the at least one human cell with an expression vector of any one of claims 20-29.
79. The method of claim 62, wherein the transfection is conducted using a DNA:Lipofectamine ratio of 1:1.
80. The method of claims 62 or 79, wherein the transfection is conducted using between about 0.15 µg and 150µg expression vector DNA/1 million cells.
81. The method of any one of claims 74-80, wherein the recovering is conducted between about 6 hours and about 14 hours after transfection of the at least one human cell.
82. The method of any one of claims 74-81, wherein the recovering comprises isolating the retinoschisin protein from the media in contact with the cells.
83. The method of any one of claims 74-82, wherein the recovering comprises isolating the retinoschisin protein from the at least one human cell or a media in contact with the cells by affinity chromatography.
84. The method of any one of claims 74-82, further comprising purifying the retinoschisin protein by affinity chromatography, size-exclusion chromatography, or a combination of both.

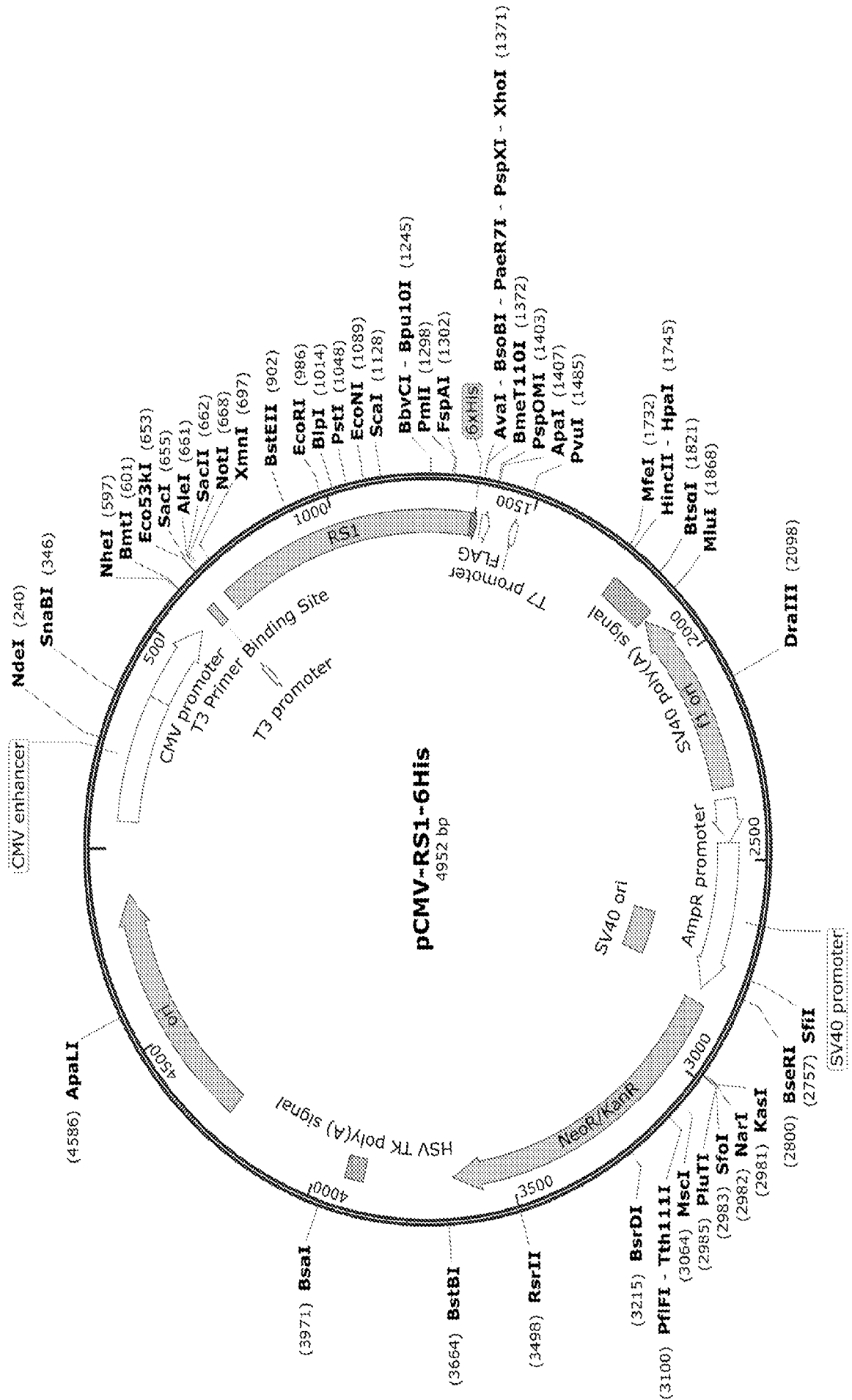


FIG. 1

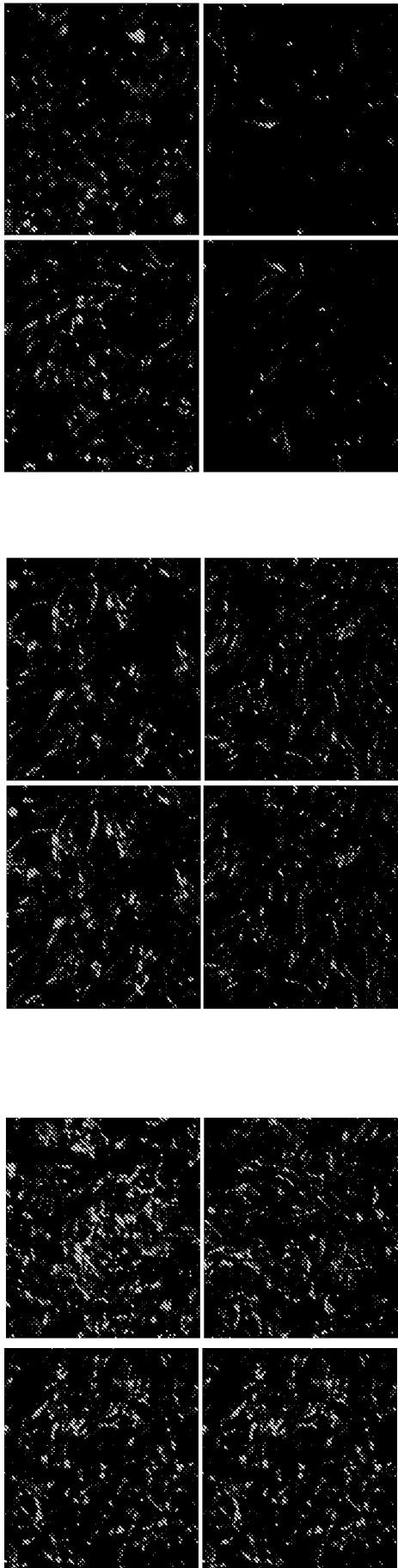


FIG. 2A

FIG. 2B

FIG. 2C

Un-transfected ARPE-19

Transfected ARPE-19 (1:1)

Transfected ARPE-19 (1:2)

Transfected ARPE-19 (1:3)

