NOVEL TARGETS FOR THE TREATMENT OF RETINA DISEASES

This invention relates to novel treatments of ocular diseases. In particular, the invention provides methods of treating a retinal disorder in a mammal comprising modulating the amount of a polypeptide encoded by a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes, in a retinal cell. The invention also relates to methods of diagnosing a retinal disorder comprising identifying an abnormal expression of a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes.

BACKGROUND OF THE INVENTION

The vertebrate retina is a complex network of neural and non-neuronal cells that lines the back of the eye. The electrical signals generated in the photoreceptor cells are transmitted to the brain. The cones and rods (*i.e.*,the photoreceptor cells) are highly specialized and polarized neurons that are divided into several morphologically and functionally distinct compartments. Photoreceptor outer segments (OS) contain hundreds of flattened membrane disks, which are continually renewed throughout an individual's lifetime according to a circadian rhythm. Newly synthesized membrane is added at the base of the OS by expansion of the plasma membrane, whereas disks shed at the distal tip are phagocytized by the adjacent pigment epithelial cells.

The retinal pigment epithelium (RPE) is a neuroectoderm-derived cell monolayer located between the neuroretina and the choroid. The RPE helps to maintain homeostasis of the outer retina and assists the photoreceptors in visual transduction. RPE cells regulate the transport of retinoids and nutrients to the photoreceptors, the regeneration of the visual pigments, the phagocytosis and digestion of old rod outer segments, the

absorption of stray light and contribute to retinal adhesion. In addition, the RPE cells are thought to play active roles in the immune response and in retinal wound healing. During the last decade, it has become clear that the critical role of RPE cells in maintaining visual functions depends on the production of many secreted proteins. For example, RPE cells produce interleukins such as IL-1α, IL-1β, IL-6, IL-8 and IL-15, which are involved in the regulation of ocular immunity and inflammation processes [for review, Holtkamp *et al. Prog Retin Eye Res* **20**, 29-48 (2001); Campochiaro *et al. Prog Ret Eye Res* **15**, 547-567 (1996)]. The RPE also produces trophic agents such as FGF-2, PEDF, BDNF, NGF and NT-3, which are involved in the development and functional maintenance of the neuroretina [for review, Campochiaro *et al. Int Rev Cytol* **146**, 75-82 (1993); Steinberg *et al. Curr Opin Neurobiol* **4**, 515-24 (1994)]. The identification of several genes expressed by the RPE greatly improved the understanding of the biology of the retina and vision; however this knowledge is still incomplete.

RPE cells are also involved in a number of ocular diseases. Indeed, the RPE and the photoreceptors form a functional visual unit, meaning that the dysfunction of RPE cells can lead to photoreceptor degeneration. Mutations in at least nine genes expressed in RPE cells are associated with progressive photoreceptor degeneration [see RetNet, Daiger *et al.* http://www.sph.uth.tmc.edu/RetNet/; Saleem *et al. Clin Genet* **61**, 79-88 (2002)]. Mutations in the RPE65, RLBP1, MERTK, LRAT and RGR genes are associated with retinitis pigmentosa [for review Phelan *et al. Mol Vis* **6**, 116-24 (2000); Clarke *et al. Clin Genet* **57**, 313-29 (2000)]. Mutations in the VMD2, TIMP3, MYO7A and RDH5 genes are responsible for Best's vitelliform macular dystrophy, Sorsby's fundus dystrophy, Usher syndrome and fundus albipunctatus, respectively. The MERTK (a tyrosine kinase receptor), TIMP3 (a tissue inhibitor of metalloproteinases) and RDH5 (11-cis retinol dehydrogenase) genes encode secreted proteins carrying a putative peptide signal sequence. Thus, the identification of new proteins

secreted by the RPE cells may lead to the identification of additional genes involved in important retinal functions or retinal disorders.

Thus, it is an object of the present invention to provide new genes and proteins involved in retinal disorders, and especially new secreted proteins synthesized in the RPE.

It is another object of the present invention to provide methods of treating retinal disorders.

It is another object of the present invention to provide methods of diagnosing retinal disorders.

SUMMARY OF THE INVENTION

The invention provides a method of treating a retinal disorder comprising modulating the amount of a polypeptide encoded by a target gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes in a retinal cell.

In a another embodiment, the method comprises administering in the eye of a mammal, an effective amount of a gene transfer vector, carrying a nucleic acid comprising one of the gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes; said gene being operably linked to a promoter active in said retinal cell.

In another embodiment, the method comprises administering to a retinal cell of a mammal, an effective amount of a gene transfer vector, carrying an antisense nucleic acid of the gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes; said antisense being capable of inhibiting *in vivo* the expression of said target gene.

The invention also provides a composition comprising cells, which produce and secrete a polypeptide encoded by a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes.

The invention further provides a method of diagnosing a retinal degeneration in a mammal, comprising identifying an abnormal expression of a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes, wherein said abnormal expression is indicative that said mammal suffers from a retinal degeneration. In a another embodiment, the method of diagnosing comprises identifying an inhibition of expression of the gene encoding prosaposin in a retinal cell of said mammal, wherein said inhibition is indicative that said mammal suffers from a retinal degenerative disorder.

The invention also provides a method for screening compounds that modulate the expression of the gene encoding prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* or *Pcdh-γ-C3* and a method for screening ligands that binds to the expression product of the gene encoding prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* or *Pcdh-γ-C3.*

BRIEF DESCRIPTION OF THE DRAWINGS

Fig 1 (A) is a partial nucleotide and deduced amino acid sequences of the pig prosaposin cDNA. The clone selected contained a 172-bp cDNA insert with an open reading frame coding for the first 51 amino acids of the pig prosaposin protein. The AUG start codon at position 20 is indicated in **bold**. The signal sequence is underlined. **(B)** Alignment of the amino-terminal sequences of pig, human, mouse, rat and bull prosaposin. Dots indicate

conserved amino acids. The vertical arrow indicates the predicted cleavage site of the signal peptide.

Fig 2 shows the distribution of prosaposin mRNA in the retina of a 14-day-old normal rdyp$^+$ rat. (**A, B, C**) *In situ* hybridization of the prosaposin mRNA using an antisense probe in a pigmented rat retina. Labeled prosaposin mRNA appeared as *dark blue* or *purple* precipitates, as indicated by arrows (**B**) Higher magnification of the outer portion of the retina (**C**) Higher magnification of the inner portion of the retina (**D**) Hybridization with a control sense probe. (**E**) *In situ* hybridization of the prosaposin mRNA using an antisense probe in an unpigmented rat retina.

The following abbreviations with their corresponding meanings are set forth in this figure:

GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; is, inner segment; os, outer segment; RPE, retinal pigment epithelial cell layer; and CC, Choroid.

Fig 3 shows the distribution of prosaposin mRNA in the retina of a 21-day-old normal rdy rat. (**A, B, C**) *In situ* hybridization of the prosaposin mRNA using an antisense probe in a pigmented rdy$^-$ rat retina. (**B**) Higher magnification of the outer portion of the retina (**C**) Higher magnification of the inner portion of the retina (**D**) Hybridization with a control sense probe. (**E**) *In situ* hybridization of the prosaposin mRNA using an antisense probe in an unpigmented rat retina.

Fig 4 shows the distribution of prosaposin mRNA in the retina of a two-month-old unpigmented Wistar rat. (**A, B** and **C**) *In situ* hybridization of the prosaposin mRNA using an antisense probe. (**D**) Hybridization with a control sense probe.

Fig 5 shows the immunohistochemical localization of prosaposin in the retina of a 45-day-old unpigmented RCS rdy+ (**A, B, C and D)** and rdy- rats (**E and F**). The prosaposin staining appears in brown. (**B and F**) Higher magnification of the outer portion of the retina. (**C**) Higher magnification of the inner portion of the retina. (**D**) Control section of rat retina incubated with nonimmune serum.

Fig 6 (**A**) shows the relative amounts ascertained by RT-PCR of prosaposin and cyclophilin (internal control) mRNA in RCS rdy+ and rdy- rat pigment epithelial cells 14, 21, and 45 days after birth. The 858-bp and the 311-bp bands correspond to prosaposin and cyclophilin PCR products, respectively. (**B**) As a control, the same experiment was performed on mRNA extracted from the cerebellum of the two rat strains.

Fig 7 is a RT-PCR analysis of *TMS-2* mRNA transcript in control *rdy+* and dystrophic *rdy-* RCS rat strain. The amount of *TMS-2* transcript (920 bp, *top*) was compared with cyclophilin A transcript (internal control, 310 bp, *bottom*) expressed in the same samples. A representative sample is shown for experiments performed in duplicate. Lane M, 100 bp or 1 kb DNA ladder (Promega), lane Cp, control without primer pairs.

Fig. 8 is a RT-PCR analysis of *Pcdh-γ-C3* mRNA transcript in control *rdy+* and dystrophic *rdy-* RCS rat strain. Level of *Pcdh-γ-C3* transcript (600 bp, *top*) was normalized with cyclophilin A as an internal control (310 bp, *bottom*). A representative sample is shown for experiments performed in duplicate. Lane M, 100 bp or 1 kb DNA ladder (Promega), lane Cc, control without cDNA.

Fig. 9 shows the localization of *TMS-2* mRNA in RCS rat tissues by *in situ* hybridization. Tissue sections hybridized with antisense riboprobes (B-C, E-F), showed posititve dark blue precipitates compared to sense probes (A, D). Arrowheads indicate *TMS-2* signals in Leydig cells located in the

interstitial layer and along the seminiferous epithelium of a testicular fragment from a one-year-old *rdy+* rat testicular fragment (B-C). Purkinje cells in the cerebellum showed strong expression of *TMS-2* transcripts in wild-type RCS *rdy+* (E, *arrows*) as well as in dystrophic RCS *rdy-* (F, *arrows*) at 21-postnatal-day-old rats, respectively. Scale bar, 1 cm = 200 μm in A, F.

Fig. 10 shows the localization of *Pcdh-γ-C3* mRNA expression in RCS rat tissues by *in situ* hybridization. Tissue sections hybridized with antisense riboprobes (B-C, E-F), appeared as dark blue precipitates compared to sense probes (A, D). Weak *Pcdh-g-C3* signals were found in the seminiferous tubules of testicles from one-year-old *rdy+* rat testicular fragment (B-C). In the cerebellum, all the Purkinje cells showed strong expression of *Pcdh-γ-C3* transcripts in wild-type RCS *rdy+* (E, arrows) as well in dystrophic RCS *rdy-* (F, arrows) at 21-postnatal-day-old rats, respectively. Scale bar, 1 cm = 200 μm in A-F.

Fig. 11 shows the expression of *TMS-2* mRNA from PN14 to PN37 of wild-type *rdy+* (A-D) and dystrophic *rdy-* (E-H) RCS rat strain. Hybridization with the sense probes (A, E) showed negative signal compared the dark blue precipitates with antisense riboprobes at PN14 (E-F), PN22 (A-B) and PN37 (C-D, G-H). In both normal and dystrophic RCS rat retinas, intense staining was predominantly found in the outer nuclear layer of photoreceptors (onl). Moderate signals were detected in the ganglion cell layer (gcl) and inner nuclear layer (inl). Higher magnification images of 37-day-old RCS rats showed thinning of the outer nuclear layer (onl) in dystrophic RCS retinas (G, H, *double arrows*) compared to normal retinas (D, *large double arrows*) and a debris region (d) of non-phagocytized outer segment membranes (G, H, *asterisk*). The following abbreviations and their meanings are set forth in this figure ch, choroid; gcl, ganglion cell layer; inl, inner nuclear layer; ipl, inner plexiform layer; is, inner

segments of photoreceptors; onl, outer nuclear layer; os, outer segments of photoreceptors; rpe, retinal pigment epithelium; and s, sclera. Scale bar (0.7 cm): 64 µm in B, D, G, H and 160 µm in A, C, E-F.

