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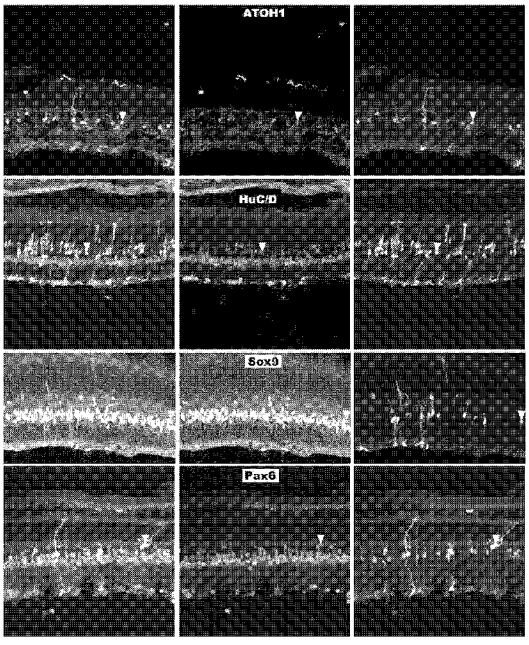
(54) **Title:** METHODS AND COMPOSITIONS FOR REPROGRAMMING MÜLLER GLIA

(57) **Abstract:** Nucleic acid molecules and compositions, and methods using the same are provided herein for intraocular gene-based delivery and expression of two or more proneural bHLH transcription factors in the retina. The nucleic acid molecules, compositions and methods disclosed herein stimulate regeneration of retinal interneurons from retinal Müller glia (MG) and reprogram the MG into bipolar, amacrine, horizontal, and/or ganglion cells. Such methods and nucleic acid molecules are used for vision restoration and/or treatment of a range of ocular diseases involving retinal degeneration after injury, disease, or vison loss.

Figure 2



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METHODS AND COMPOSITIONS FOR REPROGRAMMING MÜLLER GLIA

REFERENCE TO A SEQUENCE LISTING SUBMITTED VIA EFS-WEB

[0001] The content of the ASCII text file of the sequence listing named "UW70WOU1_seq" which is 93 kb in size was created on April 28, 2020, and electronically submitted via EFS-Web herewith the application is incorporated herein by reference in its entirety.

ACKNOWLEDGEMENT OF GOVERNMENT SUPPORT

[0002] This invention was made with government support under Grant Nos. F31 EY028412 and R01 EY021482, awarded by the National Institutes of Health. The government has certain rights in the invention.

BACKGROUND

[0003] Some of the leading causes of blindness involve the loss of one or more types of retinal neurons. Age-related macular degeneration, glaucoma, ischemia, retinal arterial occlusion and inherited retinal diseases, such as Retinitis Pigmentosa or Usher's syndrome all ultimately lead to irreversible degeneration of photoreceptors or other retinal neurons. In mammalian retinas, lost neurons are not spontaneously regenerated, and currently, there are no effective therapies to replace the degenerated neurons in patients with retinal disease. By contrast, retinas of nonmammalian vertebrates, such as fish and amphibians, show a robust regenerative response upon retinal damage. Upon injury to the retina, fish Müller Glia (MG) re-enter the cell cycle to generate a progenitor, which proliferates, and generates different types of retinal neurons to replace those that were lost.

[0004] One key difference between fish and mouse MG in the response to retinal injury is the expression of the proneural transcription factor, Ascl1. This factor is necessary for regeneration in fish retina, but is not expressed in mammalian MG after injury. When we over-expressed Ascl1 in mouse MG with a lentivirus, we found that this single factor could reprogram them into neurogenic progenitors *in vitro*. When Ascl1 expression is induced in adult MG, the combination of Ascl1 and histone deacetylase (HDAC) inhibition can stimulate new neuron production from MG in adult mice after neuronal damage. The MG-derived neurons form connections with the existing retinal circuitry. Patch-clamp recordings from MG-derived neurons in retinal slices shows that they have neuronal-like light responses. Epigenetic analyses show that chromatin remodelers can produce a neurogenic potential to adult MG by making previously inaccessible neuronal genes open to proneural transcription factors, like Ascl1. In addition, a specific micro-RNA, miR-124, can induce expression of Ascl1 in Müller glial cells, and reprogram them to a neurogenic state, resulting in neuron

production. Only a small percentage of MG are reprogrammed using these previous methods, however, limiting their promise for therapeutic applications. We also showed that Ascl1 expression and the resulting neurogenesis from MG can be significantly enhanced by inhibition of the Jak/STAT pathway, and/or by use of other reprogramming potentiating agents such as RNAi-based Ascl1 activators. However, these approaches require both retinal injury and co-administration of compositions comprising reprogramming potentiating agents (e.g., small molecules) which complicate and limit commercial application.

[0005] There are numerous diseases that cause the loss of specific neuronal populations in the retina resulting in blindness. As such, there remains a need to stimulate regeneration in the human retina for the development of new types of regenerative therapies for patients.

SUMMARY

[0006] Described herein are compositions, nucleic acid molecules and methods for inducing retinal regeneration and reprogramming of Müller glia (MG) into retinal neurons in a subject.

[0007] Disclosed herein is a nucleic acid molecule comprising a nucleic acid sequence encoding two or more proneural bHLH transcription factors. Representative examples of proneural bHLH transcription factors include, but are not limited to, Ascl1, Atonal7 (also known as Math5), Atoh1 (also known as Math1), Neurogenin-2, and Neuronal Differentiation 1 (Neurod1).

[0008] In one embodiment, the nucleic acid molecule comprises a nucleic acid sequence encoding Ascl1 and Atoh1. In another embodiment, the nucleic acid molecule comprises a nucleic acid sequence encoding Ascl1 and Atoh7. In another embodiment, the nucleic acid molecule comprises a nucleic acid sequence encoding Atoh1 and Atoh7. In another embodiment, the nucleic acid molecule comprises a nucleic acid sequence encoding Ascl1, Atoh1 and Atoh7. In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence encoding two, three, four, or all five of Ascl1, Atonal7, Atoh1, Neurogenin-2, and Neurod1.

[0009] Also disclosed herein is a method for inducing retinal regeneration in a subject comprising: a) administering to a retina of the subject a nucleic acid molecule comprising a nucleic acid sequence encoding two or more proneural bHLH transcription factors selected from the group consisting of Ascl1 + Atoh1, Ascl1 + Atoh7, Atoh1 + Atoh7, and Ascl1 + Atoh1 + Atoh7. In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence encoding two, three, four, or all five of Ascl1, Atonal7, Atoh1, Neurogenin-2, and Neurod1.

[0010] In additional embodiments, the nucleic acid molecules and methods disclosed herein stimulate production of functional neurons from reprogrammed MG. In another embodiment, the number of the MG-derived functional neurons is increased. In another embodiment, the number of functional neurons is increased by 40%. In another embodiment, the subject is treated for retinal disease, damage or degeneration in the retina. In another embodiment, the subject is an adult. In another embodiment, a vector comprises the nucleic acid molecule. In one embodiment, the vector is a non-viral vector or a viral vector, and the viral vector is an adeno-associated viral (AAV) vector or a lentiviral vector. In an additional embodiment, a promoter sequence is in operable linkage with the nucleic acid encoding Ascl1. In one embodiment, the promoter is a retinal or MG-specific promoter. In one embodiment, administering to the retina is intravitreal or subretinal injection.

[0011] Also, in another embodiment, the nucleic acid molecules disclosed herein comprise an MG-specific promoter sequence. In one embodiment, the MG-specific promoter sequence is a Rbpl1 promoter sequence or a portion thereof. In other embodiments, the nucleic acid sequence comprises an IRES or 2A self-cleaving sites situated between the sequences encoding the proneural bHLH transcription factors, for example, in a multicistronic or polycistronic configuration. In another embodiment, the proneural bHLH transcription factors are expressed as a fusion protein.

[0012] Also provided herein are methods for inducing retinal regeneration comprising administering to a subject a composition as described herein. In some embodiments, the methods are effective to increase the number of Müller glial-derived neurons, to induce Müller glial cells to enter the mitotic cell cycle, and/or to generate new retinal neurons. In some embodiments, the new retinal neurons are bipolar neurons. In some embodiments of the method, the number of retinal neurons increases by at least 40% relative to a baseline level or other reference amount representative of an untreated retina. In some embodiments, the number of retinal neurons increases by 10%, 20%, 25%, 50%, 100%, 150%, 200%, or more.