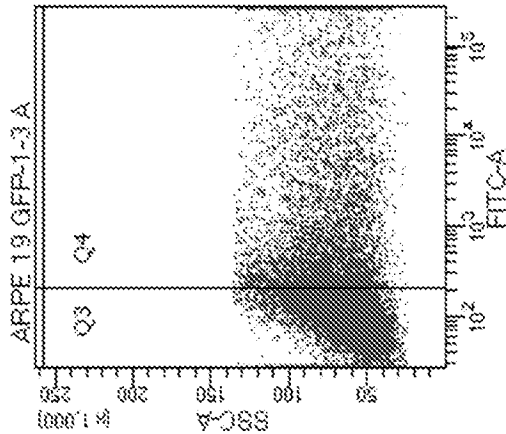
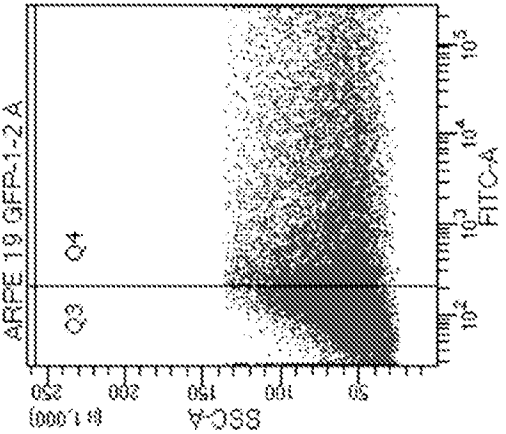
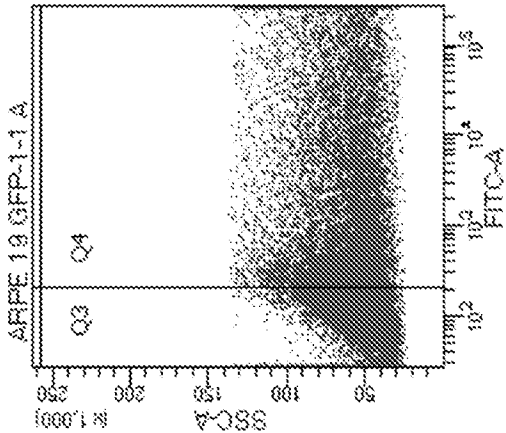
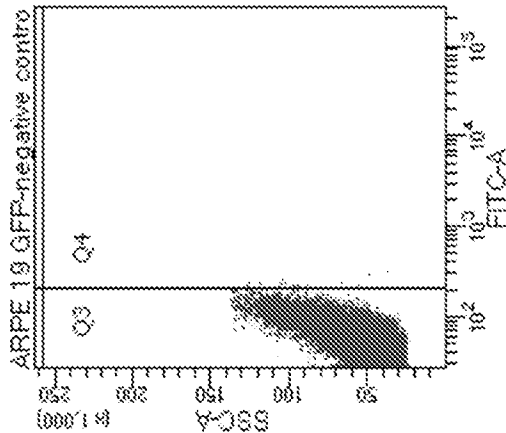