Fig. 12 shows the expression of *Pcdh-γ-C3* mRNA from PN14 to PN37 in wild-type *rdy+* (A-D) and dystrophic *rdy-* RCS (E-H) rat strain. Retinas of 14-day-old rat hybridized with antisense riboprobes (B, F) showed dark blue positive signals compared to sense probes (A,E). In both rat strains, intense staining was predominantly found in the outer nuclear layer of photoreceptors (onl). Moderate signals were detected in the ganglion cell layer (gcl), inner nuclear layer (inl). At PN 37, dystrophic RCS rat retinas showed thinning of the outer nuclear layer (onl) (H, *double arrows*) of than that of normal retinas (D, *large double arrows*) and a debris region (d) of non-phagocytized outer segment membrane was visible (G, H, *asterisk*).

Fig. 13 shows a partial amino acid sequences alignment of the rat, mouse and human USAG-1 protein. The signal sequence is underlined. The vertical arrow indicates the predicted cleavage site of the signal peptide.

Fig. 14 shows the transfection of RPE D407 cells using a USAG-1/HA chimer. This indicates that USAG-1 is a secreted protein.

Fig. 15. Illustrates the tissue distribution of USAG-1 mRNA, achieved by RT-PCR. This figure illustrates that USAG-1 is highly expressed in kidney, forebrain and cerebellum, and in the eye (particularly in the retinal pigment epithelial cells). USAG-1 is also present in the neuro retina.

Fig. 16 shows the in situ hybridization to study the distribution of USAG-1 mRNA in paraffin-embedded sections of rdy^+ pigmented rat eyes at different postnatal stages (P14, P21 and P45) using DIG-labeled sense and antisense riboprobes. The presence of USAG-1 in the eye and the retina is

demonstrated, the following abbreviations and their meanings are set forth in this figure; gcl, ganglion cell layer; inl, inner nuclear layer; onl, outer nuclear layer; rpe, retinal pigment epithelium; s, sclera.

Fig. 17 is a semi-quantitative RT-PCR to compare the amounts of USAG-1 mRNA in RPE cells of normal *rdy*$^+$ and pathologic *rdy*$^-$ RCS rats at various developmental ages (P14, P21 and P45). As a control, the same experiment is performed on mRNAs extracted from the cerebellums of the two rat strains at the same ages . Cyclophilin A mRNA is used as an internal control. In the normal *rdy*$^+$ retina, the amount of USAG-1 mRNAs in retinal pigment epithelial cells is clearly higher at P14 and P21 than at P45, whereas in the *rdy*$^-$ retina, the amount of USAG-1 mRNAS in retinal pigment epithelial cells is lower at P14 than at P21 and P45.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

As used herein the terms "polynucleotides" and "nucleic acids" are used interchangeably and include, but are not limited to RNA, DNA, RNA/DNA sequences more than one nucleotide, in either single strand or duplex form. The polynucleotides of the invention may be prepared from any known appropriate method including, but not limited to, any synthetic method, any recombinant method, any *ex vivo* generation method and the like, as well as combinations thereof.

The term "polypeptide" means herein a polymer of amino acids having no specific length. Thus, peptides, oligopeptides and proteins are included in the definition of "polypeptide" and these terms are used interchangeably throughout the specification, as well as in the claims. The term "polypeptide" does not exclude post-translational modifications such as

polypeptides having covalent attachment of glycosyl groups, acetyl groups, phosphate groups, lipid groups and the like. Also encompassed by this definition of "polypeptide" are homologs thereof.

The term "sequence identity" refers to the identity between two peptides or between two nucleic acids. Identity between sequences can be determined by comparing a position in each of the sequences which may be aligned for the purposes of comparison. When a position in the compared sequences is occupied by the same base or amino acid, then the sequences are identical at that position. A degree of sequence identity between nucleic acid sequences is a function of the number of identical nucleotides at positions shared by these sequences. A degree of identity between amino acid sequences is a function of the number of identical amino acid sequences that are shared between these sequences. Since two polypeptides may each (i) comprise a sequence (i.e., a portion of a complete polynucleotide sequence) that is similar between two polynucleotides, and (ii) may further comprise a sequence that is divergent between two polynucleotides, sequence identity comparisons between two or more polynucleotides over a "comparison window" refers to the conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference nucleotide sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences.

To determine the percent identity of two amino acids sequences or two nucleic acid sequences, the sequences are aligned for optimal comparison. For example, gaps can be introduced in the sequence of a first amino acid sequence or a first nucleic acid sequence for optimal alignment with the second amino acid sequence or second nucleic acid sequence. The amino

acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, the molecules are identical at that position.

The percent identity between the two sequences is a function of the number of identical positions shared by the sequences. Hence % identity = number of identical positions / total number of overlapping positions X 100.

In this comparison the sequences can be the same length or may be different in length. Optimal alignment of sequences for determining a comparison window may be conducted by the local homology algorithm of Smith and Waterman (*J. Theor. Biol.,* 91 (2) pgs. 370-380 (1981), by the homology alignment algorithm of Needleman and Wunsch, *J. Miol. Biol.*, 48(3) pgs. 443-453 (1972), by the search for similarity via the method of Pearson and Lipman, *PNAS, USA*, 85(5) pgs. 2444-2448 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetic Computer Group, 575, Science Drive, Madison, Wisconsin) or by inspection.

The best alignment (*i.e.*, resulting in the highest percentage of identity over the comparison window) generated by the various methods is selected.

The percentage of sequence identity of a nucleic acid sequence or an amino acid sequence can also be calculated using BLAST software (Version 2.06 of September 1998) with the default or user defined parameter.

The term "sequence similarity" means that amino acids can be modified while retaining the same function. It is known that amino acids are classified according to the nature of their side groups and some amino acids such as

the basic amino acids can be interchanged for one another while their basic function is maintained.

The term "variants" when referring to, for example, polynucleotides encoding a polypeptide variant of a given reference polypeptide are polynucleotides which encode polypeptides that differ from the reference polypeptide but generally maintain their functional characteristics of the reference polypeptide. A variant of a polynucleotide may be a naturally occurring allelic variant or it may be a variant that is known naturally not to occur. Such non-naturally occurring variants of the reference polynucleotide can be made by, for example, mutagenesis techniques, including those mutagenesis techniques that are applied to polynucleotides, cells or organisms.

Generally, differences are limited so that the nucleotide sequences of the reference and variant are closely similar overall and, in many regions identical.

Variants of polynucleotides according to the present invention include, but are not limited to, nucleotide sequences which are at least 95% identical after alignment to the reference polynucleotide encoding the reference polypeptide. These variants can also have 96%, 97%, 98% and 99.999% sequence identity to the reference polynucleotide.

Nucleotide changes present in a variant polynucleotide may be silent, which means that these changes do not alter the amino acid sequences encoded by the reference polynucleotide.

Substitutions, additions and/or deletions can involve one or more nucleic acids. Alterations can produce conservative or non-conservative amino acid substitutions, deletions and/or additions.

A "functional variant" of a polynucleotide of a target gene of the invention for the treatment of retinal disorder, possesses the same function as the one from which it derives. An example of functional variants are the "biologically active fragments" referring to fragments retaining essentially the same function as the corresponding native gene. Such fragment preferably comprise at least 100, 200, 300, 400, or up contiguous nucleotides identical to the native gene sequence.

The present invention provides methods of treating a retinal disorder in a mammal.

As used herein, "a retinal disorder" is a disorder resulting in injury or death of photoreceptor or other retinal cells. Examples of retinal disorders include ocular degeneration, retinopathies, retinitis and the like. Examples of retinal degeneration disorder include retinitis pigmentosa, macular degenerations, Leber congenital amaurosis, gyrate atrophy, Norrie disease, choroideremia, cone-rod and rod-cone dystrophies of any types, Usher's syndrome, Sorsby's fundus dystrophy, Stargardt disease, Best dystrophy and North Carolina Macular dystrophy. In addition, there are numerous inherited systemic diseases such as abetalipoproteinemia, mucopolysaccharidosis VI, Bardet-Biedle syndromes, Batten disease and Refsum disease that include retinal degeneration among the symptoms. More specifically, a retinal disorder include any retinal degenerations, syndromic or non-syndromic degenerations or inherited retinal degenerations. There are also retinal diseases resulting from abnormal expression of retinal specific genes that are required for retinal development/differentiation. Also encompassed are congenital abnormalities of the eye. The invention provides methods of treating congenital abnormalities in a mammal comprising modulating the amount of a polypeptide encoded by *USAG-1* in a retinal cell.

Preferably, the method is used for treating a retinal disorder resulting from an abnormal expression of a gene consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* or *Pcdh-γ-C3.*

As used herein, the term "abnormal expression" refers to an expression which is reduced or increased in a retinal cell of a subject suffering from the retinal disorder when compared to the expression in a retinal cell of a subject who does not suffer from said disorder. Preferably, an abnormal expression is considered when a reduction or an increase of at least 10% of the amount of target gene mRNA is detected, and more preferably a reduction or an increase of at least 50% of target gene mRNA is detected. In another embodiment, it refers to the expression of a non-functional polypeptide in the retinal cell, in particular, resulting from the expression of a pathological allelic form of the gene encoding said polypeptide.

According to the invention, a mammal is "treated" if one or more symptoms of the retinal disorder is eliminated or reduced, or prevented from progressing or developing further. For the specific purpose of treating retinal degeneration, the method of treatment of the invention refers to the ability to keep a retinal cell viable or alive for a period of time greater than is observed without application of said method.

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic or pet animals such as dogs, cats, sheep, pigs, cattle, etc. Preferably, the mammal is human.

The method of the invention comprises modulating the amount of a polypeptide encoded by a target gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes in a retinal cell of said mammal.

As used herein the "retinal cell" refers to a cell of a tissue of the retina, and more preferably, the photoreceptor cells, cones and rods, cells of the inner

nuclear layer including muller cells, bipolar neurons, horizontal cells and amacrine cells, the ganglionic cells of ganglion layer of the retina, and especially the retinal pigment epithelium cells.

The term "gene consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3*" encompass the nucleic acids comprising the coding sequence of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes as defined hereafter and any functional variants. For easy reading of the text, the genes consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* and their functional variants will be hereafter referred as the "target genes".

Indeed, all these genes have been shown in the present invention to express polypeptides which are secreted in the retinal pigment epithelium, thereby providing first evidence that these genes are involved in the hemato-ocular barrier provided by the RPE and are target genes for diagnosing or treating retinal disorders.

Preferably, a gene consisting of prosaposin comprises the following coding sequence of SEQ ID NO:1, or a nucleic acid having at least 90%, preferably 95% and more preferably 99% identity to the coding sequence of SEQ ID NO:1, or a biolobically active fragment thereof.