[0013] The subject in the methods disclosed herein is typically a mammal, such as a human or veterinary subject. In one embodiment, the subject is an adult. The subject, in some embodiments, has a retinal degenerative disease. Examples of such retinal degenerative diseases include, but are not limited to, Age-related macular degeneration, glaucoma, ischemia, central retinal arterial occlusion and inherited retinal diseases, such as Retinitis Pigmentosa or Usher's syndrome.

BRIEF DESCRIPTION OF THE DRAWINGS

[0014] **Figure 1** shows the overexpression of Atoh1 and Ascl1 in adult mice. The experimental paradigm described is in Example 1 (Glast-CreER; Glox-stop-LNL-tTA; tetO-Ascl1-IRES-GFP; tetO-Atoh1 mice being injected with 5 consecutive daily intraperitoneal injections of tamoxifen; retinas collected 3-6 weeks after last tamoxifen injection). Figure 1 shows Atoh1 (upper panel), GFP (middle panel), and DAPI (lower panel) staining in the same tissue. All GFP+ cells with MG morphology fail to express Atoh1. All GFP+ cells with neuronal morphology express Atoh1.

[0015] **Figure 2** shows Atoh1 and Ascl1-overexpressing cells in adult mice stained for a subset of amacrine markers. Arrows indicate examples of cells that express Atoh1, HuC/D, and Pax6.

DETAILED DESCRIPTION

[0016] The bHLH transcription factors play a key role in development and cell activity. The proteins are characterized by a basic helix-loop-helix (bHLH), the majority of which are heterodimeric because their activity is highly regulated by the dimerization of the subunits.

[0017] The proneural Achaete-scute family bHLH transcription factor 1 (Ascl1) gene encodes a member of the basic helix-loop-helix (BHLH) family of transcription factors. The protein activates transcription by binding to the E box (5'-CANNTG-3'). Dimerization with other bHLH proteins is required for efficient DNA binding.

[0018] An exemplary nucleic acid sequence encoding human Ascl1 can be found at NCBI Reference Sequence number NG_008950.1 and also provided herein as SEQ ID NO: 1. In another embodiment disclosed herein is a nucleic acid sequence encoding a human Ascl1 amino acid sequence or portion thereof of UniProtKB/Swiss-Prot: P50553.2, provided herein as SEQ ID NO: 2. Ascl1 homologues, e.g., derived from species such as murine, canine, equine, are included herein, without limitation.

[0019] The Protein Atonal Homolog 1 (Atoh1) is a proneural member of the family of bHLH transcription factors. The protein activates a slightly different E box than the Ascl1 gene.

[0020] An exemplary nucleic acid sequence encoding human Atoh1 can be found at NCBI Reference Sequence number NM_005172.1 and also provided herein as SEQ ID NO: 3. In another embodiment disclosed herein is a nucleic acid sequence encoding a human Atoh1 amino acid sequence or portion thereof of NP_005163.1, provided herein as SEQ ID NO: 4. Atoh1 homologs, orthologs and/or paralogs, e.g., derived from species such as murine, canine, equine, are included herein, without limitation.

[0021] The Atoh7 family bHLH transcription factor 7 (Atoh7) gene encodes a proneural member of the basic helix-loop-helix (BHLH) family of transcription factors.

[0022] An exemplary nucleic acid sequence encoding human Atoh7 can be found at NCBI Reference Sequence number NM_008553.4 and also provided herein as SEQ ID NO: 5. In another embodiment disclosed herein is a nucleic acid sequence encoding a human Atoh7 amino acid sequence or portion thereof of NP_660161.1, provided herein as SEQ ID NO: 6. Atoh7 homologs, orthologs, and/ paralogs, e.g., derived from species such as murine, canine, equine, are included herein, without limitation. An exemplary nucleic acid sequence encoding Neurogenin-2 (also known as NEUROG2 and NGN-2) can be found at NCBI Reference Sequence number NM_024019 and also provided herein as SEQ ID NO: 50. An exemplary nucleic acid sequence encoding Neurod1 can be found at NCBI Reference Sequence number KR709666 and also provided herein as SEQ ID NO: 51.

[0023] Exemplary nucleic acid sequences of the Ascl1, Atoh1, and/or Atoh7 or other proneural bHLH transcriptionfactor for use herein include, without limitation, portions thereof of the corresponding sequences of Ascl1, Atoh1, and/or Atoh7, for the purposes of configurating multicistronic, bicistronic, and/or tricistronic constructs, plasmids, and/or expression vectors.

[0024] The invention described herein is based on the discovery that intraocular gene-based delivery and expression of two or more proneural bHLH transcription factors in the retina stimulates regeneration of retinal interneurons from retinal Müller glia (MG) and reprograms the MG into bipolar, amacrine, horizontal, and/or ganglion cells.

[0025] Specifically, expression of Ascl1 and Atoh1 in the retina produced a robust reprogramming effect in MG. Surprisingly, we show that MG reprogramming occurs in adult mammals in the absence of retinal injury or additional small molecule-type reprogramming potentiating agents (e.g., HDAC inhibitors and/or Jak/STAT inhibitors and/or RNAi-based Ascl1 activators). Additionally, we show that expressing these proneural bHLH transcription factors in injured retinas similarly results in reprogramming of MG into new neurons which are able to integrate into the retinal circuitry and respond to light stimulus. We have characterized these Müller glial-derived retinal neurons with immunohistochemical staining, single-cell RNA-sequencing, electrophysiological whole-cell recordings, and various epigenetic assays and have confirmed that the new neurons highly resemble nascent retinal neurons. Atoh1, like Ascl1, belongs to the bHLH family of transcription factors. Atoh1, however, is in a different subclass of bHLH factors and binds to a related but not identical E-box sequence in the DNA to activate transcription. It is known that Atoh1 is important for hair cell development in the inner ear but it is not normally expressed in the developing or

mature retina. It is also known that the highly related gene to Atoh1, Atoh7, is expressed in the developing retina and is important for ganglion cell development in mice. Accordingly, disclosed herein is the intraocular vector-based delivery of two or more genes encoding these families of proneural bHLH transcription factors into the retina of blind patients to infect Müller glial (MG) cells so that reprogramming of the MG to retinal neurons treats retinal degenerative disease as well as restoration of vision after injury, disease, or loss.

[0026] Reprogramming of MG and regeneration of retinal neurons is particularly important for developing therapeutic products and methods for a range of degenerative ocular diseases such as, for example, and without limitation, retinal degeneration caused by diabetic retinopathy, glaucoma, and age-related macular degeneration. One such retinal degenerative disease is known as central retinal artery occlusion (CRAO), wherein blood flow through the central retinal artery is blocked or occluded often resulting in loss of vision. CRAO is caused by thromboembolus, carotid artery atherosclerosis, giant cell arteritis, aneurysms or arterial spasms. Current treatment paradigms for many of these types of degenerative diseases of the retina, particularly for CRAO, show little to no definitive improvement in outcomes.

Definitions

[0027] All scientific and technical terms used in this application have meanings commonly used in the art unless otherwise specified. As used in this application, the following words or phrases have the meanings specified.

[0028] As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but do not exclude others. As used herein, the transitional phrase "consisting essentially of" (and grammatical variants) is to be interpreted as encompassing the recited materials or steps "and those that do not materially affect the basic and novel characteristic(s)" of the recited embodiment. Thus, the term "consisting essentially of" as used herein should not be interpreted as equivalent to "comprising." "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions disclosed herein. Aspects defined by each of these transition terms are within the scope of the disclosure herein.

[0029] As used herein, "retinal neuron" refers to any of the five types of neurons in the retina: photoreceptors, bipolar cells, ganglion cells, horizontal cells, and amacrine cells. In some particular embodiments, the retinal neurons are bipolar neurons, amacrine, horizontal, and ganglion cells.

[0030] As used herein, the terms "nucleic acid sequence" or "polynucleotide" refers to nucleotides of any length which are deoxynucleotides (i.e. DNAs), or derivatives thereof;

ribonucleotides (i.e. RNAs) or derivatives thereof; or peptide nucleic acids (PNAs) or derivatives thereof. The terms include, without limitation, single-stranded, double-stranded, or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, oligonucleotides (oligos), or other natural, synthetic, modified, mutated or non-natural forms of DNA or RNA.

[0031] MicroRNAs, or "miRNAs", or "miRs", are short, non-coding RNAs that regulate gene expression by post-transcriptional regulation of target genes.