FIG. 2D

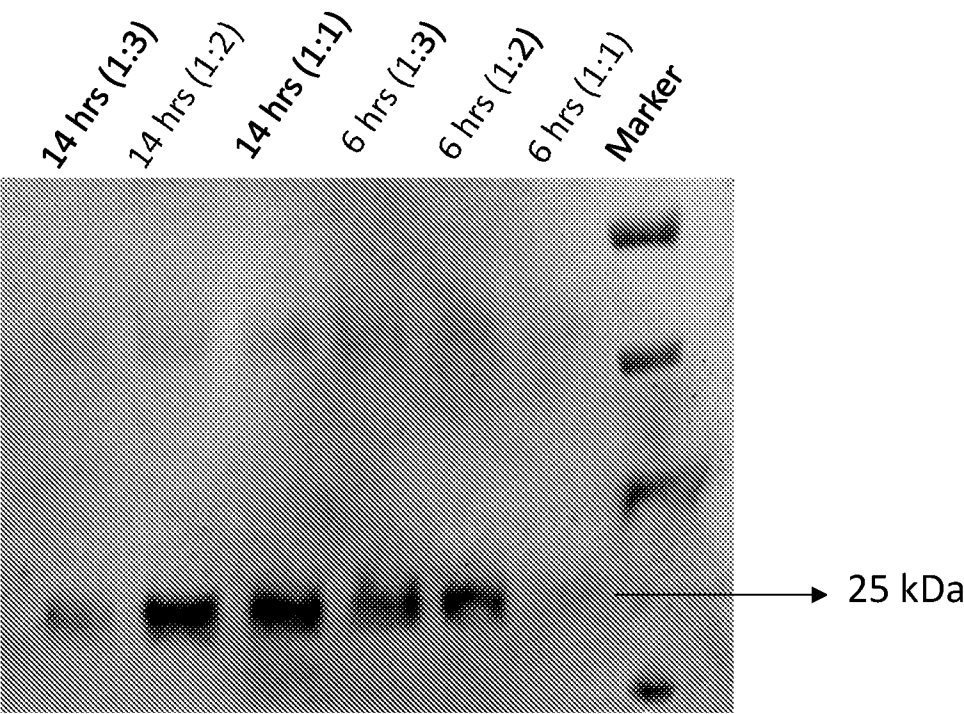


FIG. 3A

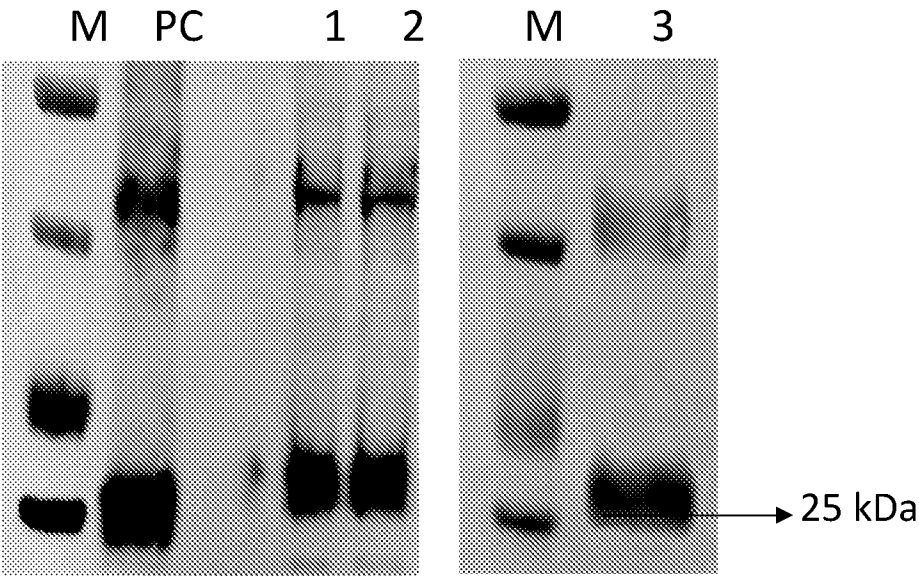


FIG. 3B

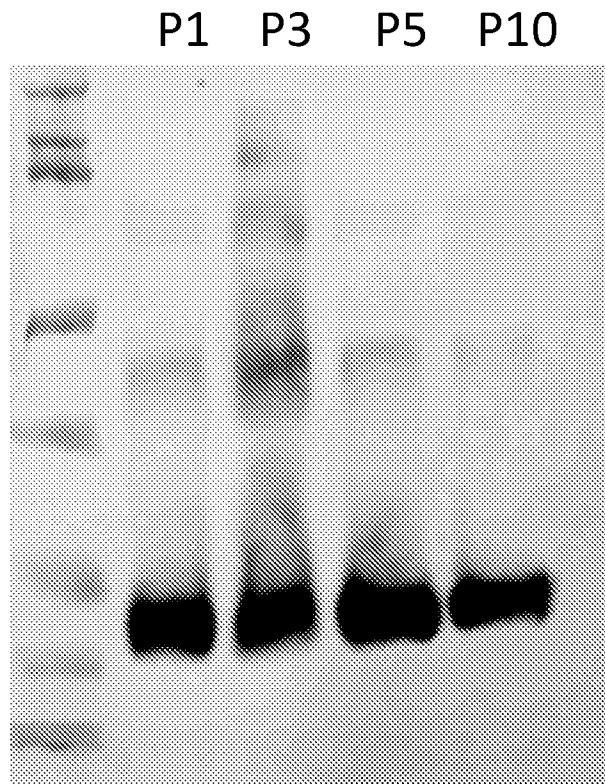


FIG. 4A

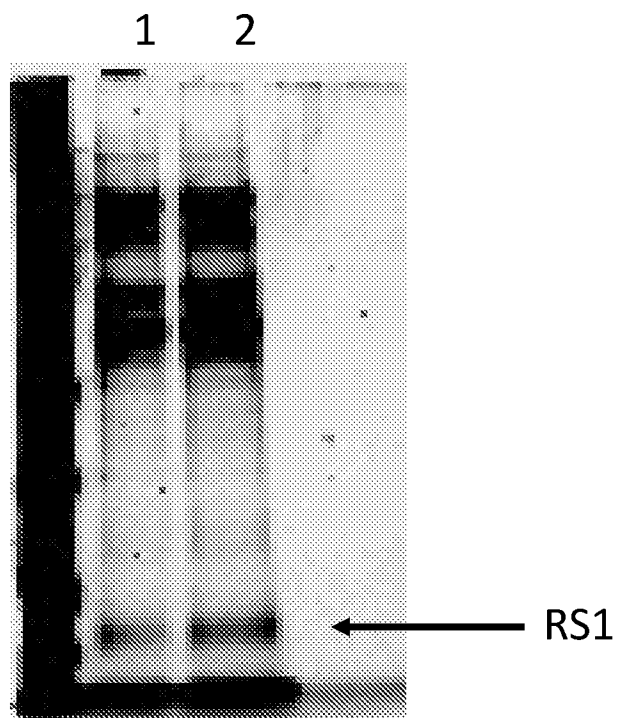
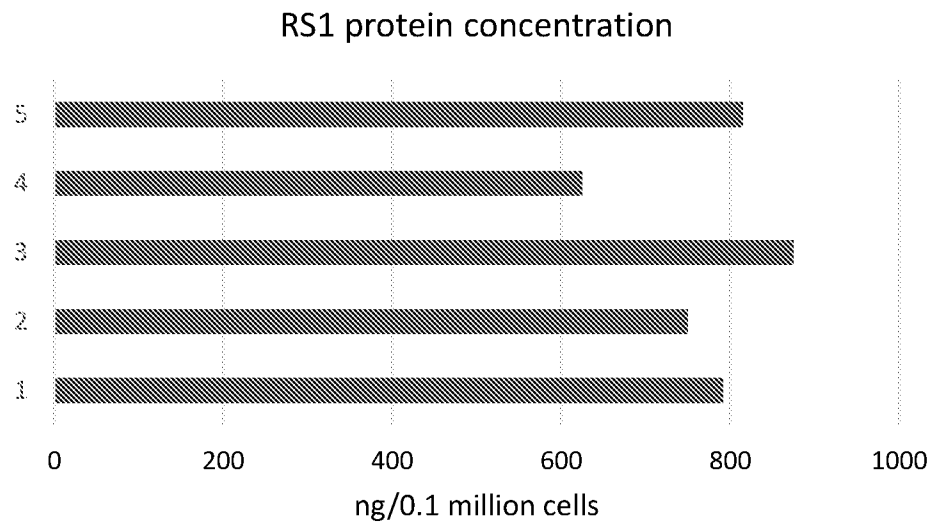
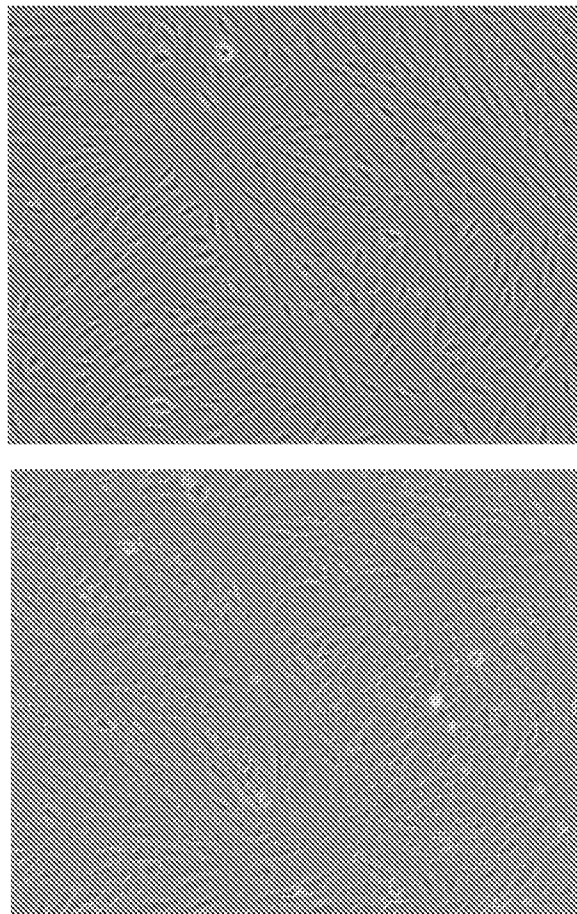
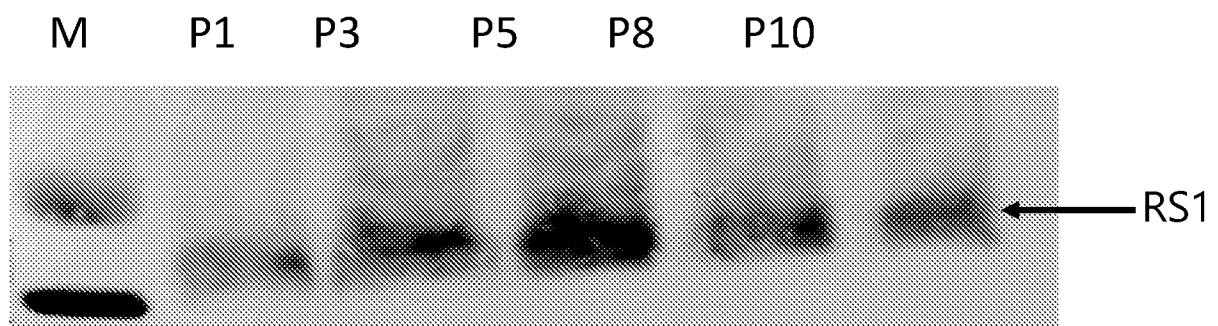