A gene consisting of *TMS-2* comprises the following coding sequence of SEQ ID NO:2, or a nucleic acid having at least 90%, preferably 95% and more preferably 99% identity to the coding sequence of SEQ ID NO:2, or a biolobically active fragment thereof.

A gene consisting of Pcdh-γ-C3 comprises the following coding sequence of SEQ ID NO:3, or a nucleic acid having at least 90%, preferably 95% and more preferably 99% identity to the coding sequence of SEQ ID NO:3, or a biologically active fragment thereof.

A gene consisting of *OAT1*, *OAT2* or *OAT3* comprises the following coding sequences of SEQ ID NO:17, SEQ ID NO:18 and SEQ ID NO:19 respectively, or a nucleic acid having at least 90%, preferably 95% and more preferably 99% identity to SEQ ID NO:17, SEQ ID NO:18 or SEQ ID NO:19 respectively, or a biologically active fragment thereof. Especially, the term *OAT1*, *OAT2* or *OAT3* refers to the corresponding human gene sequence and functional variants thereof.

A gene consisting of *OAT4* comprises the following coding sequence of SEQ ID NO:20, or a nucleic acid having at least 90%, preferably 95% and more preferably 99% identity to SEQ ID NO:20, or a biologically active fragment thereof.

A gene consisting of USAG-1 comprises the following coding sequence of SEQ ID NO: 22 (see, Fig 13), or a nucleic acid having at least or a nucleic acid having at least 90%, preferably 95% and more preferably 99% identity to SEQ ID NO: 22 (see, Fig 13), or a biologically active fragment thereof.

A gene consisting of SOST comprises the following coding sequence of SEQ ID NO:21,or a nucleic acid having at least or a nucleic acid having at least 90%, preferably 95% and more preferably 99% identity to SEQ ID NO:21, or a biologically active fragment thereof.

The term "modulating" means either reducing or increasing the amount of the synthesized polypeptide in the cell as compared to the physiological expression of the target gene in the cell. Modulation can be performed for example by inhibiting or increasing RNA synthesis or by inhibiting or increasing protein synthesis, or by administering *ex vivo* synthesized polypeptide to the cell. The term "modulating" also refers to increasing specifically non-pathological expression of the target gene, or reducing specifically the expression of pathological form of the gene, when said target gene is expressed under a pathological form in the retinal cell of the subject suffering from the retinal disorder.

In a specific embodiment, the method comprises modulating the expression of the target genes in a retinal cell of a mammal.

"Expression of a gene" refers to the cellular mechanism of transcription and/or translation enabling the production of a polypeptide encoded by a gene.

When increase of the protein synthesis is requested, for example, because the protein is synthesized at a low level in a patient suffering from a retinal disorder, the method of the invention preferably comprises administering to said retinal cell, an effective amount of a gene transfer vector, carrying a nucleic acid comprising the target gene; said target gene being operably linked to a promoter active in said retinal cell.

An "effective amount" is an amount sufficient to achieve the required expression of the target gene in the retinal cell.

A "gene transfer vector" refers to a vector appropriate for transferring a gene in the genome of a cell and expressing said gene in the transformed cell. *In vivo* gene transfer to retinal cells have been accomplished using adenovirus, adeno-associated virus, gutted adenovirus and guttless adenovirus, as well as integrative and non integrative lentivus-based vector, (Miyoshi *et al.*, 1997 PNAS USA 94: 10319 and Takahashi *et al.*, 1999, J Virol 73:7812). Trans-viral gene transfer vectors for in vivo gene transfer to retinal cells are also described in detail in US patent application 20030003582, January 2, 2003, incorporated herein by reference. Preferably, the gene transfer vector comprises a proviral genome which is selected among the group consisting of adenovirus, adenovirus associated virus and lentivirus.

The invention also encompasses a composition which comprises an effective amount of a gene transfer vector carrying a nucleic acid comprising one of the gene selected among the group consisting of

prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes; said gene being operably linked to a promoter active in said retinal cell.

Said composition can further comprise a neurotrophic factor or an anti-angiogenic factor, or a gene transfer vector comprising a nucleic acid encoding a neurotrophic factor or an anti-angiogenic factor or cells producing and secreting a neurotrophic factor or an anti-angiogenic factor.

This composition can be used for the treatment of retinal disorders as disclosed in the present application.

The invention also encompasses the use of an effective amount of a gene transfer vector in the manufacture of a compound for the treatment of retinal disorders, retinal degenerations and/or retinopathies as disclosed in the present invention, said vector carrying a nucleic acid comprising one of the gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes, said gene being operably linked to a promoter active in said retinal cell.

In another embodiment, the method comprises administering to the eye, a composition comprising an effective amount of a polypeptide encoded by a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes.

In another embodiment, the method comprises administering to the eye, a composition comprising an effective amount of cells, wherein said cells produce and secrete a polypeptide encoded by a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes. The composition comprising the cells are also encompassed by the present invention.

Preferably, the cells are eucaryotic cells, more preferably mammalian cells. The cells are compatible with biological use *in vivo* and, in particular, do not exhibit known pathogenic activity. Examples of cells include, but not limited to fibroblasts, muscle cells, hepatocytes, neural cells and the like, CHO cells, kidney cells, PC12 cell lines, MDCK cells, astrocytes and the like. The cells can be autologous, allogenic or xenogenic.

Specific examples of cells to be used include fibroblasts, such as murine fibroblasts, in particular NIH-3T3 cells, C2C12 cells, PC12 cells and the like.

In a preferred embodiment, stable cell lines producing the polypeptide are prepared. These cells usually contain one or several copies of the nucleic acid construct encoding the polypeptide incorporated into their genome.

In order to avoid inflammatory reactions, the cells are preferably encapsulated with an appropriate biocompatible material. Methods of encapsulating cells appropriate for administration to the eye are described, for example, in US patent 6,500,449.

The polypeptides or the cells can be administered in different sites of the eye, preferably in the vitreous body of the eye. A preferred implantation route is the injection in the intravitreous cavity.

Other polypeptides can also be administered in combination or separately to the eye, such as neurotrophic and/or anti angiogenic factor. Examples of neurotrophic and/or anti angiogenic factor include ciliary neurotrophic factor, glial-derived neurotrophic factor, nerve growth factor, brain-derived neurotrophic factor, fibroblast growth factors, endostatin, ATF, fragments of thrombospondin, and functional variants thereof. In a preferred embodiment, the method further comprises administering to the eye, a composition comprising a neurotrophic or anti-angiogenic factor, or a gene transfer vector comprising a nucleic acid encoding a neurotrophic or anti-

angiogenic factor or cells producing and secreting a neurotrophic or anti-angiogenic factor.

In a specific embodiment wherein inhibition of the target gene is requested, for example because the target gene is over-expressed in patients suffering from a retinal disorder, or because a pathological form of the target gene is expressed in patients suffering from a retinal disorder, the method comprises administering to a retinal cell of a mammal, a gene transfer vector carrying an antisense of the target gene. In a preferred embodiment, the antisense nucleic acid is selected to inhibit specifically the expression of a mutated pathological form of the target gene.

College of Surgeons (RCS) *rdy-* (*retinal dystrophy*) rat strain was the first spontaneous animal model described with inherited rpe dystrophy [Bourne et al. (1938) *Brit J Ophthalmol* **22** : 613-623; Dowling *et al.* (1962) *J Cell Biol* **14**: 73-109]. Congenic (genetically similar) dystrophic RCS rat strains were subsequently developed [LaVail *et al.* (1975) *J Hered* **66**(4): 242-4; LaVail (1981) *Invest Ophthalmol Vis Sci* **20**(5) : 671-5]. The primary deficiency in the dystrophic RCS rat affects the rpe cells [Mullen *et al.* (1976) *Science* **192**(4241): 799-801], which are unable to phagocytize and shed rod outer segments [Custer et al. (1975) *Exp Eye Res* **21**(2): 153-66; Goldman *et al.* (1978) *Science* **201**(4360): 1023-5]. This dysfunction leads to the gradual accumulation of undigested OS debris and concomitant photoreceptor cell death. In RCS rats, photoreceptor loss starts on postnatal day (PN) 18 and is extensive one month later. This autosomal recessive disorder affecting rpe cells was recently elucidated in RCS dystrophic rats [D'Cruz et al. (2000) *Hum Mol Genet* **9**(4) : 645-51; Nandrot *et al.* (2000) *Neurobiol Dis* **7**(6 Pt B): 586-99] and in humans affected by retinal degeneration diseases [Gal *et al.* (2000) *Nat Genet* **26**(3); 270-1].

Abnormal expression of the gene encoding prosaposin has been shown in RCS dystrophic rats, providing evidence that the expression of this gene might be affected also in human retinal degenerations.

Thus, the invention provides a method for diagnosing a retinal disorder in a mammal, comprising identifying an abnormal expression of a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes in retinal cells of said mammal, wherein said abnormal expression is indicative that said mammal suffers from a retinal disorder.

In one embodiment, the method for diagnosing a retinal degeneration in a mammal, comprises identifying an inhibition of expression of the prosaposin gene in retinal cells of said mammal, wherein said inhibition is indicative that said mammal suffers from a retinal degeneration.

Abnormal expression can be detected according to any appropriate techniques known in the Art. When referring to abnormal structural expression, probes specific of the pathological form of the gene or RNA can be designed accordingly, as well as antibodies or ligand specific of the abnormal structure of the corresponding polypeptide. When referring to increase or reduction of the level of synthesized polypeptide, any appropriate methods to quantify gene expression can be used. These methods include quantitative or semi-quantitative RT-PCR amplification. Examples of primers which can be used for RT-PCR amplification of the target gene are described in the Examples below.

Compounds capable of modulating *in vivo or in vitro* the expression of a target gene in a retinal cell of a mammal are useful for treating retinal disorders according to the method of the invention, and can be used in a pharmaceutical composition.

Compounds capable of modulating *in vivo or in vitro* the amount of a polypeptide encoded by a target gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes in a retinal cell of said mammal can also be used in a pharmaceutical composition of the present invention.

The invention thus further pertains to a method of screening for a compound capable of modulating the expression of the gene encoding prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3*, comprising:

a. providing cells expressing a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes,

b. culturing said cells in the presence of a compound,

c. determining the expression of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* or *Pcdh-γ-C3* genes, and comparing said expression to the expression of the same gene in a control cell culture in the absence of the compound, or to the expression of a control constitutive gene, wherein an higher or lower expression in the presence of the compound is indicative that said molecule is capable of modulating the expression of said prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3*.

Drug candidates for the treatment of retinal disorder can also be screened among molecules which bind to active site of the expression product of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes. These binding compounds may act as a cofactor, as an inhibitor, as antibodies, as a competitive inhibitor, as an activator or have agonistic or antagonistic activity on the active site of the expression product.

Any known methods in the Art for screening for binding molecules can be used.

The genes encoding polypeptide which interacts in the retinal cells with a target polypeptide are also candidate gene for the treatment or the diagnosis of retinal disorders. Such genes can be screened according to any known methods, including double-hybrid screening methods (Fields et al., Nature 340:245-246, US 5,283,173) and similar methods as described for example in Takacs *et al.*, PNAS USA, 1993, 90: 10375-79).

The invention thus provides a method of screening for a compound capable of binding to the expression product of the gene encoding prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3*, comprising

a. contacting a candidate compound with the expression product of a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes,

b. determining whether said candidate compound binds to the expression product.