[0032] "Short hairpin RNAs" or "shRNAs" are synthetic or non-natural RNA molecules. shRNA refers to RNA with a tight hairpin turn used to silence (via RNA interference or RNAi) target gene expression in a cell. An shRNA is typically delivered via an expression vector such as a DNA plasmid or via viral vectors.

[0033] The term "vector" refers to, without limitation, a recombinant genetic construct or plasmid or expression construct or expression vector that retains the ability to infect and transduce non-dividing and/or slowly-dividing cells and integrate into the target cell's genome. The vector may be derived from or based on a wild-type virus. Aspects of this disclosure relate to an adeno-associated virus vector, an adenovirus vector, and a lentivirus vector.

[0034] The term "expression control element" as used herein refers to any sequence that regulates the expression of a coding sequence, such as a gene. Exemplary expression control elements include but are not limited to promoters, enhancers, microRNAs, post-transcriptional regulatory elements, polyadenylation signal sequences, and introns. Expression control elements may be, without limitation, constitutive, inducible, repressible, or tissue-specific. A "promoter" is a control sequence that is a region of a polynucleotide sequence at which initiation and rate of transcription are controlled. It may contain genetic elements at which regulatory proteins and molecules may bind such as RNA polymerase and other transcription factors. In some embodiments, expression control by a promoter is tissue-specific. An "enhancer" is a region of DNA that can be bound by activating proteins to increase the likelihood or frequency of transcription. Non-limiting exemplary enhancers and posttranscriptional regulatory elements include the CMV enhancer and WPRE.

[0035] The term "multicistronic" or "polycistronic" or "bicistronic" or tricistronic" refers to mRNA with multiple, i.e., double or triple coding areas or exons, and as such will have the capability to express from mRNA two or more, or three or more, or four or more, etc., proteins from a single construct. Multicistronic vectors simultaneously express two or more separate proteins from the same mRNA. The two strategies most widely used for constructing multicistronic configurations are through the use of 1) an IRES or 2) a 2A self-cleaving site. An "IRES" refers to an internal ribosome entry site or portion thereof of viral,

prokaryotic, or eukaryotic origin which are used within polycistronic vector constructs. In some embodiments, an IRES is an RNA element that allows for translation initiation in a cap-independent manner. The term "self-cleaving peptides" or "sequences encoding self-cleaving peptides" or "2A self-cleaving site" refer to linking sequences which are used within vector constructs to incorporate sites to promote ribosomal skipping and thus to generate two polypeptides from a single promoter, such self-cleaving peptides include without limitation, T2A, and P2A peptides or sequences encoding the self-cleaving peptides.

[0036] The term "substantially complementary," when used to define either amino acid or nucleic acid sequences, means that a particular sequence, for example, an oligonucleotide sequence, is substantially complementary to the sequence of miR-214 referenced. As such, typically the sequences will be highly complementary to the microRNA "target" sequence, and will have no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 base mismatches throughout the sequence. In many instances, it may be desirable for the sequences to be exact matches, i.e. be completely complementary to the sequence to which the nucleic acid specifically binds, and therefore have zero mismatches along the complementary stretch. As such, highly complementary sequences will typically bind quite specifically to the target sequence region and will therefore be highly efficient in reducing, and/or even inhibiting the biological activity of the target sequence.

[0037] Substantially complementary nucleic acid sequences will be greater than about 80 percent complementary (or '% exact-match') to the corresponding target sequence to which the nucleic acid specifically binds, and will, more preferably be greater than about 85 percent complementary to the corresponding target sequence to which the nucleic acid specifically binds. In certain aspects, as described above, it will be desirable to have even more substantially complementary nucleic acid sequences for use in the practice of the invention, and in such instances, the nucleic acid sequences will be greater than about 90 percent complementary to the corresponding target sequence to which the nucleic acid specifically binds, and may in certain embodiments be greater than about 95 percent complementary to the corresponding target sequence to which the nucleic acid specifically binds, and even up to and including 96%, 97%, 98%, 99%, and even 100% exact match complementary to the target to which the designed nucleic acid specifically binds.

[0038] "Homology" or "identity" or "similarity" refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of homology between sequences is a function of the number of matching or homologous positions shared by the

sequences. An "unrelated" or "non-homologous" sequence shares less than 40% identity, or alternatively less than 25% identity, with one of the sequences of disclosed herein.

[0039] Percent similarity or percent complementary of any of the disclosed sequences may be determined, for example, by comparing sequence information using the GAP computer program, version 6.0, available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (1970). Briefly, the GAP program defines similarity as the number of aligned symbols (i.e., nucleotides or amino acids) which are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess (1986), (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

[0040] "Nucleotide sequence" refers to a heteropolymer of deoxyribonucleotides, ribonucleotides, or peptide-nucleic acid sequences that may be assembled from smaller fragments, isolated from larger fragments, or chemically synthesized de novo or partially synthesized by combining shorter oligonucleotide linkers, or from a series of oligonucleotides, to provide a sequence which is capable of specifically binding to a target molecule and acting as an antisense construct to alter, reduce, or inhibit the biological activity of the target.

[0041] As used herein, "directed against", in the context of antisense oligonucleotides, means the antisense oligonucleotide binds to a target miRNA and blocks or suppresses activity of the target.

[0042] As used herein, the terms "protein", "peptide", and "polypeptide" refer to amino acid subunits, amino acid analogs, or peptidomimetics. The subunits may be linked by peptide bonds. In another aspect, the subunit may be linked by other bonds, e.g., ester, ether, etc. As used herein the term "amino acid" refers to either natural and/or unnatural or synthetic amino acids.

[0043] As used herein, the term "recombinant expression system" or "recombinant expression vector" refers to a genetic construct for the expression of certain genetic material formed by recombination.

[0044] The term "effective amount" or "therapeutically effective amount" or "prophylactically effective amount", refer to an amount of an active agent described herein that is effective to provide the desired/intended result and/or biological activity. Thus, for example, in various embodiments, an effective amount of a composition described herein is an amount that is

effective to result in regeneration of retinal neurons, and/or to improve or to ameliorate symptoms of and/or to treat retinal degenerative diseases.

[0045] When the disclosure herein relates to a small molecule, polypeptide, protein, polynucleotide, nucleic acid, oligonucleotide, antisense, or miRNA, an equivalent or a biologically equivalent of such is intended within the scope of this disclosure. As used herein, the term "biological equivalent thereof" is intended to be synonymous with "equivalent thereof" when referring to a reference small molecule, polypeptide, protein, polynucleotide, nucleic acid, oligonucleotide, antisense, or miRNA even those reference molecules having minimal homology while still maintaining desired structure or functionality. Unless specifically recited herein, it is contemplated that any nucleic acid, polynucleotide, oligonucleotide, antisense, miRNA, polypeptide, or protein mentioned herein also includes equivalents thereof. For example, an equivalent intends at least about 70% homology or identity, or at least 80 % homology or identity and alternatively, or at least about 85 %, or alternatively at least about 90 %, or alternatively at least about 95 %, or alternatively 98 % percent homology or identity and exhibits substantially equivalent biological activity to the reference protein, polypeptide or nucleic acid. Alternatively, when referring to polynucleotides, an equivalent thereof is a polynucleotide that hybridizes under stringent conditions to the reference polynucleotide or its complement.

[0046] In some embodiments disclosed herein, the polypeptide and/or polynucleotide sequences are provided herein for use in gene and protein transfer and expression techniques described below. Such sequences provided herein can be used to provide the expression product as well as substantially identical sequences that produce a protein that has the same biological properties. These "biologically equivalent" or "biologically active" or "equivalent" polypeptides are encoded by equivalent polynucleotides as described herein. They may possess at least 60%, or alternatively, at least 65%, or alternatively, at least 70%, or alternatively, at least 75%, or alternatively, at least 80%, or alternatively at least 85%, or alternatively at least 90%, or alternatively at least 95% or alternatively at least 98%, identical primary amino acid sequence to the reference polypeptide when compared using sequence identity methods run under default conditions. Specific polynucleotide or polypeptide sequences are provided as examples of particular embodiments. Modifications may be made to the amino acid sequences by using alternate amino acids that have similar charge. Additionally, an equivalent polynucleotide is one that hybridizes under stringent conditions to the reference polynucleotide or its complement or in reference to a polypeptide, a polypeptide encoded by a polynucleotide that hybridizes to the reference encoding polynucleotide under stringent conditions or its complementary strand. Alternatively, an equivalent polypeptide or protein is one that is expressed from an equivalent polynucleotide.