FIG. 4B

**FIG. 4C****FIG. 4D**

**FIG. 4E****FIG. 4F**

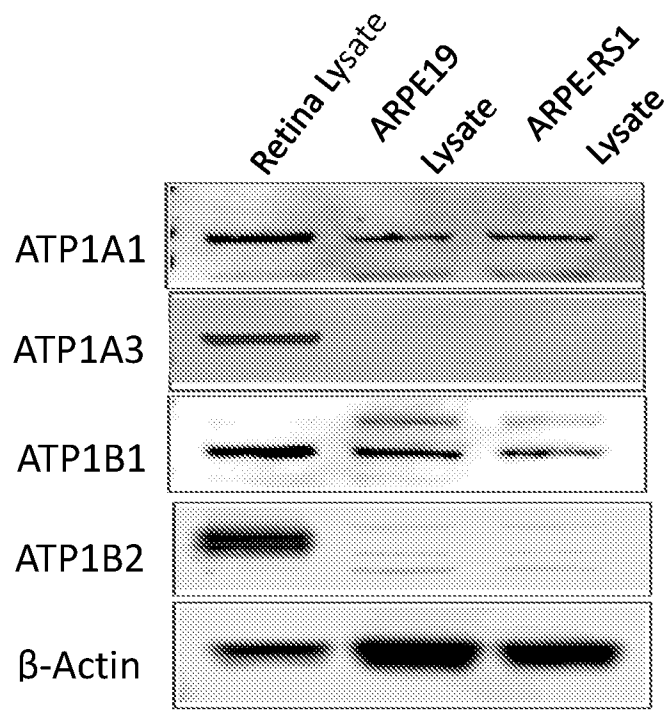


FIG. 4G

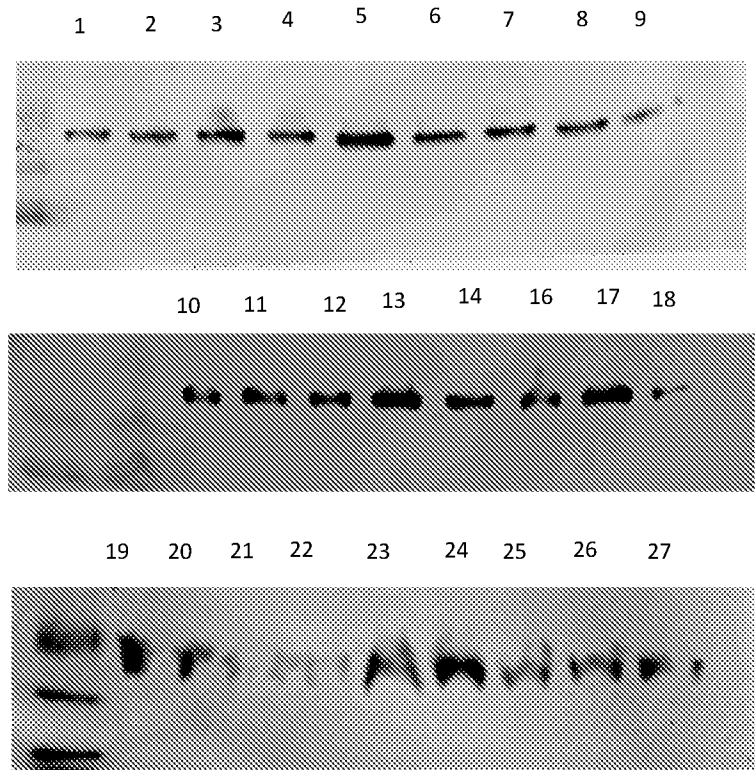


FIG. 5

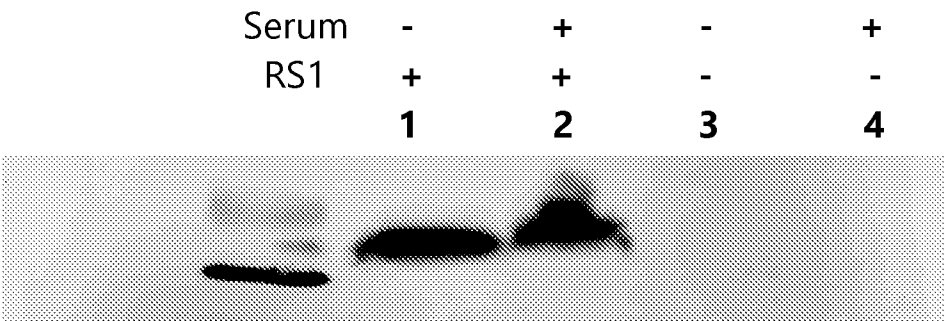


FIG. 6A

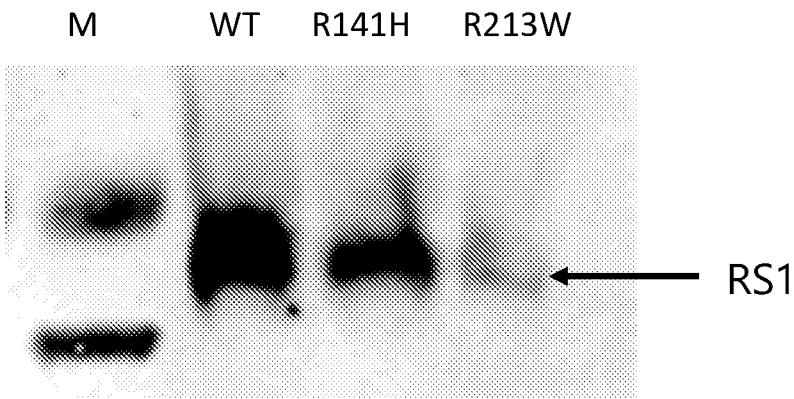