In a specific embodiment, step (a) consists of culturing cells expressing a nucleic acid comprising a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes and incubating said cells with a candidate molecule.

The invention also relates to a method for screening for a pathological allelic form of a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3*, comprising

a. isolating a polynucleotide comprising a specific fragment of a gene selected among the group consisting of prosaposin, *TMS-2, OAT1,*

OAT2, *OAT3*, *OAT4*, *USAG-1*, *SOST* and *Pcdh-γ-C3* from a retinal cell of a subject suffering from a retinal degeneration,

b. determining the sequence of said polynucleotide and comparing with a wild-type sequence

wherein the detection of one or more mutation is indicative that said polynucleotide is a pathological allelic form of the gene.

The probes specific for detecting a pathological form of a target gene are also encompassed in the present invention as well as a kit for detecting said pathological form, comprising the corresponding probes.

Other advantages and embodiments of this invention will be disclosed in more details in the following experimental section, which should be regarded as illustrative and not limiting the scope of the invention. All references cited in the present application are incorporated herein by reference.

EXAMPLES

1. PROSAPOSIN AS A TARGET GENE FOR DIAGNOSING AND/OR TREATING RETINAL DEGENERATIVE DISEASES

Prosaposin (PSAP) is synthesized as a 53-kDa precursor protein that is post-translationally modified to a 65-kDa form that is associated with the Golgi membranes and targeted to lysosomes. Proteolytic cleavage of PSAP in the lysosome releases four smaller active peptides of between 8 and 11 kDa, known as saposin A, B, C, and D [for review, Kishimoto *et al. J Lipid Res* **33**, 1255-67 (1992); Sandhoff *et al. In Scriver, C.R., Beaudet A.L., Sly, W.S., Valle, D. and Childs, B., The Metabolic and Molecular Bases of Inherited Disease* 8th edn. Mc Graw-Hill, New york, NY, 3371-3388 (2001)]. The dissociated mature protease, cathepsin D, participates in the maturation of PSAP. The saposins activate specific lysosomal hydrolases including cerebrosidases, ceramidases, sphingomyelinase, galactosidase and arylsulfatase A, which are required for the *in vivo* degradation of various glycosphingolipids, components of the plasma membrane.

A significant portion of PSAP is glycosylated leading to a 70-kDa secreted form that is found in several extracellular fluids such as cerebrospinal fluid, maternal milk, seminal plasma and pancreatic secretions. Secreted PSAP can be subsequently targeted to the lysosomal compartment via receptor-mediated endocytosis. Moreover, in addition to its role as a lysosomal precursor protein, this secreted form can act as a neurotrophic, neuroprotective, reparative and myelinotrophic factor. PSAP is thought to have specific effects on the development, maintenance and differentiation of the male reproductive organs and may also play a role in lysosomal residual body degradation in Sertoli cells.

The physiological *in vivo* importance of saposins has been demonstrated thanks to the identification of several mutations in the saposin gene that are

responsible for lysosomal disorders such as metachromatic leukodystrophy and Gaucher's disease, both of which are characterized by the accumulation of undigested glycosphingolipids in cells. Furthermore, the total inactivation of the SAP precursor leads to death during fetal development or early childhood in humans.

It is shown hereafter for the first time by *in situ* hybridization, immunohistochemistry and RT-PCR that prosaposin mRNA and protein are detected within the retina. It is further shown hereafter that prosaposin mRNA and protein are mainly present in the RPE layer and that the levels differ between normal (RCS rdy+) and pathological dystrophic (RCS rdy-) rat retinas.

1.1 MATERIALS AND METHODS:

Animals. All animals were handled in strict accordance with the Helsinki Declaration and with the Association for Research in Vision Ophthalmology (ARVO) Statement for Use of Animals in Ophthalmic and Vision Research. The animals were kept at 21°C with a 12h light (100 lx): 12h dark cycle and fed *ad libitum.* Normal pigmented (p+) and unpigmented (p-) Royal College of Surgeons (RCS) rats with (RCS rdy- (for *R*etinal *Dy*strophy)] and without retinal dystrophy (RCS rdy+) were kindly provided by Dr. M.M. LaVail (UCSF, San Francisco, USA). Adult Wistar rats (Iffa Credo Enterprise, L'Arbresle, France) were used between the ages of 12 and 14 weeks.

Construction of the pig RPE cDNA library. Pigs obtained from the SOCOPA slaughterhouse (Evron, France) were killed by electrocution. The eyes were enucleated carefully, and adherent muscles and fat bodies were removed. The anterior parts of the eyeballs, including the iris, cornea and lens, were discarded. Neural retina tissues were removed from the posterior eye cups. RPE cells were scraped from the posterior eye cups into the RLT lysis buffer from the RNeasy RNA extraction kit (Qiagen, Chatsworth, CA). Total

RNA and then mRNA were extracted from RPE cells by use of the RNeasy and Oligotex kits (Qiagen, Chatsworth, CA) respectively following manufacturer's instructions. Samples were treated with DNase I to remove genomic DNA before washing. The poly A+ RNA (1 µg) was used to synthesize cDNA using the SuperScript Choice System (Life Technologies, Grand Island, USA). For the reverse transcriptase step, the degenerate primer 5'-CGATTGAATTCTAGACCTGCCTCGAGNNNNNNNNN-3' (SEQ ID NO:16) (where N denotes G, A, T, or C and the *Xho*I site is underlined) was used. After second-strand synthesis, the resulting products were ligated to *Eco*RI adapters, digested with *Xho*I and products of between 300 and 900 bp were selected. The double stranded cDNAs were then ligated into the *Eco*RI and *Xho*I sites of pSUC2T7M13ORI [Jacobs *et al. Gene* **198**, 289-96 (1997)] before being fused to the invertase gene lacking its signal sequence. ElectroMax *E. coli* DH10B cells (Life Technologies, Grand Island, USA) were transformed with the resulting cDNA library by electroporation.

Semi-quantitative RT-PCR. Total RNA was extracted from rat RPE cells and cerebellum at different post-natal ages (P14, P21, P45) with TRIzol (Life Technologies, Grand Island, USA) according to the manufacturer's instructions. Briefly, RPE cells collected from ten eyes or 50 mg of cerebellum tissues were homogenized with 500 µl of TRIzol reagent. Total RNA was separated from DNA and protein using a phenol/chloroform phase separation and the RNA was precipitated with isopropanol. The RNA pellet was then washed with 80% ice-cold ethanol, dried and dissolved in RNase-free water. The yield of total RNA was determined by measuring the absorption at 260 nm. One microgram of total RNA was reverse transcribed to give single stranded cDNA in a 20 µl reaction volume using an oligodT primer, according to the manufacturer's instructions (Life Technologies, Grand Island, USA). The primers, total RNA and H_2O were heated at 70°C for 10 min before adding the other components and incubating at 42°C for

50 min. Finally, the mixture was incubated at 70°C for 15 min to inactivate the reverse transcriptase. For semi-quantitative PCR, the number of cycles, amount of cDNA and annealing temperature were optimized (data not shown). Cyclophilin was co-amplified with the target gene as an internal control for comparative purposes. PCRs were conducted in a 20 µl volume containing 1 µl of cDNA, 1 µl of DMSO, 1 µl of 10X PCR buffer (Promega, Madison, WI), 50 pM each 5' and 3' prosaposin primers, 25 pM each 5' and 3' cyclophilin primers, 0.2 mM dNTP, 1.5 mM $MgCl_2$ and 0.5 U of Taq DNA polymerase. The initial denaturation step at 92°C for 2 min was followed by 25 cycles of 15 s at 92°C, 1 min at 55°C and 1 min 30 at 72°C. The PCR products were separated in 1% agarose gels and stained with ethidium bromide. The prosaposin primers (5'-TGCAAGGAGGTGGTTGAC-3' SEQ ID NO:4 and 5'-CGGGTTGGCAGAACAGAG-3' SEQ ID NO:5) amplified an 858-bp product; and the cyclophilin primers (5'-TGGTCAACCCCACCGTGTTCTTCG-3' (SEQ ID NO:6) and 5'-TCCAGCATTTGCCATGGACAAGA-3') (SEQ ID NO:7) amplified a 311-bp product.

Signal Sequence Trap Screening. The yeast signal sequence trap screening method was carried out as previously described Jacobs *et al.* *Gene* **198**, 289-96 (1997)]. Briefly, yeast strain YTK12 was transformed with the pig RPE cDNA library by the lithium acetate method and plasmids were isolated from colonies that survived on medium containing sucrose. Inserts from positive clones were sequenced and analyzed for similarity to known genes.

Plasmid construction. pCDNA-PROSAP: the full-length prosaposin cDNA was isolated by RT-PCR (Superscript kit, Life Technologies) from total rat RPE RNA. The primers used were PROSAP5' (5'-AAA**GAATTC**ATGTATGCTCTCGCTCTCCT-3' (SEQ ID NO:8) where the *Eco*RI site is in bold and the ATG start codon is underlined) and PROSAP3' (5'-TTT**CTCGAG**CTAGTTCCACACATGGCGTT-3' (SEQ ID NO:9) where

the *Xho*I site is in bold and the TAG stop codon is underlined). The 1684-bp PCR product was digested with *Eco*RI and *Xho*I and subcloned into the *Eco*RI-*Xho*I sites of pCDNA3 (Invitrogen, San Diego, CA).

In situ *Hybridization*.

Tissue preparation: After CO_2 asphyxiation, eyes were collected from rats at different post-natal ages (P14, P21, P45 and P60), enucleated and immediately fixed in 3% paraformaldehyde for three hours at 4°C before being embedded in paraffin. Five-micrometer sections were cut and mounted on pre-coated Superfrost/Plus slides. The slides were then heated at 37°C for 24 hours to allow a better adhesion of the tissue sections on slides.

Riboprobe preparation: Sense and antisense riboprobes were synthesized from the pCDNA-PROSAP vector linearized with *Bgl*I. The probes were labeled using the digoxigenin (DIG) RNA labeling Kit (Boehringer-Mannheim, Indianapolis, USA). The transcription reaction was carried out with SP6 (antisense) and T7 (sense) RNA polymerases according to the manufacturer's instructions. The integrity of the probes was checked by agarose gel electrophoresis.

***In situ* Hybridization:** For *in situ* hybridization, sections were deparaffinized, rehydrated, postfixed with 4% paraformaldehyde for 20 min and washed in TBS buffer (50 mM Tris-HCL (pH 7.5), 150 mM NaCl). Sections were treated for 10 min with 0.2 M HCl to denature proteins, washed in TBS buffer and digested with proteinase K (0.1 mg/ml) for 20 min at 37°C. Sections were then washed in TBS, dehydrated and air-dried. After prehybridization at 65°C for 15 min, hybridization was carried out at 65 °C for 16 hours in 50% formamide, 10% dextran sulfate, 1X Denhardt's solution, 2X SSC, 0.1 mg/ml sheared salmon sperm DNA, 1 mg/ml *E. coli* tRNA, and DIG-labeled antisense or sense probes (15 µl for 150 µl of hybridization buffer). The slides were subsequently washed three times in

2X SSC, 50 % formamide, 0.1% Tween for 30 min at 65 °C, rinsed twice in PBS-Triton (1%) and incubated with blocking reagent (Boehringer-Mannheim, Indianapolis, USA) containing 10% non immune goat serum for 1 hour at RT. Tissue sections were incubated overnight with alkaline-phosphatase-conjugated anti-DIG antibody at 4°C and washed five times in PBS-Triton. Bound probes were detected by incubating with the alkaline phosphatase substrate NBT/BCIP (Boehringer-Mannheim, Indianapolis, USA) at 37°C in the dark. The reaction was stopped by rinsing with distilled water before mounting in Aquatex mounting medium (Merck, Darmstadt, Germany).