[0047] "Hybridization" refers to a reaction in which one or more polynucleotides react to form a complex that is stabilized via hydrogen bonding between the bases of the nucleotide residues. The hydrogen bonding may occur by Watson-Crick base pairing, Hoogstein binding, or in any other sequence-specific manner. The complex may comprise two strands forming a duplex structure, three or more strands forming a multi-stranded complex, a single self-hybridizing strand, or any combination of these. A hybridization reaction may constitute a step in a more extensive process, such as the initiation of a PC reaction, or the enzymatic cleavage of a polynucleotide by a ribozyme.

[0048] Examples of stringent hybridization conditions include: incubation temperatures of about 25°C to about 37°C; hybridization buffer concentrations of about 6x SSC to about 10x SSC; formamide concentrations of about 0% to about 25%; and wash solutions from about 4x SSC to about 8x SSC. Examples of moderate hybridization conditions include: incubation temperatures of about 40°C to about 50°C; buffer concentrations of about 9x SSC to about 2x SSC; formamide concentrations of about 30% to about 50%; and wash solutions of about 5x SSC to about 2x SSC. Examples of high stringency conditions include: incubation temperatures of about 55°C to about 68°C; buffer concentrations of about lx SSC to about 0.1x SSC; formamide concentrations of about 55% to about 75%; and wash solutions of about lx SSC, 0.1x SSC, or deionized water. In general, hybridization incubation times are from 5 minutes to 24 hours, with 1, 2, or more washing steps, and wash incubation times are about 1, 2, or 15 minutes. SSC is 0.15 M NaCl and 15 mM citrate buffer. It is understood that equivalents of SSC using other buffer systems can be employed.

[0049] As used herein, "treating" or "treatment" of a retinal degenerative disease in a subject refers to (1) preventing the symptoms or disease from occurring in a subject that is predisposed or does not yet display symptoms of the disease; (2) inhibiting the disease or arresting its development; or (3) ameliorating or causing regression of the disease or the symptoms of the disease. As understood in the art, "treatment" is an approach for obtaining beneficial or desired results, including clinical results. For the purposes of the compositions, combination therapy, nucleic acid molecules, and methods disclosed herein for inducing neurogenesis from MG and/or generating functional neurons from MG, beneficial or desired results can include one or more, but are not limited to, alleviation or amelioration of one or more symptoms of retinal degeneration, diminishment of extent of a retinal degenerative condition (including a retinal degenerative disease), stabilized (*i.e.*, not worsening) state of a retinal degenerative condition (including disease), delay or slowing of a retinal degenerative condition (including disease), progression, amelioration or palliation of a retinal degenerative condition (including disease), states of and remission of (whether partial or total) retinal degeneration, whether detectable or undetectable.

[0050] As used herein, the term "isolated" means that a naturally occurring DNA fragment, DNA molecule, coding sequence, or oligonucleotide is removed from its natural environment, or is a synthetic molecule or cloned product. Preferably, the DNA fragment, DNA molecule, coding sequence, or oligonucleotide is purified, i.e., essentially free from any other DNA fragment, DNA molecule, coding sequence, or oligonucleotide and associated cellular products or other impurities.

[0051] The term "cell" as used herein refers to either a prokaryotic or eukaryotic cell, optionally obtained from a subject or a commercially available source. Cells treated, transfected, transformed, or otherwise in contact with compositions and/or nucleic acid molecules disclosed herein, include without limitation, cells of a human, non-human animal, mammal, or non-human mammal, including without limitation, cells of murine, canine, or non-human primate species. Cells treated, transfected, transformed, or otherwise in contact with compositions and/or nucleic acid molecules disclosed herein are, without limitation, retinal cells, Müller glia (MG), and/or retinal neuronal cells such as retinal neurons, bipolar neurons, amacrine cells, horizontal cells, ganglion cells and/or glia. The term "Müller glial" cells "or "Müller glia" or "MG" refer to cells which are found in the vertebrate retina and are support cells for neurons. MG are the most common type of glial cells in the retina. While MG cell bodies are located in the inner nuclear layer of the retina, MG span across the entire retina.

[0052] As used herein, the term "subject" includes any human or non-human animal. The term "non-human animal" includes all vertebrates, e.g., mammals and non-mammals, such as non-human primates, horses, sheep, dogs, cows, pigs, chickens, and other veterinary subjects.

[0053] As used herein, "a" or "an" means at least one, unless clearly indicated otherwise.

[0054] As used herein, to "prevent" or "protect against" a condition or disease means to hinder, reduce or delay the onset or progression of the condition or disease.

[0055] The term "encode" as it is applied to nucleic acid sequences refers to a polynucleotide which is said to "encode" a polypeptide, an mRNA, or an effector RNA if, in its native state or when manipulated by methods well known to those skilled in the art, can be transcribed and/or translated to produce the effector RNA, the mRNA, or an mRNA that can for the polypeptide and/or a fragment thereof. The antisense strand is the complement of such a nucleic acid, and the encoding sequence can be deduced therefrom.

[0056] As used herein, the term "expression" or "gene expression" refers to the process by which polynucleotides are transcribed into mRNA and/or the process by which the transcribed mRNA is subsequently translated into peptides, polypeptides, or proteins. If the

polynucleotide is derived from genomic DNA, expression may include splicing of the mRNA in a eukaryotic cell. The expression level of a gene may be determined by measuring the amount of mRNA or protein in a cell or tissue sample; further, the expression level of multiple genes can be determined to establish an expression profile for a particular sample.

[0057] As used herein, the term "functional" may be used to modify any molecule, biological, or cellular material to intend that it accomplishes a particular, specified effect.

[0058] As used herein, the term "combined therapy" refers to two or more compositions and/or nucleic acid molecules, delivered in combination, for example and without limitation, sequentially, concurrently, simultaneously, and/or step-wise, in order to achieve a therapeutic effect.

[0059] As used in the description of the invention and the appended claims, the singular forms "a," "an" and "the" are intended to include the plural forms as well, unless the context clearly indicates otherwise.

[0060] The term "about," as used herein when referring to a measurable value such as an amount, level or concentration, for example and without limitation, is meant to encompass variations of 20%, 10%, 5%, 1 %, 0.5%, or even 0.1 % of the specified amount, or fold differences in levels of a quantifiable comparison with a standard or control or reference material, such as 1-fold, 2-fold, 3-fold, 4-fold…10-fold, 100-fold, etc. of the specified level of comparison.

[0061] In some embodiments, enhancing expression levels of the two or more proneural bHLH transcription factors, endogenous and/or exogenous, refers to an increase in the amount of expressed as compared to a control sample or explant levels of endogenous and/or exogenous Ascl1, and/or Atoh1, and/or Atoh7 such as, without limitation, untreated, or Ascl1 expression alone. In some embodiments, neurogenesis is increased and/or the production of functional neurons is increased as compared to a control. In some embodiments, expression levels and/or functional neurons are increased about 1.1 fold, about 1.2 fold, about 1.3 fold, about 1.4 fold, about 1.5 fold, about 1.6 fold, about 1.7 fold, about 1.8 fold, about 1.9 fold, about 2 fold, about 2.5 fold, about 3 fold, about 4 fold, about 5 fold, about 6 fold, about 7 fold, about 8 fold, about 9 fold, about 10 fold, about 20 fold, about 50 fold, about 100 fold, about 1000 fold, or about 10,000 fold relative to the control.

[0062] In some embodiments, the terms "reprogramming potentiator" or "reprogramming potentiating agent", used herein interchangeably, refers to a small molecule, polypeptide, protein, polynucleotide, nucleic acid, oligonucleotide, antisense, miRNA, or an equivalent or a biologically equivalent thereof which assists in the process of stimulating and/or boosting neurogenesis from MG in a manner such that functional neurons from the MG are produced.

In one embodiment, one or more reprogramming potentiators assist in the process of stimulating neurogenesis and producing functional neurons from MG by inhibiting the HDAC pathway. In another embodiment, one or more reprogramming potentiators assist in the process of stimulating neurogenesis and producing functional neurons from MG by inhibiting the Jak/STAT pathway. In another embodiment, one or more reprogramming potentiators assist in the process of stimulating neurogenesis and producing functional neurons from MG by inhibiting the HDAC + Jak/STAT pathways. In another embodiment, one or more reprogramming potentiators assist in the process of stimulating neurogenesis and producing functional neurons from MG by enhancing and/or increasing endogenous and/or exogenous Ascl1 expression levels. See also our previous work in WO2019/210320, incorporated herein by reference in its entirety.