FIG. 6B

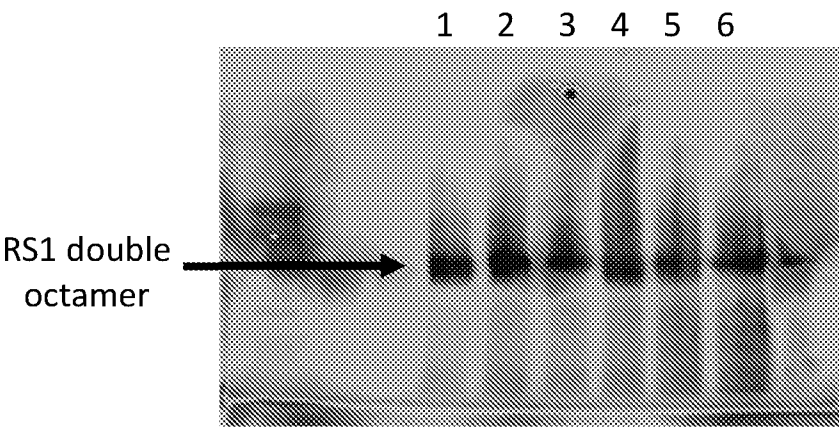


FIG. 6C

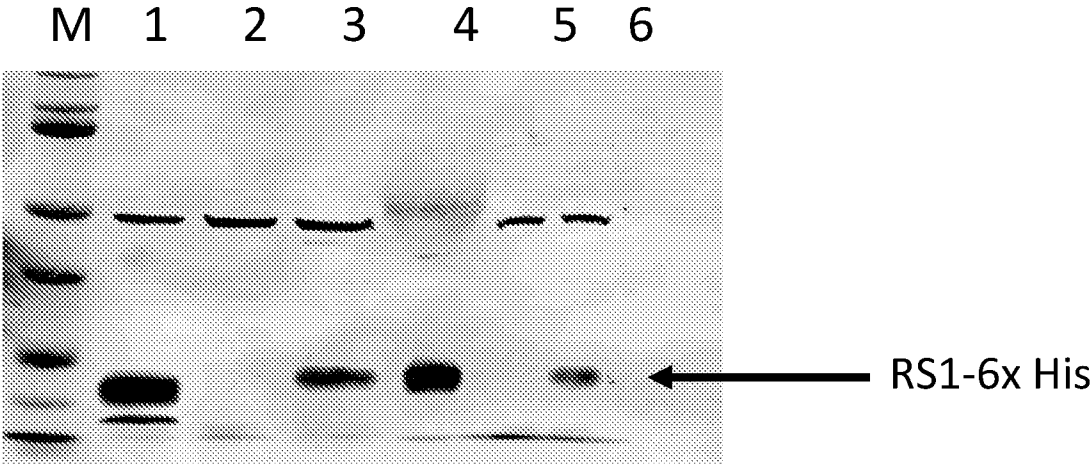


FIG. 6D

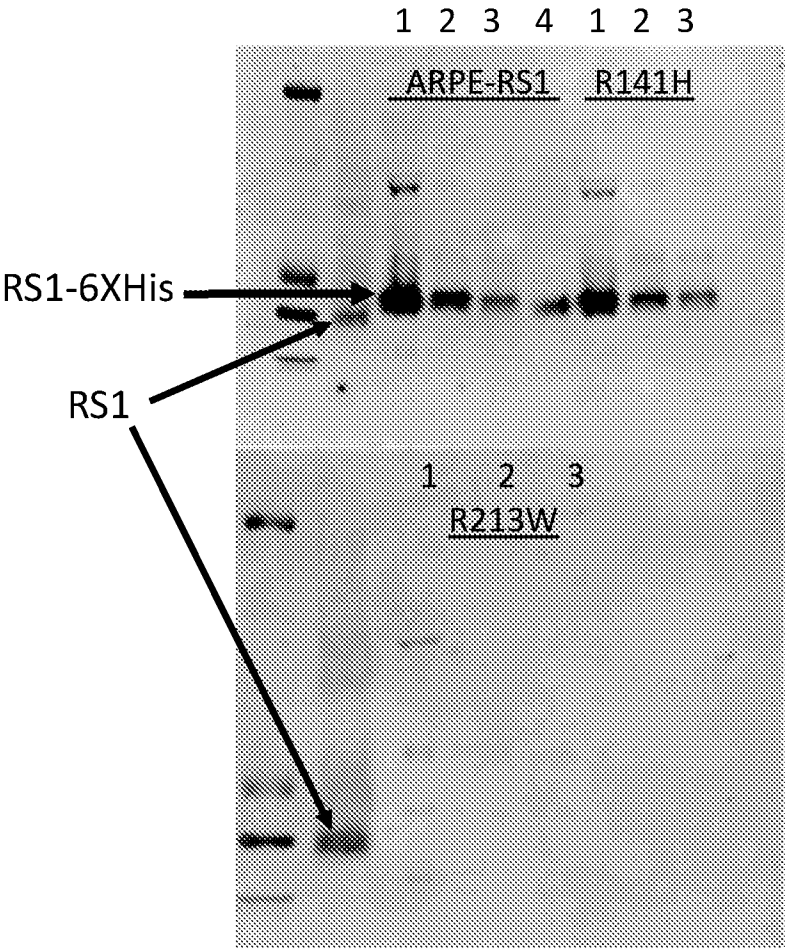
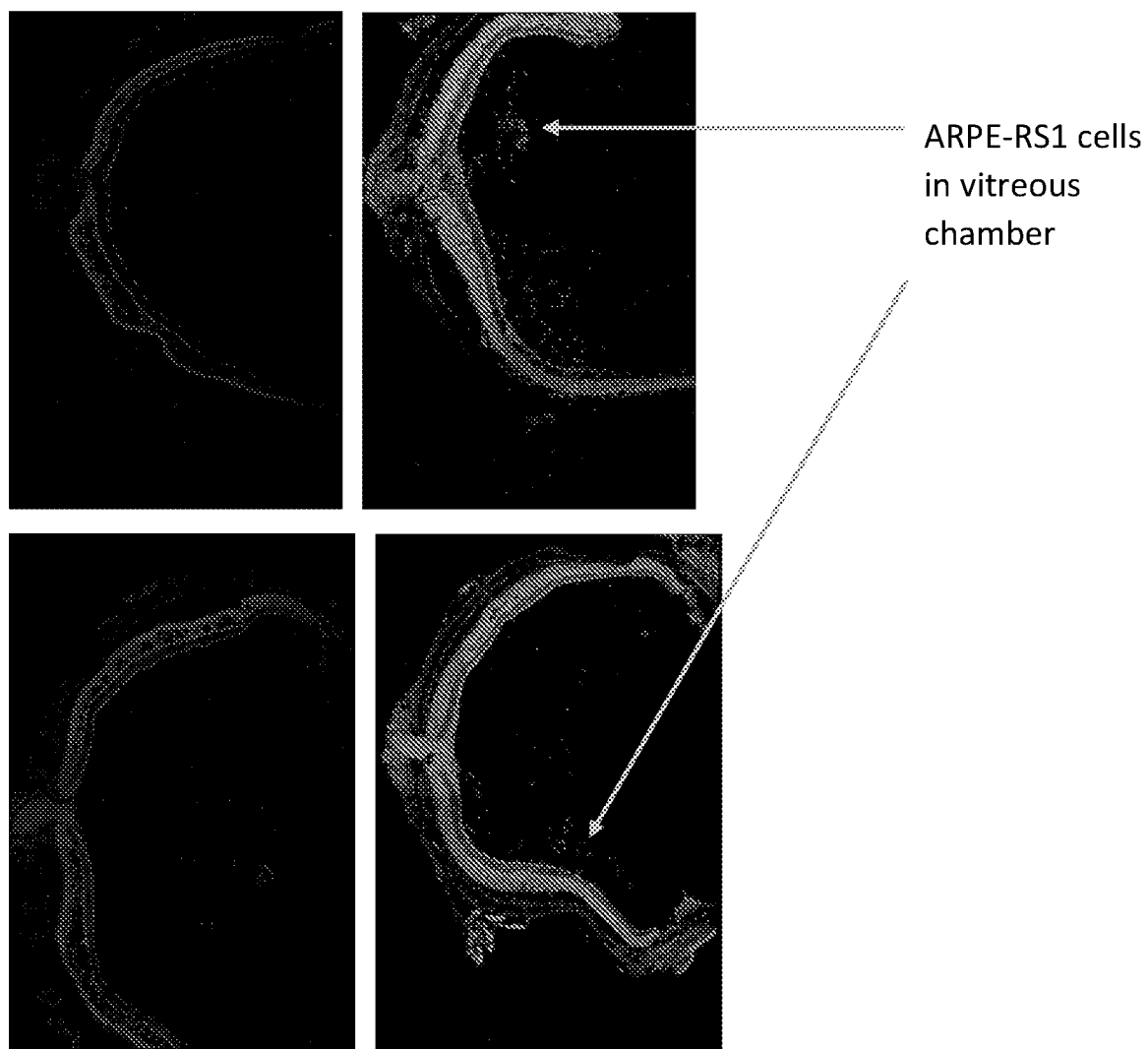
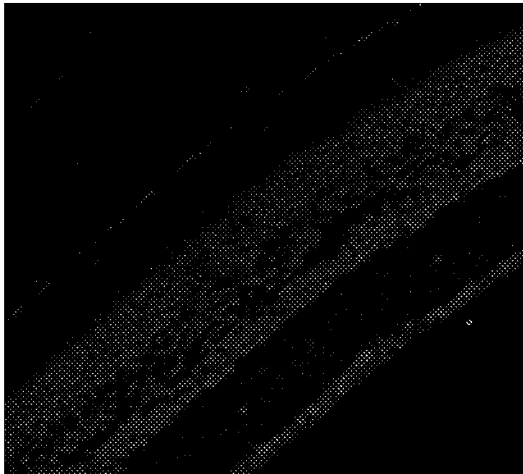