Immunohistochemistry. Saposin was detected by incubating 5-μm paraffin-embedded retina sections from rdy+ and rdy- RCS rats with the SGP-1 antibody Sylvester *et al. Biol Reprod* **41**, 941-8 (1989)]. The ChemMate peroxidase/DAB Rabbit/Mouse detection kit was used to detect bound antibodies (Dako, Glostrup, DK). Sections were deparaffinized, rehydrated and boiled for 20 minutes in citrate buffer (10 mM, pH6). Endogenous peroxidase was inhibited by treating with 3% H_2O_2, 1X SSC and 5% formamide. Tissues were then treated with 0.3% Triton in TBS buffer (0.5 M Tris-HCl, 1.5 M NaCl, pH 7.6) for 5 minutes. After incubation in TBS buffer containing 10% pig serum (Dako, Glostrup, DK) for 10 minutes, sections were incubated with SGP-1 primary antibody at a dilution of 1/100 in antibody diluent overnight at 4°C, then, with biotinylated secondary antibodies for 30 minutes at room temperature. Subsequently, the sections were incubated with a peroxidase staining kit for 30 minutes at room temperature. The sections were washed three times with PBS and treated with diaminobenzidine (DAB) solution (0.01% 3',3-diaminobenzidine tetrahydrochloride, Tris-HCl (pH 7.5), and 0.6% H_2O_2). Tissue sections were then counterstained with hematoxylin. In control experiments, sections were incubated with nonimmune serum.

Results

Isolation of prosaposin cDNA from pig RPE cDNA library.

The yeast "sequence signal trap" system was used to identify proteins secreted by retinal pigmented epithelial cells. mRNAs were extracted from the pig RPE monolayer and reverse-transcribed using random primers. The resulting cDNAs were size-selected and cloned into an pSUC2T7M13ORI vector such that they were fused with the invertase gene lacking its signal peptide Jacobs *et al. Gene* **198**, 289-96 (1997)]. The secretion of invertase allows the yeast to grow on media containing sucrose. The pig RPE cDNA library was used to transform the YTK12 yeast strain and clones surviving on sucrose were selected and the inserts sequenced. One of these clones contained a 172-bp insert that was 86% identical to the 5' end of the gene encoding for human prosaposin. This insert contained a 19-bp upstream noncoding sequence and 153-bp coding sequence including the ATG codon and the sequence encoding for the signal peptide of the prosaposin protein (Fig 1A). As expected, the sequence encoding prosaposin was in-frame with the invertase gene.

The first 51 amino acids of the pig prosaposin protein were 88% identical with the human prosaposin protein, 86% identical with the bovine prosaposin protein and 58% identical with the mouse and rat prosaposin proteins (Fig 1B).

RPE cells contain high levels of prosaposin mRNA

In situ hybridization was used to study the distribution of prosaposin mRNA in paraffin-embedded sections of rdy+ pigmented and unpigmented rat eyes at different post-natal stages (P14, P21, P45 and P60) using sense and antisense DIG-labeled riboprobes.

At P14, high levels of prosaposin mRNA were observed in the ganglion cell layer of the rdy+ pigmented rat retina (Fig 2A, 2C at higher magnification). A weak prosaposin mRNA signal was also detected in the inner nuclear

layer (Fig 2A, 2C at higher magnification). No labeling was observed in the outer nuclear layer, inner or outer segments or in the inner or outer plexiform layers. As expected, no specific labeling was detected in sections hybridized with the prosaposin sense probe (Fig 2D). A strong signal was also observed in the RPE cell layer (Fig 2B, higher magnification), however the labeling could not be clearly distinguished from the natural pigmentation of these cells. Thus, to confirm this observation, we performed *in situ* hybridization on unpigmented rat retina sections. The labeling was the strongest in ganglion (Fig 2E) and RPE (Fig 2F) cells.

At P21, a strong prosaposin signal was observed in the ganglion cells of the rdy+ pigmented rat retina (Fig 3A and 3C, higher magnification). As at P14, the inner nuclear layer was labeled, but a signal was also detected in the outer nuclear layer (Fig 3A and 3C, higher magnification). No labeling was observed in the inner or outer segments or in the inner or outer plexiform layers. A strong signal was also clearly detected in the RPE cell layer (Fig 3B, higher magnification). The same results were obtained with 21-day-old rdy+ unpigmented rats (Fig 3E higher magnification).

At P45 and P60, the distribution of prosaposin mRNA was similar to that observed at P21 (data not shown).

To confirm that the signals detected are not specific to the RCS strain, we repeated these experiments on retina sections from Wistar rats. The distribution of prosaposin mRNA in the Wistar rat retina was generally similar to that observed in the RCS rdy+ strain, with very high levels of prosaposin in the cytoplasm of RPE cells (Fig 4 A, B and C). No specific labeling was detected in sections hybridized with the prosaposin sense probe (Fig 4D).

These results indicate that the pigment epithelial cell layers and the ganglion cells of rdy and Wistar rats at different developmental stages contain high levels of prosaposin mRNA.

RPE cells produce high levels of prosaposin protein.

To confirm the *in situ* hybridization results, immunohistochemistry was used to determine the prosaposin protein profile in retinal sections from normal unpigmented RCS rdy+ rats at P45 using the SGP-1 antibody. Strong prosaposin immunostaining was detected in ganglion cells (Fig. 5A) and more precisely in the cytoplasm of these cells (Fig 5C). Intense prosaposin immunostaining was also observed in the cytoplasm of RPE cells (Fig 5A and 5B). The saposin immunostaining seemed to be concentrated on the apical side that faces the photoreceptor cells. Moreover, weak staining was observed in the outer plexiform layer, near to the RPE cells (as indicated by arrows in fig 5B). A very weak saposin immunostaining was also observed in the inner nuclear layer. No signal was observed with nonimmune serum confirming that the immunostaining was specific to prosaposin (Fig 5D).

The same analysis was performed on retinal sections from unpigmented dystrophic RCS rdy- rats, which is the first spontaneous animal model of inherited retinal pigment epithelium defect to be described [Bourne *et al. Br. J. Ophthalmo.* **22**, 613-623 (1938)], and is one of the major animal models for human retinitis pigmentosa. The cyclic circadian phagocytosis of shed POS by the RPE is abolished in the RCS rdy- rat retina [Mullen *et al. Science* **192**, 799-801 (1976)]. Consequently, POS fragments accumulate, leading to the degeneration of photoreceptor cells between 18 days and 3 months after birth and the loss of vision [Dowling *et al. J. Cell Biol.* **14**, 73-109 (1962)]. In this RCS rdy- rat strain, the phagocytosis defect is caused by a deletion in the *c-mer* gene [D'Cruz *et al. Hum Mol Genet* **9**, 645-51 (2000)]; [Nandrot *et al. Neurobiol Dis* **7**, 586-99 (2000)]. The prosaposin protein pattern was similar to that observed in retina of normal rdy+ rats (Fig 5E). However, although the immunohistochemical analysis is not quantitative, it appeared that the staining was lighter in the RCS rdy- retina than in the rdy+ rat retina (Fig 5E compared to Fig 5A). Moreover, the prosaposin immunostaining was stronger in the outer plexiform layer, near

the RPE cells, of the RCS rdy- rat retina than in the equivalent layer of the rdy+ rat retina (Fig 5F compared to Fig 5B).

Thus, these immunohistochemical data confirmed the results of the *in situ* hybridization analysis and demonstrate that prosaposin is principally localized in the RPE and ganglion cells of the rat retina.

The RPE cell layers of RCS rdy+ and RCS rdy- rats contain different amounts of prosaposin mRNA.

Semi-quantitative RT-PCR was used to compare the amounts of prosaposin mRNA in RPE cells of normal rdy+ and pathological rdy- RCS rats at various developmental ages (Fig 6A). As a control, the same experiment was performed on mRNA extracted from the cerebellum of the two rat strains at the same ages (Fig 6B). Cyclophilin mRNA was used as an internal control. As expected, an 818-bp band corresponding to prosaposin mRNA was observed in all samples tested (Fig 6). In the normal rdy+ retina, the amount of prosaposin mRNA in pigment epithelial cells was clearly higher at P21 and P45 than at P14 (Fig 6A). In contrast, in RPE cells from the dystrophic RCS rdy- rat strain, the prosaposin mRNA level remained constant over the time (Fig 6A). The amounts of prosaposin mRNA in the cerebellums of the two strains remained stable over the time (Fig 6B).

Thus, in normal physiological conditions, the prosaposin mRNA levels appear to increase with age between P14 and P45 and prosaposin gene transcription and/or mRNA stability appears to be altered in the RPE cells of RCS rdy- rats.

Discussion

The semi-quantitative RT-PCR analysis demonstrates that the amount of prosaposin in the RPE of normal rdy+ rats progressively increases with

age. Although the *in situ* hybridization technique is not quantitative, similar conclusions could be drawn from the intensity of labeling at P14 and P21 (compare Fig 3E and Fig 2F). The increase in the amount of prosaposin mRNA in normal RPE cells coincided with the maturation of the photoreceptor cells, especially the elongation of the photoreceptor outer segments and the beginning of the phagocytosis process [for review, [Nguyen-Legros *et al. Int Rev Cytol* **196**, 245-313 (2000); [LaVail *et al. Trans Ophthalmol Soc U K* **103 (Pt 4)**, 397-404 (1983)]. This suggests that prosaposin participates in the renewal of photoreceptor outer segments and/or in the regulation of their degradation. In accordance with this suggestion, unlike in the normal rdy+ rat strain, the amount of prosaposin mRNA did not increase between P14 and P45 in the pathological RCS rdy- rat strain (Fig 6A), which is characterized by the abrogation of the ingestion phase of photoreceptor outer segments. These results suggest that the RPE cells of adult RCS rdy- rats present a deficiency in prosaposin expression and that this deficiency is closely associated with the RCS rat pathology as the levels of prosaposin mRNA were identical in the cerebellums of the two rat strains.

The retinal pigment epithelium produces large amounts of many cysteine proteases such as cathepsins B, D, H, L and S, which have important functions in the catabolism of peptides and proteins. Cathepsin S affects the activity of cathepsin D, which is the main protease directly involved in the proteolytic processing of diurnally shed photoreceptor outer segments [Rakoczy *et al. Invest Ophthalmol Vis Sci* **35**, 4100-8 (1994); Rakoczy *et al. Biochem J* **324 (Pt 3)**, 935-40 (1997)]; Rakoczy *et al. Invest Ophthalmol Vis Sci* **39**, 2095-104 (1998)]. Regulators of cathepsin activities such as cystatin C, which efficiently inactivates some cysteine proteases such as cathepsin S, are also expressed in RPE cells [Wasselius *et al. Invest Ophthalmol Vis Sci* **42**, 1901-6 (2001)]. Thus, these enzymes may be directly or indirectly involved in the regulation of photoreceptor outer

segment degradation. As prosaposin activates the hydrolysis of sphingolipids by lysosomal hydrolases, we hypothesize that it plays a complementary role in the process of photoreceptor outer segment degradation. This assumption is supported by the fact that, like for cystatin C, the increase in prosaposin mRNA concentration in the RPE cells coincides with the opening of the eyelids in rat pups (P14) [Wasselius *et al. Invest Ophthalmol Vis Sci* **42**, 1901-6 (2001)]. It is noteworthy that the opening of the rat eyelids is associated with an increase in phagocytic activity in the RPE cells.