[0063] The terms "acceptable," "effective," or "sufficient" when used to describe the selection of any components, ranges, dose forms, etc. disclosed herein intend that said component, range, dose form, etc. is suitable for the disclosed purpose.

[0064] The term "adeno-associated virus" or "AAV" as used herein refers to a member of the class of viruses associated with this name and belonging to the genus dependoparvovirus, family Parvoviridae. Multiple serotypes of this virus are known to be suitable for gene delivery; all known serotypes can infect cells from various tissue types. At least 11 or 12, sequentially numbered, are disclosed in the prior art. Non-limiting exemplary serotypes useful in the methods disclosed herein include any of the 11 or 12 serotypes, e.g., AAV2, AAV5, and AAV8, or variant serotypes, e.g. AAV-DJ. The AAV structural particle is composed of 60 protein molecules made up of VP1, VP2, and VP3. Each particle contains approximately 5 VP1 proteins, 5 VP2 proteins and 50 VP3 proteins ordered into an icosahedral structure.

Compositions/ Nucleic acid Molecules

[0065] Provided are compositions and/or nucleic acid molecules for retinal regeneration, the potentiation of retinal regeneration, restoration of vision, and for treatment of retinal degenerative disease, damage, or injury.

[0066] Disclosed herein is a nucleic acid molecule comprising a nucleic acid sequence encoding two or more proneural bHLH transcription factors. In one embodiment, the nucleic acid molecule comprises a nucleic acid sequence encoding Ascl1 and Atoh1. In another embodiment, the nucleic acid molecule comprises a nucleic acid sequence encoding Ascl1 and Atoh7. In another embodiment, the nucleic acid molecule comprises a nucleic acid sequence encoding Atoh1 and Atoh7. In another embodiment, the nucleic acid molecule comprises a nucleic acid sequence encoding Ascl1, Atoh1 and Atoh7. In another

embodiment, the nucleic acid molecule comprises a nucleic acid sequence encoding two or more proneural bHLH transcription factors selected from the group consisting of Ascl1 + Atoh1, Ascl1 + Atoh7, Atoh1 + Atoh7, and Ascl1 + Atoh1 + Atoh7. In another embodiment, the nucleic acid molecule comprises a nucleic acid sequence encoding three or more proneural bHLH transcription factors (e.g., Ascl1 + Atoh1 + Atoh7). In some embodiments, the nucleic acid molecule comprises one, two, or three of the proneural bHLH transcription factors selected from Ascl1, Atoh1, and Atoh7, in combination with another proneural bHLH transcription factor. In some embodiments, the nucleic acid molecule comprises a nucleic acid sequence encoding two, three, four, or all five of Ascl1, Atonal7, Atoh1, Neurogenin-2 (also known as NEUROG2 and NGN-2; Accession No. NM_024019; SEQ ID NO: 50), and Neurod1 (Accession No. KR709666; SEQ ID NO: 51).

[0067] In one embodiment disclosed herein is a nucleic acid molecule comprising a nucleic acid sequence encoding the two or more proneural bHLH transcription factors in operable linkage with a MG-specific promoter. In another embodiment, a nucleic acid molecule comprising a nucleic acid sequence encoding the two or more proneural bHLH transcription factors in operable linkage with a human Rlbp1 promoter. Rlbp1 (Retinaldehyde binding protein 1) is a robust MG tissue specific promoter capable of driving expression of the two or more proneural bHLH transcription factors to MG cells at a level sufficient to induce neurogenesis from MG. In one embodiment, a portion of the Rlbp1 promoter is used for Ascl1 + Atoh1 expression in MG. In another embodiment, the portion of the Rlbp1 promoter in operable linkage with the nucleic acid sequence encoding Ascl1 and Atoh1 is the Rlbp1 sequence found in SEQ ID NO: 7.

[0068] In one embodiment, the nucleic acid molecule for use in inducing or stimulating retinal neurogenesis from MG comprises a nucleic acid sequence encoding two or more proneural transcription factors. In another embodiment, the nucleic acid sequence encoding Ascl1 + Atoh1 comprises a native Ascl1 promoter sequence. In another embodiment, the nucleic acid sequence encoding Ascl1 + Atoh1 comprises a non-native Ascl1 promoter such as a retinal specific promoter. In another embodiment, the promoter is a MG-specific promoter such as, for example and without limitation, a retinaldehyde binding protein 1 (Rlbp1) promoter or portion thereof, glial fibrillary acidic protein (GFAP) promoter, vimentin (VIM) promoter, Hes1 promoter, or CD44 promoter. In another embodiment, the promoter is a ubiquitous promoter such as, for example and without limitation, a CMV promoter, CAG promoter or miniCMV promoter.

[0069] Such nucleic acid molecules may be delivered by viral or non-viral means. One example of viral delivery is adeno-associated virus (AAV). Other examples include retrovirus, lentivirus, and baculovirus delivery. One example of a non-viral method of miR delivery is

cell penetrating peptide (CPP). Polynucleotide constructs may also be modified, such as through chemical modification, to improve their stability and/or suitability for delivery. In some embodiments, the oligonucleotide is modified by locked nucleic acids and/or phosphorothioate linkages. In some embodiments, a delivery system is selected for improved bioavailability, such as PEGylated liposomes, lipidoids, or biodegradable polymers, as examples.

Nucleic Acid Molecules and Combined Therapy Compositions

[0070] In some embodiments, the composition further comprises one or more additional potentiating or therapeutic agents, including, for example, reprogramming potentiating agents. In some embodiments, the composition is free of reprogramming potentiating agents. Optionally, a composition comprising one or more small molecule reprogramming potentiating agents can be administered sequentially or concurrently with the nucleic acid molecules disclosed herein. In another embodiment, one or more protein/peptide or miR-based reprogramming potentiators can be incorporated into the nucleic acid molecules disclosed herein. Such one or more reprogramming potentiators are selected from HDACi, STATi, Jak/STATi and RNAi-based Ascl activators. See also our previous work in WO2019/210320, incorporated herein by reference in its entirety.

[0071] In one embodiment, the HDAC signaling pathway inhibitor is selected from the group consisting of peptidomimetics, small molecule inhibitors, oligonucleotides, peptides and proteins. Representative examples of small molecule HDACi include, but are not limited to, trichostatin A (TSA), Istodax™ also known as (Pro)/romidepsin, Beleodaq™, also known as (Pro)/belinostat, Farydak™, also known as (Pro)/panobinostat, and Zolinza™, also known as (Pro)/vorinostat, Quisinostat, Abexinostat, Givinostat, Resminostat, Phenylbutyrate, Valproic Acid, Depsipeptide, Entinostat, Mocetinostat, and Tubastatin A. Exemplary HDACi peptides are, without limitation, 16cyc-HxA, 16lin-HxA and 16KA (SEQ ID NO: 8-10).

[0072] In one embodiment, the STAT signaling pathway inhibitor or Jak/STATi is selected from the group consisting of natural compounds, peptidomimetics, peptides, proteins, small molecules and oligonucleotides. Examples of endogenous STAT pathway inhibitors, include, but are not limited to, suppressor of cytokine signaling (SOCS) proteins, phosphatases, and protein inhibitor of activated STAT (PIAS) proteins. Such endogenous inhibitors provide a basis for therapeutic molecules and compounds for STAT inhibition. In one embodiment, protein or peptide inhibitors of STAT include, for example and without limitation, Socs1 (SEQ ID NO: 11), Socs2 (SEQ ID NO: 13), Socs3 (SEQ ID NO: 15), Socs4 (SEQ ID NO: 17), Socs5 (SEQ ID NO: 19), Socs6 (SEQ ID NO: 21), Socs7 (SEQ ID NO: 23), CIS, and/or XpYL. In one embodiment, a peptidomimetic inhibitor of STAT is ISS610. In another

embodiment, small molecule inhibitors of STAT and/or Jak/STAT include, without limitation, STA-21, LLL3, S31-201, Stattic, OPB-31121, OPB-51602, SH-4-54, Tofactinib, Ruxolitinib, Baricitinib, Oclacitinib, AZD1480, and Dasatinib. In one embodiment, the STAT signaling pathway inhibitor is an inhibitor of STAT3. An exemplary STAT3 inhibitor includes, but is not limited to, SH-4-54. See also Fagard et al. JAKSTAT. 2013 Jan 1; 2(1): e22882. In another embodiment, STAT and/or Jak/STAT inhibitors include natural compounds such as Butein and Capsaicin.