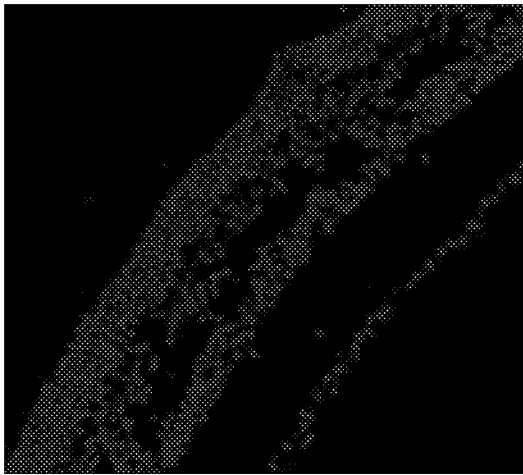
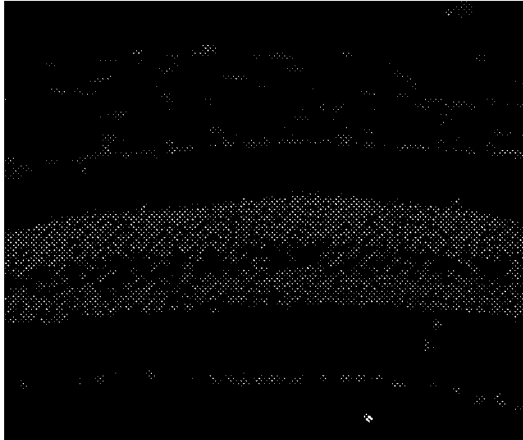
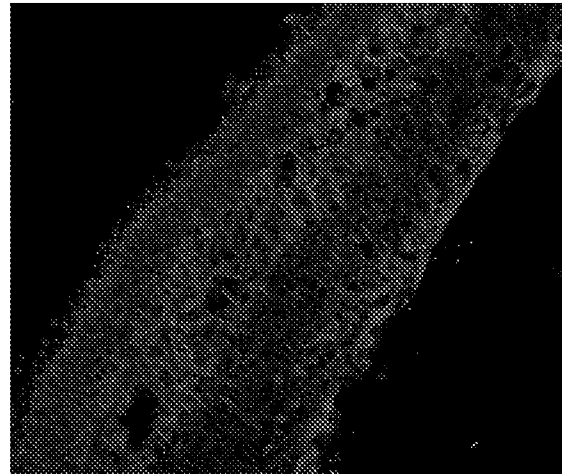
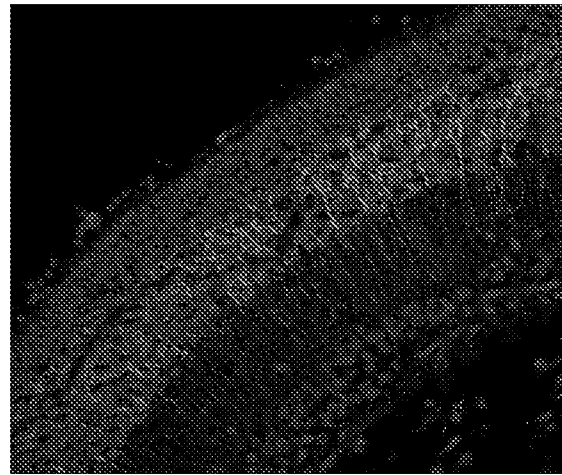
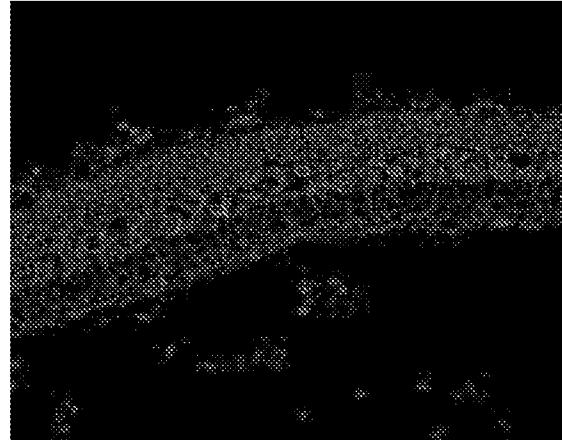
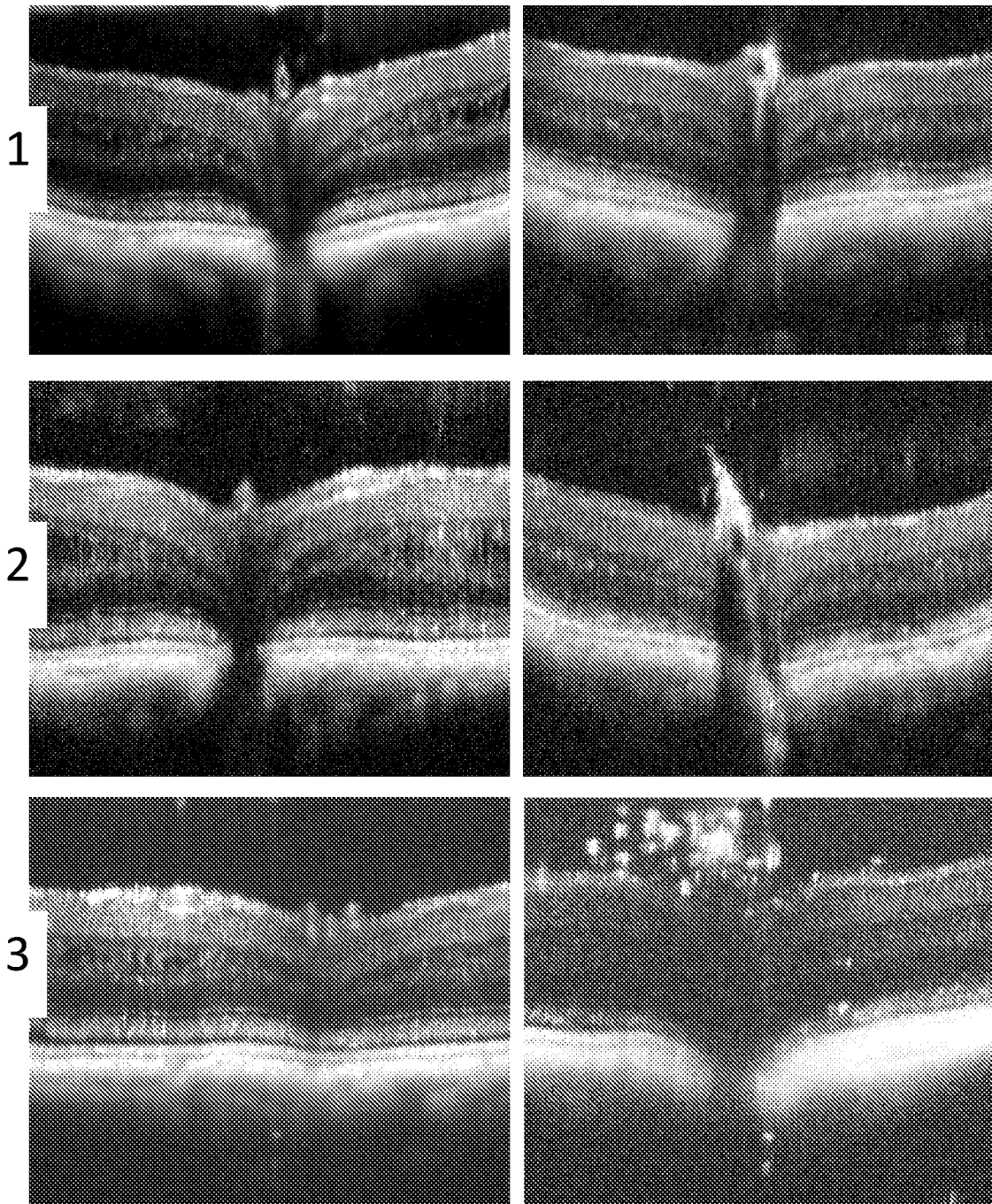