In addition to a well-documented role in the cellular metabolism of glycosphingolipids, the saposin precursor may mediate neurotrophic activities *in vitro* and *in vivo*. Prosaposin stimulates neurite outgrowth and prevents programmed cell death of a variety of neuronal cells [O'Brien *et al. Proc Natl Acad Sci U S A* **91**, 9593-6 (1994); Tsuboi *et al. Brain Res Dev Brain Res* **110**, 249-55 (1998); Misasi *et al. Faseb J* **15**, 467-74 (2001)]. Moreover, prosaposin can protect hippocampal neurons against ischemic damage when infused intracerebroventrically before ischemia [Sano *et al. Biochem Biophys Res Commun* **204**, 994-1000 (1994)] and the amount of prosaposin mRNA in the hippocampus increases after ischemia [Yokota *et al. Stroke* **32**, 168-74 (2001)]. Gillen *et al.* showed that the amount of SGP-1 (rat prosaposin) mRNA increases by up to six-fold 7 days after crushing the sciatic nerve [Gillen *et al. J Neurosci Res* **42**, 159-71 (1995)]. Direct application of prosaposin to transected sciatic nerves promotes nerve regeneration and/or prevents retrograde neuronal peripheral cell death after injury [Kotani *et al. J Neurochem* **66**, 2197-200 (1996)], suggesting that prosaposin is as an endogenous modulator of nerve sprouting and regeneration. Prosaptide, a linear 18-mer prosaposin derivative comprising the hydrophilic sequence of the rat saposin C domain, is a potent neuritogenic factor for sensory and motor neurons of the adult peripheral nerve [Campana *et al. J Peripher Nerv Syst* **5**, 126-30 (2000)] and protects

against slow progressing neuronal degeneration after brief ischemia [Morita *et al. J Cereb Blood Flow Metab* **21**, 1295-302 (2001)]. The high levels of prosaposin in RPE cells are compatible with the hypothesis that some of the synthesized prosaposin protein is secreted to play neurotrophic and protective roles on photoreceptor cells.

Mutations in the SAP precursor-encoding gene result in phenotypes that are similar to metachromatic leukodystrophy (MLD) or variants of Gaucher disease (for review, Sandhoff *et al. In Scriver, C.R., Beaudet A.L., Sly, W.S., Valle, D. and Childs, B., The Metabolic and Molecular Bases of Inherited Disease* **8th edn. Mc Graw-Hill, New york, NY**, 3371-3388 (2001)). Saposin-deficient patients suffer from various ophthalmic disorders. Some patients with *sap*-B deficiencies have pallid optic disks. The eyes of adult *sap*-B deficient patients sometimes display a macular grayness, consistent with an RPE abnormality. As well as suffering from frequent oculo-motor problems, some *sap*-C-deficient patients have irregularly pigmented retinas. The first patients to be described with sap precursor deficiencies (combined SAP deficiency, SAPD, prosaposin deficiency) consistently display precocious optic nerve atrophy [for review, [Sandhoff *et al. In Scriver, C.R., Beaudet A.L., Sly, W.S., Valle, D. and Childs, B., The Metabolic and Molecular Bases of Inherited Disease* **8th edn. Mc Graw-Hill, New york, NY**, 3371-3388 (2001)]. Thus, there is clearly a link between the ophthalmic clinical manifestations of saposins and prosaposin deficiencies and the retinal sites found to express the prosaposin gene in our study: RPE and ganglion cells. Prosaposin and/or saposin deficiencies may alter the function of these cells, thus resulting in retinal pigment alterations and optic nerve atrophy. Moreover, neuropathological studies on a *sap*-C-deficient patient revealed massive intraneuronal lipid storage and electron microscopy revealed the presence of different types of storage bodies including lipofuscin granules [Pampols *et al. Acta Neuropathol (Berl)* **97**, 91-7 (1999)]. Interestingly, several ocular

diseases such as age-related macular degeneration (AMD), Best's disease and Stargardt's disease are characterized by the massive accumulation of lipofuscin in the RPE cells, resulting in the atrophy of the photoreceptor layer of the retina [Hogan *et al. Trans Am Acad Ophthalmol Otolaryngol* **76**, 64-80 (1972)]. Although several genes involved in these genetic diseases have been identified, many remain to be characterized. Moreover, juvenile neuronal ceroid lipofuscinosis (NCL), which is a group of neurodegenerative disorders characterized by the intralysosomal accumulation of autofluorescent lipopigments in neurons and other cell types, is associated with retinal degeneration leading to blindness. Some diseases from this group are caused by mutations in genes encoding lysosomal enzymes (PPT1, CLN2, CLN3). These observations provide evidence that prosaposin is a potential candidate gene for ocular diseases, and that some ocular disease can be detected by determining an abnormal expression of the gene encoding prosaposin.

2. TMS-2 AND PCDH-γ-C3 AS A TARGET FOR TREATING RETINAL DISEASE.

2.1Materials and Methods

Animals

All experiments described in this report were carried out in strict accordance with the Association for Research in Vision Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The animals were maintained at 21°C with a 12-h light/dark cycle and fed *ad libitum*. The wild-type pigmented RCS rdy^+p^+ rat strain and dystrophic RCS rdy^- p^+ were used for RT-PCR analysis and for *in situ* hybridization (ISH). All animals were killed by asphyxiation in a CO_2 chamber.

Construction of human cDNA library

The human adult normal retina poly A+ RNA used in this study was isolated from a pool of 14 donors aged between 21 and 43 years old (BioChain Institute, Inc., San Leandro, CA). The GeneRacer kit (Invitrogen Corporation, San Diego, CA) was used to generate a human retina cDNA library for secreted proteins. Briefly, 250 ng of poly(A)+ mRNA were dephosphorylated, decaped and ligated with a oligo/ARN primer (5'-CGACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAUUC AAA-3', SEQ ID NO:10) with a flanking *Eco*RI site. Poly(A)+ mRNA was reverse transcribed to first strand cDNA using a oligo/random nonamer primer (5'-GCTGTCAACGATACGCTACGTAACGGCATGACACAGTGCTCGAGNNN NNNNNN-3', SEQ ID NO:11) with a *Xho*I site and the SUPERSCRIPT™ II Rnase H- Reverse Transcriptase Choice System (Invitrogen Corporation, California, USA). These random nonamers reduced the 3' bias and the likelihood of capturing full-length sequences, while enriching the selection of the 5' functional domain of the transcripts, which include putative signal peptide sequences. The resulting cDNAs were amplified with custom adapters (GeneRace primer) containing an EcoRI and XhoI site by a PCR Touch Down reaction for 12 cycles, with an initial denaturing phase at 94°C for 30 sec, annealing temperature phase at 65°C for 45 sec and a final extension of 1m30 at 72°C. After second strand synthesis, the cDNAs were size-fractionated on a polyacrylamide gel to isolate cDNAs between 500 and 1000 bp. The protocol of Jacobs et al., modified as described subsequently, was then followed to screen the retina cDNA library (Jacobs, Collins-Racie et al., 1997). Selected base pair fragments were ligated into pSUC2T7M13ORI vector pre-digested with EcoRI and XhoI restriction enymes. The library was amplified by transformation into electrocompetent Escherichia coli (ElectroMax DH10B; Invitrogen). Yeast strain transformed using lithium acetate was selected by growth on complete minimal medium

lacking tryptophan. Yeast colonies were prepared to islate plasmid DNA. The cDNA inserts were PCR amplified, purified and sequenced with an automated ABI 373A DNA sequencer (Applied Biosystems, Norwalk, CT) by using the BigDye-Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, The Netherlands). Public databases were searched to compare the cDNA sequences with those obtained.

Rat tissue preparation

For *in situ* hybridization, congenic control or dystrophic RCS rats were killed at the following developmental ages: postnatal day (PN) 14, PN 22, PN 37and month 5. Tissues (testis, brain, kidney) were removed and fixed in Davidson's AFA Fixative (Prolabo, Briare, France) for 24 hours at 4°C. After enucleation, the cornea, lens and vitreous body were removed from the eyeballs and the posterior eyecups fixed as described above. All the tissues were dehydrated in ethanol, embedded in paraffin, cut into 5-µm sections and mounted onto precoated SuperFrost/Plus slides (Fisher Scientific, Pittsburgh, PA). The slides were heated at 37°C overnight to optimize the adhesion of the paraffin sections on slides.

For RNA extraction, the testis and cerebellum of 21- and 60-day-old control and dystrophic RCS rats were rapidly removed and frozen in dry ice at -80°C. A portion of the testis and cerebellum tissues was used as a positive control. Two pigmented rat strains were killed at PN14, PN21 and PN45 and the eyes were enucleated. The cornea, iris, lens and vitreous were immediately removed. The neural retina was removed from the remaining RPE-choroid-eyecup complex and frozen in liquid nitrogen. RPE cells were isolated by scraping with a small spatula, homogenized in lysis buffer and frozen. Tissues from four retinas and ten RPE were pooled for each analysis. Total RNAs were isolated from each of these tissues using the TRIZOL® Reagent (Invitrogen Corporation, California, USA) according to the manufacturer's protocol. The quality of the total RNA was verified on a 1%

agarose gel and the yield was assessed by measuring absorption at 260 and 280 nm with a spectrophotometer.

***In situ* hybridization**

To prepare the probe, the rat PCR amplified fragment were cloned into the pcDNA3 vector (Invitrogen Corporation, California, USA) containing dual SP6 and T7 promoters. The size and orientation of the cDNA inserts was checked by sequencing. Antisense and sense riboprobes were transcribed with SP6 RNA or T7 RNA polymerases (Riboprobe® Combination System-SP6/T7 RNA Polymerase, Promega Corporation, Madison, USA) from the appropriate linearized plasmid and labeled using a digoxigenin (DIG) dUTP RNA labeling kit (Roche Diagnostics, Mannheim, Germany). Each labeled probe was purified from the DNA template and unincorporated DIG-UTP, and then resuspended at a final concentration of 10 ng/µl in 1X TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4).

For *in situ* hybridization, the paraffin sections were deparaffinized, rehydrated and post-fixed in 4% paraformaldehyde for 20 min. Tissue sections were washed in 1X TBS buffer (50 mM Tris-HCL (pH 7.5), 150 mM NaCl), treated with 200 mM HCl for 10 min, washed and permeabilized further with proteinase K (100 µg/ml) in 1X TBS for 20 min at 37°C. The proteinase K was deactivated by rinsing the slides three times in ice-cold **???**, dehydrated in a graded ethanol series and air-dried. Sections were hybridized with 150 µl of hybridization mix buffer containing 150 ng of DIG-labeled antisense or sense probes, 50% de-ionized formamide, 10% dextran sulfate, 1X Denhardt's solution, 2X SSC, 0.1 mg/ml sheared and denatured salmon sperm DNA, 1 mg/ml *E. coli* tRNA. The slides were incubated at 65°C for 16 hours in a humidified chamber. They were washed three times for 30 min with 2X SSC supplemented with 50% deionized formamide, 0.1% Tween 20 at 65°C and then rinsed twice in 0.1% Triton X-100 in 1X PBS for 30 min at room temperature.