[0073] In some embodiments, the inhibitor, mimic, activator, or antagomir is an oligonucleotide or a nucleotide sequence. The invention thus provides nucleotide constructs for use in the compositions or combined therapy or nucleic acid molecules and methods described herein.

[0074] The reprogramming potentiating agents, in some embodiments, are selected from one or more STAT signaling pathway inhibitors; and one or more Ascl activators such as, without limitation, miR-25 and/or miR-124; and one or more let-7 family inhibitors. Exemplary sequences for such agents include, without limitation, SEQ ID NO: 25-32.

[0075] Optionally, provided herein is a composition comprising any one or more of the combined therapy of RNAi-based Ascl1 activators and/or HDACi + STATi, and/or a nucleic acid sequence encoding the two or more proneural bHLH transcription factors or a vector comprising the nucleic acid sequences disclosed herein, and a carrier. In some embodiments, the carrier is a pharmaceutically acceptable carrier.

[0076] Viral Vectors

[0077] In some embodiments, the vector disclosed herein is a viral vector. In some embodiments, the vector is an adenoviral vector, an adeno-associated viral (AAV) vector, or a lentiviral vector. In some embodiments, the vector is a retroviral vector, an adenoviral/retroviral chimera vector, a herpes simplex viral I or II vector, a parvoviral vector, a reticuloendotheliosis viral vector, a polioviral vector, a papillomaviral vector, a vaccinia viral vector, or any hybrid or chimeric vector incorporating favorable aspects of two or more viral vectors. In some embodiments, the vector further comprises one or more expression control elements operably linked to the polynucleotide. In some embodiments, the vector further comprises one or more selectable markers.

[0078] In some embodiments, the vector disclosed herein is an AAV vector with low toxicity. In some embodiments, the AAV vector does not incorporate into the host genome, thereby having a low probability of causing insertional mutagenesis. In some embodiments, the AAV vector can encode a range of total polynucleotides from 4.5 kb to 4.75 kb. In some embodiments, exemplary AAV vectors that may be used in any of the herein described

compositions, systems, methods, and kits can include an AAV1 vector, a modified AAV1 vector, an AAV2 vector, a modified AAV2 vector, an AAV3 vector, a modified AAV3 vector, an AAV4 vector, a modified AAV4 vector, an AAV5 vector, a modified AAV5 vector, an AAV6 vector, a modified AAV6 vector, an AAV7 vector, a modified AAV7 vector, an AAV8 vector, an AAV9 vector, an AAV.rh10 vector, a modified AAV.rh10 vector, an AAV.rh32/33 vector, a modified AAV.rh32/33 vector, an AAV.rh43 vector, a modified AAV.rh43 vector, an AAV.rh64R1 vector, and a modified AAV.rh64R1 vector and any combinations or equivalents thereof.

[0079] In some embodiments, the vector disclosed herein is a lentiviral vector. In one embodiment, the lentiviral vector is an integrase-competent lentiviral vector (ICLV). In some embodiments, the lentiviral vector can refer to the transgene plasmid vector as well as the transgene plasmid vector in conjunction with related plasmids (e.g., a packaging plasmid, a rev expressing plasmid, an envelope plasmid) as well as a lentiviral-based particle capable of introducing exogenous nucleic acid into a cell through a viral or viral-like entry mechanism. Lentiviral vectors are well-known in the art. In some embodiments, exemplary lentiviral vectors that may be used in relation to any of the herein described compositions, nucleic acid molecules and/or methods, and can include a human immunodeficiency virus (HIV) 1 vector, a modified human immunodeficiency virus (HIV) 1 vector, a human immunodeficiency virus (HIV) 2 vector, a modified human immunodeficiency virus (HIV) 2 vector, a sooty mangabey simian immunodeficiency virus (SIV_{SM}) vector, a modified sooty mangabey simian immunodeficiency virus (SIV_{SM}) vector, a African green monkey simian immunodeficiency virus (SIV_{AGM}) vector, a modified African green monkey simian immunodeficiency virus (SIV_{AGM}) vector, a equine infectious anemia virus (EIAV) vector, a modified equine infectious anemia virus (EIAV) vector, a feline immunodeficiency virus (FIV) vector, a modified feline immunodeficiency virus (FIV) vector, a Visna/maedi virus (VNV/VMV) vector, a modified Visna/maedi virus (VNV/VMV) vector, a caprine arthritis-encephalitis virus (CAEV) vector, a modified caprine arthritis-encephalitis virus (CAEV) vector, a bovine immunodeficiency virus (BIV), or a modified bovine immunodeficiency virus (BIV).

[0080] In some embodiments of the compositions and/or nucleic acid molecules and/or methods of the disclosure, a vector of the disclosure is a viral vector. In some embodiments, the viral vector comprises a sequence isolated or derived from a retrovirus. In some embodiments, the viral vector comprises a sequence isolated or derived from a lentivirus. In some embodiments, the viral vector comprises a sequence isolated or derived from an adenovirus. In some embodiments, the viral vector comprises a sequence isolated or derived from an adeno-associated virus (AAV). In some embodiments, the viral vector is

replication incompetent. In some embodiments, the viral vector is isolated or recombinant. In some embodiments, the viral vector is self-complementary.

[0081] In some embodiments of the compositions and/or nucleic acid molecules and/or methods of the disclosure, the viral vector comprises a sequence isolated or derived from an adeno-associated virus (AAV). In some embodiments, the viral vector comprises an inverted terminal repeat sequence or a capsid sequence that is isolated or derived from an AAV of serotype AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, or AAV12, or the vector and/or components are derived from a synthetic AAV serotype, such as, without limitation, Anc80 AAV (an ancestor of AAV 1, 2, 6, 8 and 9). In some embodiments, the viral vector is replication incompetent. In some embodiments, the viral vector is isolated or recombinant (rAAV). In some embodiments, the viral vector is self-complementary (scAAV).

[0082] In some embodiments of the compositions and methods of the disclosure, a vector of the disclosure is a non-viral vector. In some embodiments, the vector comprises or consists of a nanoparticle, a micelle, a liposome or lipoplex, a polymersome, a polyplex or a dendrimer.

[0083] In some embodiments, expression vector or viral vector disclosed herein is used to transfect, transform, or come in contact with a cell which is a eukaryotic cell. In some embodiments, the cell is an animal cell. In some embodiments, the cells is a zebrafish cell. In some embodiments, the cell is a mammalian cell. In some embodiments, the cell is a bovine, murine, feline, equine, porcine, canine, simian, or human cell. In particular embodiments, the cell is a retinal neuron or MG of an animal or mammal.

[0084] In some embodiments, a cell is a packaging cell or a producer cell for production of a viral particle.

[0085] In some embodiments, provided herein are viral particles comprising, consisting of, or consisting essentially of a vector comprising, consisting of, or consisting essentially of a polynucleotide sequence encoding an Ascl1 protein.

[0086] In general, methods of packaging genetic material such as RNA or DNA into one or more vectors is well known in the art. For example, the genetic material may be packaged using a packaging vector and cell lines and introduced via traditional recombinant methods.

[0087] In some embodiments, the packaging vector may include, but is not limited to retroviral vector, lentiviral vector, adenoviral vector, and adeno-associated viral vector. The packaging vector contains elements and sequences that facilitate the delivery of genetic materials into cells. For example, the retroviral constructs are packaging plasmids

comprising at least one retroviral helper DNA sequence derived from a replication-incompetent retroviral genome encoding *in trans* all virion proteins required to package a replication incompetent retroviral vector, and for producing virion proteins capable of packaging the replication-incompetent retroviral vector at high titer, without the production of replication-competent helper virus. The retroviral DNA sequence lacks the region encoding the native enhancer and/or promoter of the viral 5' LTR of the virus, and lacks both the psi function sequence responsible for packaging helper genome and the 3' LTR, but encodes a foreign polyadenylation site, for example the SV40 polyadenylation site, and a foreign enhancer and/or promoter which directs efficient transcription in a cell type where virus production is desired. The retrovirus is a leukemia virus such as a Moloney Murine Leukemia Virus (MMLV), the Human Immunodeficiency Virus (HIV), or the Gibbon Ape Leukemia virus (GALV). The foreign enhancer and promoter may be the human cytomegalovirus (HCMV) immediate early (IE) enhancer and promoter, the enhancer and promoter (U3 region) of the Moloney Murine Sarcoma Virus (MMSV), the U3 region of Rous Sarcoma Virus (RSV), the U3 region of Spleen Focus Forming Virus (SFFV), or the HCMV IE enhancer joined to the native Moloney Murine Leukemia Virus (MMLV) promoter.