FIG. 6E

**FIG. 7A**

Un-Injected Eye**ARPE-RS1 Injected eye****FIG. 7B**

Un-Injected Eye**ARPE-RS1 Injected eye****FIG. 7C**

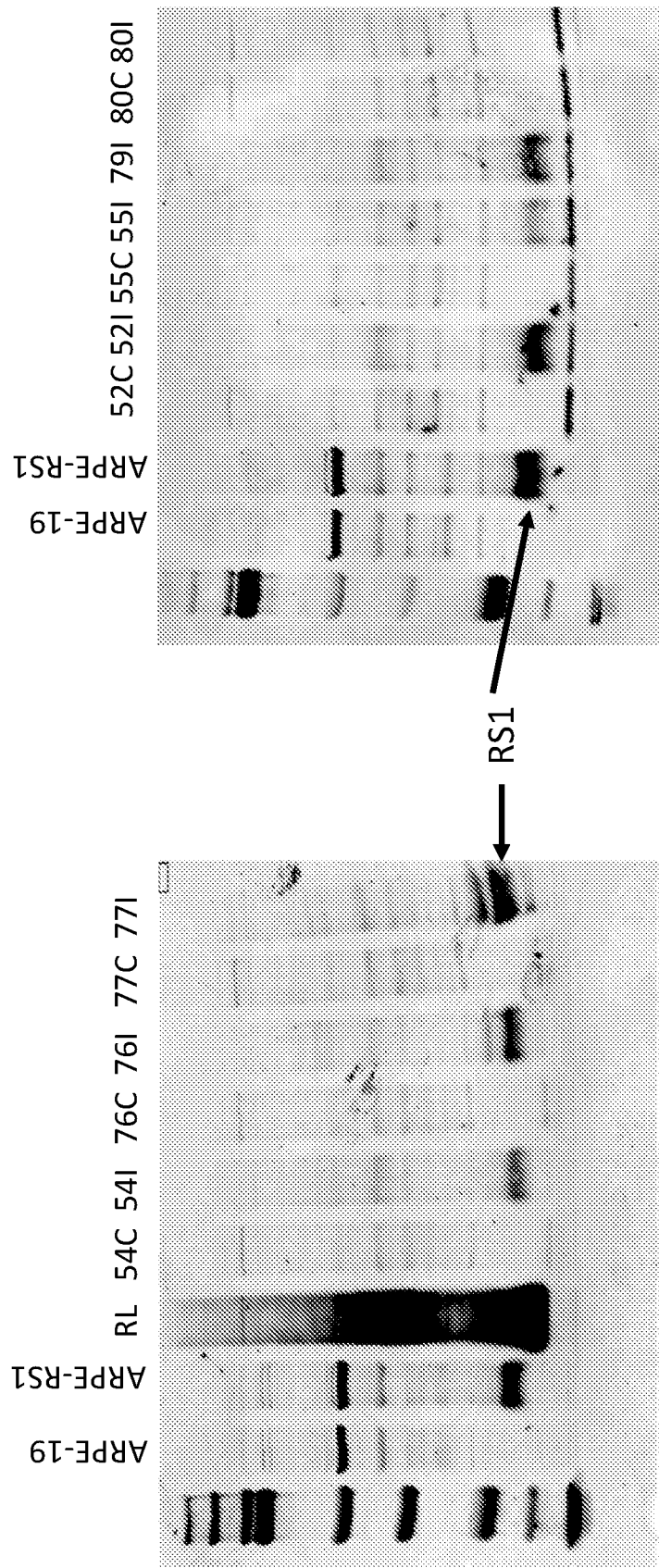


FIG. 8A

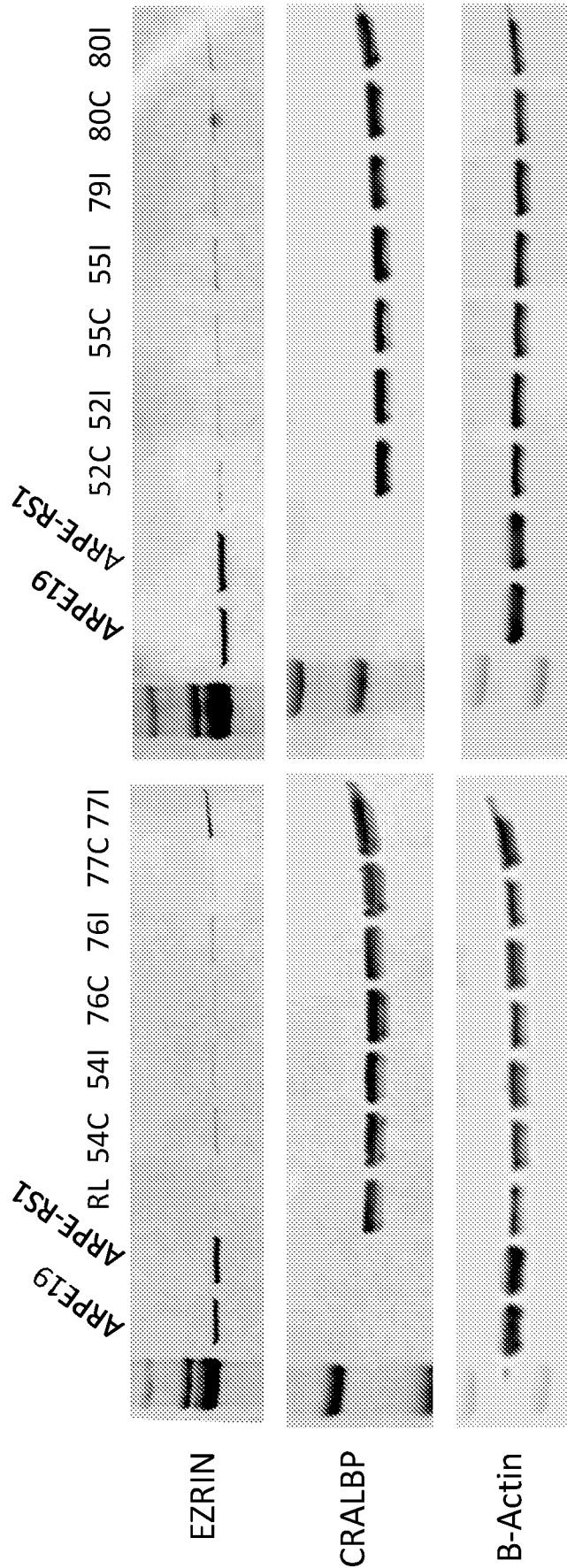


FIG. 8B

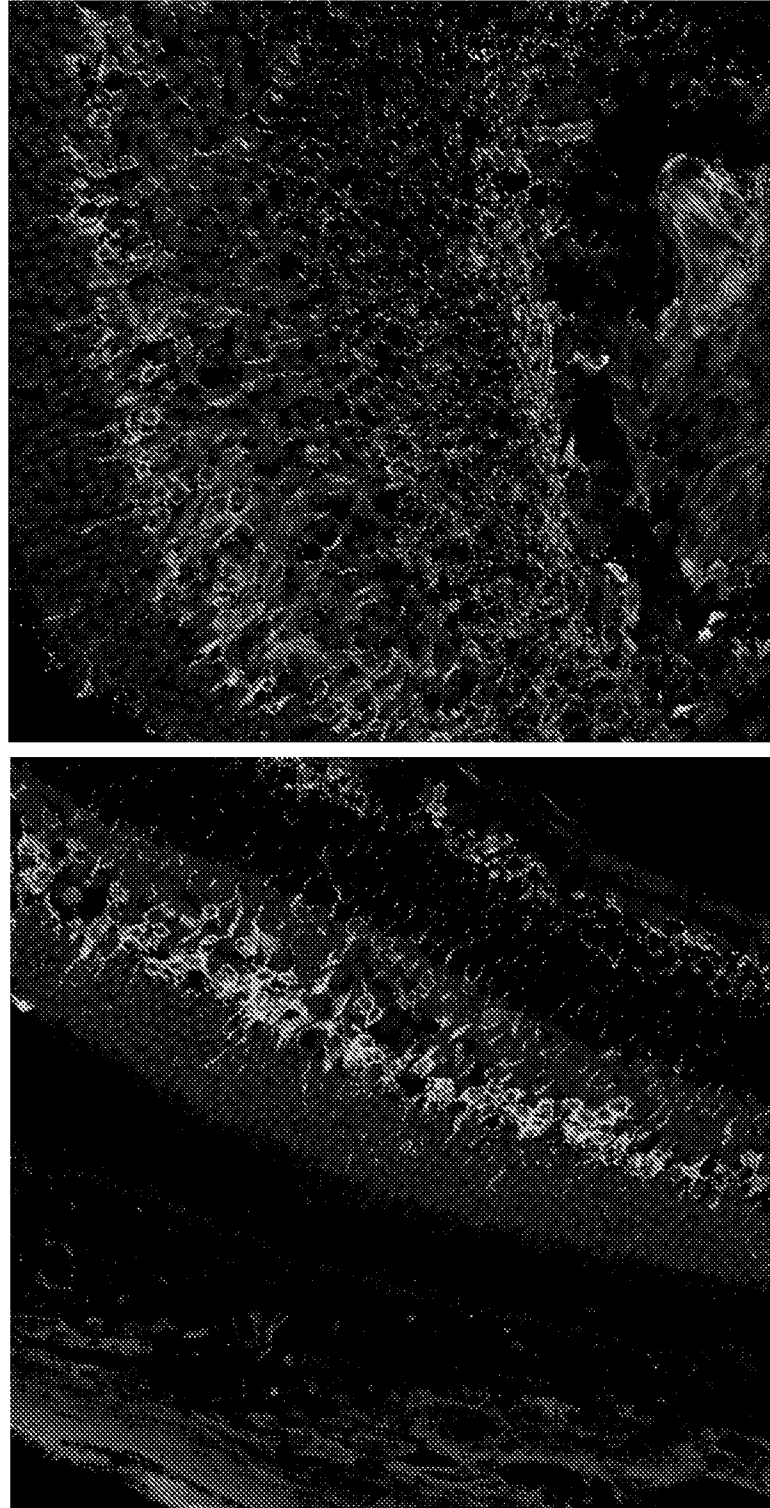


FIG. 8C

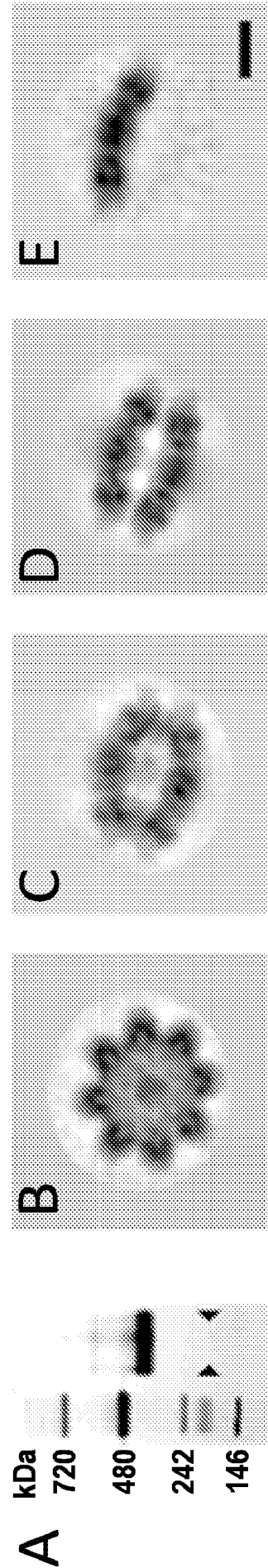


FIG. 9

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2021/040790

A. CLASSIFICATION OF SUBJECT MATTER

INV. A61K48/00 C07K14/47
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EP0-Internal, BIOSIS, Sequence Search, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2014/127196 A1 (US HEALTH [US]) 21 August 2014 (2014-08-21) cl 1-69; Fig. 1; SEQ ID NO: 2, 5; pg 12, 14-15, 34-39 -----	1-5,7, 10-25, 28-57, 59-65, 67,69, 70,77, 79,83,84
X	WO 2019/144077 A1 (THE USA AS REPRESENTED BY THE SECRETARY DEPT OF HEALTH AND HUMAN SERVI) 25 July 2019 (2019-07-25) cl 1-16; Fig. 1; SEQ ID NO: 2, 5 ----- -/--	1-5,7, 10-25, 28-57, 59-65, 67,69, 70,77, 79,83,84



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search

14 October 2021

Date of mailing of the international search report

26/10/2021

Name and mailing address of the ISA/

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Fax: (+31-70) 340-3016

Authorized officer

Behrens, Joyce

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2021/040790

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	T K PARK ET AL: "Intravitreal delivery of AAV8 retinoschisin results in cell type-specific gene expression and retinal rescue in the Rsl-KO mouse", GENE THERAPY, vol. 16, no. 7, 1 July 2009 (2009-07-01), pages 916-926, XP055113217, ISSN: 0969-7128, DOI: 10.1038/gt.2009.61 abstract; Fig. 2 pg 917 -----	1-5,7, 10-25, 28-57, 59-65, 67,69, 70,77, 79,83,84
X	APAOLAZA PAOLA STEPHANIE ET AL: "A novel gene therapy vector based on hyaluronic acid and solid lipid nanoparticles for ocular diseases", INTERNATIONAL JOURNAL OF PHARMACEUTICS, ELSEVIER, NL, vol. 465, no. 1, 24 February 2014 (2014-02-24), pages 413-426, XP028837215, ISSN: 0378-5173, DOI: 10.1016/J.IJPHARM.2014.02.038 abstract; Fig. 13; pg 414-416, 425 -----	1-5,7, 10-17, 20,23, 26,27, 30, 32-35, 37-41, 45-84