For immunologic detection of the probe, a blocking reagent (10% non immune goat serum in 1X PBS-Triton) was added to each tissue section and incubated for 1 hour at 4°C. Without further washing, sections were incubated overnight at 4°C with an alkaline phosphatase-conjugated anti-DIG Fab fragments antibody (Roche Diagnostics, Mannheim, Germany) diluted 1:2000 in the blocking mixture. Sections were rinsed five times in 1X PBS-Triton for 20 min and then incubated with the alkaline phosphatase substrate, 100 mg/ml of nitro-blue tetrazolium (NBT), 50 mg/ml of 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche Diagnostics, Mannheim, Germany) in NTMT buffer (100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM $MgCl_26H_2O$, 0.1% Triton x100, 0.5 mg/ml Levamisol). The color reaction was developed overnight in the dark at room temperature and then stopped by washing twice in 1X PBS-Triton and twice in 1X PBS. The slides were mounted in Aquatex aqueous mounting medium (Merck, Darmstadt, Germany) and visualized under a light microscope.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RCS rat RNA (1 µg) was reverse transcribed to first strand cDNA in a 20 M reaction volume containing 0.5 µg oligo $(dT)_{12-18}$ primer and 200 U of SUPERSCRIPT™ II RNase H⁻ Reverse Transcriptase (Invitrogen Corporation, California, USA) according to the manufacturer's instructions. The samples were incubated at 70°C for 10 min and then at 42°C for 50 min. The enzyme was inactivated by heating at 70°C for 15 min and the resulting cDNA was stored at -20°C until PCR. For comparison purposes, equal amounts of RNA were reverse transcribed for all samples within a given experimental group.

PCR Conditions and Primers

The following specific primer pair was used to amplify rat *TMS-2*: upstream primer, 5'-GGAGTATGCGTAGCTTGTG-3' (SEQ ID NO:12); downstream primer, 5'-CTCTATTAATGTTGACTCATCA-3' (SEQ ID NO:13). The

expected product size was 920 bp. The following specific primer pair was used to amplify rat *Pcdh-γ-C3*: upstream primer, 5'-ACGGTGGGAGTTTTGCTTCTG-3' (SEQ ID NO:14); downstream primer, 5'-GGTCAGTACCAACTGGACACT-3' (SEQ ID NO:15). The expected product size was 600 bp. The following primers were used to amplify *Cyclophilin A* (GenBank accession no. NM_017101): upstream primer, 5'-TGGTCAACCCCACCGTGTTCTTCG-3' (SEQ ID NO:6) (nt-44-67); downstream primer, 5'- TCCAGCATTTGCCATGGACAAGA-3' (SEQ ID NO:7) (nt-354-332). The expected product size was 310 bp. The concentrations of magnesium and primers, the annealing temperature and extension conditions were optimized for each primer pair. To determine the optimal number of amplification cycles (i.e. in the linear range prior to the onset of the plateau effect), 25, 30 and 35 PCR cycles were performed for each of the specific cDNAs. *Cyclophilin A* was co-amplified as an internal control to allow the comparison of target mRNA concentrations in the different tissues. PCRs were done in a 20 µl final volume containing 1 µl of the cDNA, 0.50 µM specific primers pairs and 0.02 µM *cyclophilin A* primers, 200 µM each dNTP, 1.5 mM $MgCl_2$, 1X PCR buffer and 0.5 U of *Taq* DNA polymerase (Promega Corporation, Madison, USA). The thermal cycling conditions comprised an initial denaturing step at 92°C for 2 min followed by 25 cycles at 92°C for 15 sec, 55°C for 1 min and 72°C for 1m30. Experiments were performed in duplicate for each data point with two negative controls (without specific primer pairs and without cDNA template). The PCR amplification products were separated on 1% agarose gels and visualized by ethidium bromide staining.

2.2 Results

Isolation and identification of two novel human cDNA clones

A BLAST search showed that the first novel human retina cDNA sequence was related to the human Diff33 protein mRNA (GenBank accession n°

AF164794) and the mouse membrane protein TMS-2 mRNA (GenBank accession n° AF181685). Primers derived from the 5'UTR on the basis of the mouse EST sequences were used to amplify the rat homolog of *TMS-2* mRNA from rat cerebellum cDNA by RT-PCR. The 1342-bp cDNA fragment, encompassing the entire coding region of rat *TMS-2* mRNA, was purified and cloned into pcDNA3 for sequence analysis and *in situ* hybridization experiments. The nucleotide sequence of the rat *TMS-2* homolog is 97% identical to published murine sequences (data not shown).

A BLAST search revealed that the second novel human retina cDNA clone was homologous with human protocadherin gamma C3 (*Pcdh-γ-C3*) mRNA (GenBank accession AF152337). Overlapping RT-PCR was performed with primers derived from mouse sequences (GenBank accession NM_033581) to amplify the rat *Pcdh-γ-C3* cDNA sequence using adult rat brain tissue as a template. The amplified cDNA (2940 bp), which encompassed the entire coding region, was cloned into pcDNA3 for sequence analysis. A 1200-bp cDNA fragment, encompassing the 5'coding-sequence, was subcloned into pcDNA3 for *in situ* hybridization experiments. Alignment of the rat mRNA partial coding sequence revealed that a high proportion of nucleotides were identical in human (74%) and mouse sequences (unpublished data). No in-frame stop codons were present upstream of the predicted start site.

RT-PCR analysis of *TMS-2* mRNA in RCS rat cerebellum, testis, retina and RPE cells

Relative RT-PCR was used to detect the 920-bp *TMS-2* transcript in tissue sections. This transcript was detected in all tissues, including brain and retina (fig. 7). *In situ* hybridization showed that the cerebellum contained higher levels of this transcript than the testicular fragments.

Relative RT-PCR was used to determine whether *TMS-2* mRNA was present in tissues from both pigmented wild-type rdy^+ and dystrophic rdy^-

RCS rats. *Cyclophilin A* primers were included in each run as an internal control, to normalize the amounts of transcripts present in the same samples. Both TMS-2 and *cyclophilin A* transcripts were detected at PN21 and PN60 in the testis and cerebellum (positive controls) and no significant variations were observed between control and dystrophic rats (Figure 7). The levels of the TMS-2 transcript were lower than those of the *cyclophilin A* transcript in all the tissues tested. The whole retina, rpe and neuroretina all contained similar and moderate levels of the *TMS-2* transcript from PN14 to PN45. In two independent experiments, no significant difference was found in the intensity of these bands in testis, cerebellum and retinal extracts at any of the stages analyzed. Thus, the amount of this transcript in dystrophic RCS neural retinas remained stable during the early stages of the disease.

RT-PCR analysis of *Pcdh-γ-C3* mRNA in rat cerebellum, testis, retina and RPE cells

RT-PCR was used to determine the distribution of *Pcdh-γ-C3* mRNA in pigmented wild-type *rdy*$^+$ and dystrophic *rdy*$^-$ RCS rat tissues during the early stages of retinal degeneration. *Cyclophilin A* primers were included in each run as an internal control to normalize the levels of these transcripts present in the same samples. Both *Pcdh-γ-C3* and *cyclophilin A* transcripts were detected at PN21 and PN60 in the testis and cerebellum (positive controls) and no significant variations were observed between control and dystrophic rats (Figure 8). The whole retina, rpe and neuroretina all contained similar levels of the *Pcdh-γ-C3* transcript from PN14 to PN45. In two independent experiments, no significant difference was found in the intensity of these bands in testis, cerebellum and retinal extracts at any of the stages analyzed.

In situ hybridization analysis of _TMS-2_ transcripts in RCS rat tissues

Although RT-PCR is a sensitive method, it cannot reveal which cell types within a tissue contain a given mRNA. Thus, digoxigenin *ISH* was used to localize these transcripts. Positive ISH signals appeared as a purple precipitate in the rat tissue sections. Digoxigenin, a naturally occurring plant steroid, is not found in animal tissues, so cytoplasmic localization of the immunoproduct is considered to be specific. With the antisense riboprobes (Fig. 9B-C), but not with the sense probes (Fig. 9A), a dark blue precipitate was observed. Faint *TMS-2* signals were observed in Leydig cells located in the interstitial layer and along the seminiferous epithelium of testicular fragments from one-year-old rdy+ rats (Fig. 9B-C). At PN21, the cerebellar Purkinje cells of wild-type RCS *rdy+* (Fig. 9E) and dystrophic RCS *rdy-* (Fig. 9F) rats contained high levels of *TMS-2* transcripts.

In situ hybridization analysis of _Pcdh-γ-C3_ transcripts in RCS rat tissues

Hybridization with the digoxigenin-labeled antisense riboprobe revealed a low amount of *Pcdh-γ-C3* mRNA in the primary gonocytes of seminiferous tubules of testicular fragments (Fig. 10B, C). Leydig cells located in the interstitial layer of the testis were also slightly labeled (Fig. 10C). At PN21, all the cerebellar Purkinje cells from wild-type RCS *rdy+* (Fig. 10E, arrows) and dystrophic RCS *rdy-* (Fig. 10F, arrows) rats were strongly labeled for *Pcdh-γ-C3* transcripts. Sense riboprobes (negative controls) revealed no specific signals on the testis (Fig. 10A) or cerebellum sections (Fig. 10D).

Presence of _TMS-2_ transcripts in wild-type and degenerating RCS rat retinas

To determine which cells in the neural retinal layers contained *TMS-2* mRNA, ISH analysis was performed on wild-type *rdy+* (Fig. 11A-D) and dystrophic *rdy-* (Fig. 11E-H) RCS rat eye sections (n=4) from PN14 (E-F),

PN22 (A-B) and PN37 (C-D, G-H) (*i.e.* during the pathologic retinal degeneration process). Hybridization with the sense probe (Fig. 11A, 11E) gave a negative signal whereas the antisense riboprobe gave a dark blue positive signal. In both normal and dystrophic RCS rat retinas, intense staining was predominantly found in the onl and in the rpe. Moderate signals were detected in the gcl, inner inl, choroidal cells (ch) and sclera (s). No signal was detected in the inner plexiform layer (ipl) or in the inner (is) or outer (os) segment of photoreceptors. Examination at a higher magnification showed that the onl was thinner in dystrophic RCS retinas (Fig. 11G, 11H, double arrows) than in normal retinas (Fig. 11D, large double arrows) on day 37 and revealed a debris region (d) of non-phagocytized os membranes in dystrophic retinas (Fig. 11G, 11H, asterisk). In addition, the intensity of the labeling in the onl, inl or rpe cells did not vary significantly during the 37-day study period (Fig. 11G, 11H) and was similar in dystrophic and normal retinas (Fig. 11C, 11D).