[0088] The retroviral packaging plasmid may consist of two retroviral helper DNA sequences encoded by plasmid-based expression vectors, for example where a first helper sequence contains a cDNA encoding the gag and pol proteins of ecotropic MMLV or GALV and a second helper sequence contains a cDNA encoding the env protein. The Env gene, which determines the host range, may be derived from the genes encoding xenotropic, amphotropic, ecotropic, polytropic (mink focus forming) or 10A1 murine leukemia virus env proteins, or the Gibbon Ape Leukemia Virus (GALV env protein, the Human Immunodeficiency Virus env (gp160) protein, the Vesicular Stomatitus Virus (VSV) G protein, the Human T cell leukemia (HTLV) type I and II env gene products, chimeric envelope gene derived from combinations of one or more of the above env genes or chimeric envelope genes encoding the cytoplasmic and transmembrane of the above env gene products and a monoclonal antibody directed against a specific surface molecule on a desired target cell. Similar vector-based systems may employ other vectors such as sleeping beauty vectors or transposon elements.

[0089] The resulting packaged expression systems may then be introduced via an appropriate route of administration, discussed in detail with respect to the method aspects disclosed herein.

[0090] Pharmaceutical compositions

[0091] Pharmaceutical compositions disclosed herein include one or more pharmaceutically or physiologically acceptable carriers, diluents or excipients. Such compositions may comprise buffers such as neutral buffered saline, phosphate buffered saline and the like; carbohydrates such as glucose, mannose, sucrose or dextrans, mannitol; proteins; polypeptides or amino acids such as glycine; antioxidants; chelating agents such as EDTA or glutathione; adjuvants (e.g., aluminum hydroxide); and preservatives. Compositions of the disclosure may be formulated for intraocular administration.

[0092] Cells

[0093] In some embodiments of the compositions and/or nucleic acid molecules and/or methods of the disclosure, a cell of the disclosure is a retinal cell, such as a Müller glial (MG) cell, or a rod or cone photoreceptor cell. In some embodiments, the cell is a neuronal cell. In some embodiments, a neuronal cell of the disclosure is a neuron of the retina. In some embodiments, a neuron cell of the disclosure is a neuron of an optic nerve. In some embodiments, a neuron cell of the disclosure is a neuroglial or a glial cell. In some embodiments, a cell is a bipolar neuron, a horizontal cell, a ganglion cell, or an amacrine cell. In some embodiments, a cell of the disclosure is an astrocyte. In some embodiments, cells of the disclosure are macroglia or microglia or glia.

[0094] In some embodiments of the compositions and methods of the disclosure, a cell of the disclosure is a cultured cell.

[0095] In some embodiments of the disclosure, a cell is in vivo, in vitro, ex vivo, or in situ. In some embodiments, the cells are modified ex vivo and transplanted into and/or administered to the retina of a subject in need thereof.

[0096] In some embodiments, a cell of the disclosure is autologous or allogeneic and used for transplantation.

[0097] In some embodiments, a cell of the disclosure is a stem cell-derived or an embryonic stem cell-derived retinal cell. In some embodiments, the cell is derived from an induced pluripotent stem cell (iPS cell)-dervied retinal cell.

Methods

[0098] Described herein are methods for inducing retinal regeneration in a subject. Also provided are methods for enhancing retinal regeneration, improving retinal neurogenesis, potentiating retinal regeneration, restoring vision, and treating retinal degenerative disease, damage, injury, or blindness.

[0099] In one embodiment is a method for inducing retinal regeneration in a subject comprising: a) administering to a retina of the subject the nucleic acid molecules and/or compositions disclosed herein. In one embodiment, a method for inducing retinal regeneration in a subject comprises: a) administering to a retina of the subject a nucleic acid molecule comprising a nucleic acid sequence encoding two or more proneural bHLH transcription factors, wherein expression of the proneural bHLH transcription factors stimulates regeneration of retinal interneurons from retinal Müller glia (MG) and reprograms the MG into bipolar, amacrine, horizontal, and/or ganglion cells. In particular, in one embodiment of the method disclosed herein, the nucleic acid molecule comprises a nucleic acid sequence encoding Ascl1 and Atoh1. In another embodiment of the method disclosed herein, the nucleic acid molecule comprises a nucleic acid sequence encoding Ascl1 and Atoh7. In another embodiment of the method disclosed herein, the nucleic acid molecule comprises a nucleic acid sequence encoding Atoh1 and Atoh7. In another embodiment of the method disclosed herein, the nucleic acid molecule comprises a nucleic acid sequence encoding Ascl1, Atoh1 and Atoh7. In another embodiment of the method disclosed herein, the nucleic acid molecule comprises a nucleic acid sequence encoding two or more proneural bHLH transcription factors selected from the group consisting of Ascl1 + Atoh1, Ascl1 + Atoh7, Atoh1 + Atoh7, and Ascl1 + Atoh1 + Atoh7.

[0100] In one embodiment of the method disclosed herein, the nucleic acid molecule comprising a nucleic acid sequence encoding the two or more proneural bHLH transcription factors is in operable linkage with a MG-specific promoter. In another particular embodiment of the method disclosed herein, a nucleic acid molecule comprising a nucleic acid sequence encoding the two or more proneural bHLH transcription factors (e.g., Ascl1, Atoh1 and Atoh7) is in operable linkage with a human Rlbp1 promoter. In another embodiment of the method disclosed herein, a nucleic acid molecule comprising a nucleic acid sequence encoding Ascl1 and Atoh1 is in operable linkage with a human Rlbp1 promoter. In another embodiment of the method disclosed herein, a nucleic acid molecule comprising a nucleic acid sequence encoding Ascl1 and Atoh7 is in operable linkage with a human Rlbp1 promoter. In another embodiment of the method disclosed herein, a nucleic acid molecule comprising a nucleic acid sequence encoding Ascl1, Atoh1, and Atoh7 is in operable linkage with a human Rlbp1 promoter. Rlbp1 (Retinaldehyde binding protein 1) is a robust MG tissue specific promoter capable of driving expression of Ascl1 to MG cells at a level sufficient to induce neurogenesis from MG. In one embodiment of the method disclosed herein, a portion of the Rlbp1 promoter is used for expression of the two or more proneural bHLH transcription factors in MG. In another embodiment of the method disclosed herein, the portion of the Rlbp1 promoter in operable linkage with the nucleic acid sequence

encoding the proneural bHLH transcription factors (e.g., Ascl1 and Atoh1) is the Rlbp1 sequence found in SEQ ID NO: 7.

[0101] Also provided herein are methods for inducing retinal regeneration comprising administering to a subject a composition as described herein. In some embodiments, the methods are effective to increase the number of Müller glial-derived neurons, to induce Müller glial (MG) cells to enter the mitotic cell cycle, and/or to generate new retinal neurons, including the generation of new bipolar neurons, horizontal cells, ganglion, and/or amacrine cells. In some embodiments of the method, the number of retinal neurons increases by at least 25% relative to a baseline level or other reference amount representative of an untreated retina. In other embodiments, the number of retinal neurons increases by at least 40%. In some embodiments, the number of retinal neurons increases by 10%, 20%, 50%, 100%, 150%, 200%, or more.

[0102] Optionally, methods disclosed herein may utilize combined therapy compositions comprising one or more, or two or more, small molecule reprogramming potentiating agents. The agents can be administered sequentially or concurrently with the nucleic acid molecules disclosed herein. In another embodiment, one or more protein/peptide or miR-based reprogramming potentiators can be incorporated into the nucleic acid molecules used in the methods disclosed herein. Such one or more reprogramming potentiators are selected from HDACi, STATi, Jak/STATi and RNAi-based Ascl activators. See also our previous work in WO2019/210320, incorporated herein by reference in its entirety. In some embodiments, the method is performed in the absence of such reprogramming potentiators.