X	EP 2 656 837 A1 (UNIV PAIS VASCO [ES]; UNIV MIGUEL HERNANDEZ DE ELCHE [ES]) 30 October 2013 (2013-10-30) par 67-71; ex 15-20; cl 1-14 -----	1-5,7, 10-17, 20,23, 26,27, 30, 32-35, 37-41, 45-84
X	WU WINCO W.H. ET AL: "RS1, a Discoidin Domain-containing Retinal Cell Adhesion Protein Associated with X-linked Retinoschisis, Exists as a Novel Disulfide-linked Octamer", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 280, no. 11, 1 March 2005 (2005-03-01), pages 10721-10730, XP055851187, US ISSN: 0021-9258, DOI: 10.1074/jbc.M413117200 pg 10722, 10727; Fig. 6 ----- -/--	1,5,6,8, 9

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2021/040790

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/184173 A1 (DOSE MEDICAL CORP [US]) 3 December 2015 (2015-12-03) par 2, 511; cl 1-41	4,7, 10-12, 17,20, 32-34, 41,45, 60-65, 67,69, 70,77, 79,83,84
A	LIU YANG ET AL: "Mouse models of X-linked juvenile retinoschisis have an early onset phenotype, the severity of which varies with genotype", HUMAN MOLECULAR GENETICS, vol. 28, no. 18, 15 September 2019 (2019-09-15), pages 3072-3090, XP055851197, ISSN: 0964-6906, DOI: 10.1093/hmg/ddz122 abstract; pg 3083, 3085	1-84
A	Gisele Silva ET AL: "Implants as drug delivery devices for the treatment of eye diseases", Brazilian Journal of Pharmaceutical Sciences, 1 September 2010 (2010-09-01), pages 585-595, XP055669156, DOI: 10.1590/S1984-82502010000300024 Retrieved from the Internet: URL:http://www.scielo.br/pdf/bjps/v46n3/v46n3a24.pdf [retrieved on 2021-10-14] pg 588	1-84

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2021/040790

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2021/040790

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014127196 A1	21-08-2014	AU 2014216160 A1	13-08-2015
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		JP 2017517363 A	29-06-2017
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		WO 2015184173 A1	03-12-2015