Presence of *Pcdh-γ-C3* mRNA in wild-type and degenerating RCS rat retinas

ISH analysis was performed on wild-type *rdy+* (Fig. 12A-D) and dystrophic *rdy-* RCS (Fig. 12 E-H) rats to determine the distribution of *Pcdh-γ-C3* mRNA in the neural retinal layers from PN14 (A-C, E-F) to PN37 (D, G-H) (i.e. during the course of the retinal degeneration in the dystrophic strain). At PN14, the antisense riboprobes revealed dark blue positive signals on sections from wild-type (Fig. 12B, 12C) and dystrophic animals (Fig. 12F), whereas the sense probe did not give a positive signal on rat sections (Fig. 12A, 12E). In both strains, intense staining was predominantly found in the onl. The gcl and inl were weakly labeled. Faint signals were also observed in the rpe, ch and s. No signal was detected in the ipl or in the is or os of photoreceptors. Examination at a higher magnification showed a debris region (d) of non-phagocytized os membranes in PN37 dystrophic RCS retinas (Fig. 12H, *asterisk*). Furthermore, at PN37, the onl of dystrophic rat

retinas was thinner (Fig. 12H, *double arrows*) than that of normal retinas (Fig. 12D, *large double arrows*). The intensity of the onl labeling was similar in both dystrophic (Fig. 12H, *double arrows*) and normal retinas (Fig. 12D) throughout the 37-day study period.

The SOST gene is of unknown function. However, its expression in the retinal pigment epithelium strongly suggests that mutation of this gene may affect the function of the external hemato-ocular barrier. Thus, the SOST gene is a target gene for diagnosing or treating retinal disorders.

3. OAT1, OAT2, OAT3, OAT4 and SOST as a target for treating retinal disorder

cDNA libraries were generated from mRNA of RPE cells of rat and human using the GeneRacer kit (Invitrogen Corporation, San Diego, CA).

cDNA clones including putative signal peptide sequences were isolated and sequenced.

A blast search showed that four novel rat RPE cDNA sequences were identical to the rat OAT1, OAT2, OAT3, OAT4 mRNA.

Another clone is related to SOST human mRNA.

Expression in RPE cells was confirmed by *in situ* hybridization according to the protocol detailed in example 2.

The OAT genes encode multispecific organic anionic transporter. Expression of these genes in the RPE suggest an essential role in the external hemato-ocular barrier provided by the RPE layer. The OAT genes are thus target genes for retinal disorders and especially retinal disorder associated with dysfunction of the retinal pigmentary epithelium.

The SOST gene is of unknown function. However, its expression in the retinal pigment epithelium strongly suggests that mutation of this gene may affect the function of the external hemato-ocular barrier. Thus, the SOST gene is a target gene for diagnosing or treating retinal disorders.

4. Uterine Sensitization-Associated Gene-1 (USAG-1)

The cDNA encoding USAG-1 was isolated. This is the first time this gene has been shown to be expressed in the eye. USAG-1 mRNA was detected by RT-PCR and in situ hybridization in the rat RPE cell monolayer and in the different cell layers of the retina at 14, 21, and 45 days after birth. The amount of USAG-1 mRNA decreased between days 14 and 45 after birth in normal retinas (*rdy*$^+$), but not in the pathologic retinas (*rdy*$^-$) of RCS rats

Isolation of USAG-1 cDNA from the rat RPE cDNA library

The yeast sequence signal trap system was used to identify proteins secreted by retinal pigmented epithelial cells. The USAG-1 protein is highly conserved through species (Fig 13).

Transfection of RPE D407 cells by USAG-1/HA chimer (Fig 14) shows that USAG-1 is a secreted protein.

The tissue distribution experiment of USAG-1 mRNA, achieved by RT-PCR, shows that USAG-1 is highly expressed in kidney, forebrain and cerebellum, and in eye (particularly in the retinal pigment epithelial cells). USAG-1 is also present in the neuroretina but to a lesser extent than in the RPE (Fig 15).

In situ hybridization was used to study the distribution of USAG-1 mRNA in paraffin-embedded sections of *rdy*$^+$ pigmented rat eyes at different postnatal stages (P14, P21 and P45) using DIG-labeled sense and

antisense riboprobes. The presence of USAG-1 in the eye and especially in the retina was confirmed (Fig 16).

At P14 and P21, few amounts of USAG-1 mRNAs were observed in the ganglion cell layer of the *rdy*$^+$ and *rdy*$^-$ pigmented rat retina, while a high mRNA signal was detected in the inner and outer nuclear layer (INL and ONL). A high signal was also observed in the RPE cell layer. The intensity of labelling in the neuroretina decreased considerably between P14 and P45 in *rdy*$^+$ pigmented rat, while the opposite is observed in *rdy*$^-$ pigmented rat.

A technique, using 60 mers sense and antisense oligonucleotides radiolabelled with 35S was used to study the distribution of USAG-1 mRNA in frozen sections of *rdy*$^+$ embryo at different stages (E13.5 to E20.5). USAG-1 was clearly expressed in the central nervous system (plexus choroids, 4th ventricule) and in the eye. Labelling in liver, bone (skull, spinal column and ribs), tongue and pilous follicules was also observed.

Differential USAG-1 mRNA analysis in normal and dystrophic RCS rat retina

Semiquantitative RT-PCR was used to compare the amounts of USAG-1 mRNA in RPE cells of normal *rdy*$^+$ and pathologic *rdy*$^-$ RCS rats at various developmental ages (P14, P21 and P45). As a control, the same experiment was performed on mRNAs extracted from the cerebellums of the two rat strains at the same ages . Cyclophilin A mRNA was used as an internal control. In the normal *rdy*$^+$ retina, the amount of USAG-1 mRNAs in retinal pigment epithelial cells was clearly higher at P14 and P21 than at P45. Thus, in normal physiological conditions, the USAG-1 mRNA levels appear to decrease with age in RPE. In contrast, in RPE cells from the

dystrophic RCS *rdy*$^-$ rat strain, the USAG-1 mRNA levels increased over time, and were clearly higher at P45 than at P14 and P21. Surprisingly, the amounts of USAG-1 mRNAs in cerebellum (supposed to be a control tissue during PCR reactions) of the control rat (*rdy*$^+$) were extremely lowered as age increased, while a strong increase of USAG-1 mRNAs levels was observed in dystrophic rat (*rdy*$^-$) during the same period of time : an extremely high amount of these USAG-1 mRNAs was reproducibly detected in the cerebellum of P45 dystrophic rat (*rdy*$^-$) (Fig 17).

The yeast sequence signal trap method was used to identify proteins secreted by RPE cells. This system allowed to select the cDNA encoding for USAG-1. Using in situ hybridization and RT-PCR, USAG-1 was present in RPE cells and in the INL and ONL of the rat neural retina. This is the first time that the distribution of USAG-1 in the eye, and especially in the neural retina is reported.

The semi-quantitative RT-PCR analysis demonstrated that the amount of USAG-1 in the RPE of normal *rdy*$^+$ rats progressively decreases with age. This is not occurring only in the RPE cells but is also observed in the cerebellum of the same rat strain. In contrast, increased amounts of USAG-1 mRNAS in the RPE and cerebellum of pathologic *rdy*$^-$rats were observed as the age of the animals increased. In situ hybridization also showed a more intense labelling at P14 than at P45 in the retinas of normal rats.

These results allow illustrated a major role of USAG-1 in the development of the eye and especially of the retina as well as in their normal functions. Additionally, as USAG-1 is to be an antagonist of Bone Morphogenetic Proteins which are known to play a crucial role in eye and retinal development via the TGF Beta receptor. These results show that the absence or the overexpression of USAG-1 plays a key role both in genetic

congenital abnormalities of the eye as well as in the pathophysiology of retinal degenerations.

5

CLAIMS

1. A method of treating a retinal disorder in a mammal, comprising modulating the amount of a polypeptide encoded by a target gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes in a retinal cell of said mammal.

2. The method of Claim 1, comprising modulating the expression of the gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* in a retinal cell of said mammal.

3. The method of Claim 2, comprising administering to a retinal cell of said mammal, an effective amount of a gene transfer vector carrying a nucleic acid comprising one of the gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes; said gene being operably linked to a promoter active in said retinal cell.

4. The method of Claim 3, wherein said gene transfer vector comprises a proviral genome which is selected among the group consisting of adenoviruses, adenovirus associated viruses and lentiviruses.

5. The method of Claim 1, comprising administering to said retinal cell, a composition comprising an effective amount of cells, wherein said cells produce and secrete a polypeptide encoded by a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes.

6. The method of Claim 5, wherein said secreting cells are encapsulated in a biocompatible polymer.

7. The method of Claim 3, further comprising administering a composition comprising a neurotrophic factor or an anti-angiogenic factor, or a gene transfer vector comprising a nucleic acid encoding a neutrotrophic factor or an anti-angiogenic factor or cells producing and secreting a neurotrophic factor or an anti-angiogenic factor.

8. The method of Claim 2, comprising administering to a retinal cell of said mammal, an effective amount of a gene transfer vector carrying an antisense nucleic acid of the target gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes; wherein said antisense mRNA is capable of inhibiting *in vivo* the expression of said target gene.

9. The method of Claim 1, wherein said retinal disorder is selected from the group of retinal degenerations and retinopathies.

10. The method of Claim 9, wherein said retinal degeneration is selected from the group consisting of retinitis pigmentosa and macular degenerative diseases.

11. The method of Claim 1, wherein said retinal cell is a retinal pigment epithelium (RPE) cell or a ganglion cell.

12. A composition which comprises encapsulated cells which produce and secrete a polypeptide encoded by a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes, operably linked to a promoter active in a retinal cell.

13. A method for diagnosing a retinal disorder in a mammal, comprising identifying an abnormal expression of a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4,*

USAG-1, SOST and *Pcdh-γ-C3* genes in retinal cells of said mammal.

14. A method for diagnosing a retinal degeneration in a mammal, comprising identifying an inhibition of expression of the prosaposin gene in retinal cells of said mammal.

15. The method of Claim 14, wherein said abnormal expression is detected by RT-PCR amplification.

16. The method of Claim 15, wherein said RT-PCR amplification is carried out using one of the following pair of primers: SEQ ID NO:4-5 for prosaposin amplification, or functional variants thereof.

17. A method of screening for a compound capable of modulating the expression of the gene encoding prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes, comprising

a. providing cells expressing a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes,

b. culturing said cells in the presence of a compound,

c. determining the expression of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* or *Pcdh-γ-C3* genes, and comparing said expression to the expression of the same gene in a control cell culture in the absence of the compound, or to the expression of a control constitutive gene, wherein an abnormal expression in the presence of the compound is indicative that said compound is capable of modulating the expression of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* or *Pcdh-γ-C3*.

18. A method of screening for a compound capable of binding to the expression product of a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3*, comprising

a. contacting a candidate compound with the expression product of a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes,

b. determining whether said candidate molecule binds to the expression product.

19. The method of Claim 18, wherein step a consists of culturing cells expressing a nucleic acid comprising a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* genes and incubating said cells with a candidate compound.

20. Method for screening for a pathological allelic form of a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3,* comprising

a. isolating a polynucleotide comprising a specific fragment of a gene selected among the group consisting of prosaposin, *TMS-2, OAT1, OAT2, OAT3, OAT4, USAG-1, SOST* and *Pcdh-γ-C3* from a retinal cell of a subject suffering from a retinal degeneration,

b. determining the sequence of said polynucleotide and comparing with a wild-type sequence

wherein the detection of one or more mutation in the sequence of said polynucleotide is indicative that said polynucleotide is a pathological allelic form of the gene.