[0103] The subject is typically a mammal, such as a human or veterinary subject. In one embodiment, the subject is an adult. The subject, in some embodiments, has a retinal degenerative disease. Examples of such retinal degenerative diseases include, but are not limited to, Age-related Macular Degeneration (AMD), Retinitis Pigmentosa (RP), Diabetic Retinopathy (DR), Central Retinal Artery Occlusion (CRAO), Vitreoretinopathy, and Glaucoma.

[0104] Administration and Dosage

[0105] The compositions and/or nucleic acid molecules disclosed herein are administered in any suitable manner, often with pharmaceutically acceptable carriers. Suitable methods of administering compositions, compounds, molecules, nucleic acids, and vectors in the context of the present invention to a subject's eye or retina are available, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route. For treatment of the retina, intraocular injection, such as, for example and without limitation,

intravitreal injection and subretinal injection are the most common routes of delivery to the retina. In some embodiments, however, periocular, suprachoroidal, systemic, or topical administration is more suitable for efficacy and safety of delivery.

[0106] The dose administered to a patient, in the context of the disclosure herein, should be sufficient to result in a beneficial therapeutic response in the patient over time, or to inhibit disease progression. Thus, the composition is administered to a subject in an amount sufficient to elicit an effective response and/or to alleviate, reduce, cure or at least partially arrest symptoms and/or complications from the retinal disease or injury. An amount adequate to accomplish this is defined as a "therapeutically effective dose."

[0107] Routes, order and/or frequency of administration of the therapeutic compositions disclosed herein, as well as dosage, will vary from individual to individual, and may be readily established using standard techniques. In general, an appropriate dosage and treatment regimen provides the active compound(s) in an amount sufficient to provide therapeutic and/or prophylactic benefit. Such a response can be monitored by establishing an improved clinical outcome in treated patients as compared to non-treated patients.

Example Embodiments

[0108] Embodiment 1: A nucleic acid molecule comprising a nucleic acid sequence encoding two or more proneural bHLH transcription factors.

[0109] Embodiment 2: The nucleic acid molecule of the preceding embodiment, wherein the two or more proneural bHLH transcription factors is selected from the group consisting 1) Ascl1 + Atoh1, 2) Ascl1 + Atoh7, 3) Atoh1 + Atoh7, and 4) Ascl1 + Atoh1 + Atoh7, and 5) a combination thereof.

[0110] Embodiment 3: The nucleic acid molecule of the preceding embodiments, wherein the two or more proneural bHLH transcription factors is Ascl1 and Atoh1.

[0111] Embodiment 4: The nucleic acid molecule of the preceding embodiments, wherein the nucleic acid sequence comprises an IRES or a 2A self-cleaving site.

[0112] Embodiment 5: The nucleic acid molecule of the preceding embodiments, wherein the nucleic acid sequence comprises a promoter sequence.

[0113] Embodiment 6: The nucleic acid molecule of the preceding embodiments, wherein the promoter sequence is a retinal or Müller glial (MG)-specific promoter.

[0114] Embodiment 7: The nucleic acid molecule of the preceding embodiments, wherein the MG-specific promoter is Rlbp1.

[0115] Embodiment 8: A method for inducing retinal regeneration in a subject comprising: administering to a retina of the subject a nucleic acid molecule comprising a nucleic acid sequence encoding two or more proneural bHLH transcription factors, wherein expression of the proneural bHLH transcription factors stimulates regeneration of retinal interneurons from retinal Müller glia (MG) and reprograms the MG into bipolar, amacrine, horizontal, and/or ganglion cells.

[0116] Embodiment 9: The method of the preceding embodiment, wherein the two or more proneural bHLH transcription factors is selected from the group consisting of 1) Ascl1 + Atoh1, 2) Ascl1 + Atoh7, 3) Atoh1 + Atoh7, and 4) Ascl1 + Atoh1 + Atoh7, and 5) a combination thereof.

[0117] Embodiment 10: The nucleic acid molecule or method of any of the preceding embodiments, wherein the two or more proneural bHLH transcription factors is selected from the group consisting of Ascl1, Atonal7, Atoh1, Neurogenin-2, Neurod1, and combinations thereof.

[0118] Embodiment 11: The methods of any of the preceding embodiments, wherein the number of the MG-derived functional neurons is increased.

[0119] Embodiment 11: The methods of the preceding embodiments, wherein the subject is treated for retinal disease, damage or degeneration in the retina.

[0120] Embodiment 12: The methods of the preceding embodiments, wherein the subject is an adult.

[0121] Embodiment 13: The methods of the preceding embodiments, wherein a vector comprises the nucleic acid molecule.

[0122] Embodiment 14: The methods of the preceding embodiments, wherein the vector is a non-viral vector or a viral vector.

[0123] Embodiment 15: The methods of the preceding embodiments, wherein the vector is a viral vector.

[0124] Embodiment 16: The methods of the preceding embodiments, wherein the viral vector is an adeno-associated viral (AAV) vector or a lentiviral vector.

[0125] Embodiment 17: The methods of the preceding embodiments, wherein the nucleic acid molecule further comprises a promoter sequence in operable linkage with the nucleic acid sequence encoding the two or more proneural bHLH transcription factors.

[0126] Embodiment 18: The methods of the preceding embodiments, wherein the promoter is a retinal or MG-specific promoter.

[0127] Embodiment 19: The methods of the preceding embodiments, wherein the MG-specific promoter is Rlbp1.

[0128] Embodiment 20: The methods of the preceding embodiments, wherein administering to the retina is intravitreal or subretinal injection.

[0129] Embodiment 21: The methods of the preceding embodiments, wherein the nucleotide sequence comprises an IRES or 2A self-cleaving site.

[0130] Embodiment 22: The nucleic acid molecules or methods of the preceding embodiments, wherein the two or more proneural bHLH transcription factors are expressed as a fusion protein.

[0131] Embodiment 23: The nucleic acid molecules or methods of the preceding embodiments, optionally comprising, or accompanying the combined therapy of administering reprogramming potentiators selected from the group consisting of HDACi, Jak/STATi, and RNAi-based Ascl activators.

EXAMPLES

[0132] The following examples are presented to illustrate the present invention and to assist one of ordinary skill in making and using the same. The examples are not intended in any way to otherwise limit the scope of the invention.

Example 1: Atoh and Ascl1 Overexpression in Adult Mice Induces Reprogramming of Müller Glia

[0133] Adult *Glast*-CreER: Flox-stop-*LNL*-tTA: *tetO*-Atoh1: *tetO*-Ascl1-ires-GFP mice were injected for 5 consecutive days with tamoxifen and retinas were collected 3-6 weeks later for immunohistochemistry. GFP and Atoh1 were stained to determine (1) if there were GFP+ cells present in this paradigm, and (2) if Atoh1 was expressed in MG when co-expressed with Ascl1. Abundant GFP expressing cells were observed with no obvious signs of injury or apoptosis, suggesting that the combination of Ascl1 and Atoh1 are tolerable (Figure 1). Surprisingly, over 80% of the MG had retracted their glial processes and adopted a neuronal morphology. All the MG that maintained their glial morphology failed to express detectable levels of Atoh1 (Figure 1), whereas every GFP+ cell with a neuronal morphology expressed high levels of Atoh1 (Figure 1). These data show that the combination of Atoh1 and Ascl1

expression induces MG reprogramming without the need for retinal damage or a reprogramming potentiator such as an HDAC inhibitor (e.g., TSA).

Example 2: Atoh1 and Ascl1-Overexpression Cells in Adult Mice Stain for a Subset of Amacrine Markers

[0134] To further characterize the MG-derived neurons co-expressing Ascl1/Atoh1, immunohistochemical staining with a panel of neuronal markers was used. Every GFP+ cell with a neuronal morphology stained for amacrine marker HuC/D, and none of the cells stained for bipolar markers Otx2 or Cabp5. This is in stark contrast to Ascl1 alone, wherein generated neurons primarily express Otx2 and Cabp5 and very few HuC/D positive cells are present. A subset of the neurons in the Atoh1/Ascl1 expressing cells additionally stained with amacrine marker Pax6 (Figure 2). Notably, the neurons had reduced expression of the MG marker Sox9, while the GFP+ MG that failed to undergo neurogenesis still had high levels of Sox9 (Figure 2). This is similar to previous findings from ANT and ANTSi-treated retinas, wherein MG-derived neurons downregulate glial genes.

[0135] In addition to Atoh1 and HuC/D being expressed in every GFP+ cell with neuronal morphology, NeuN expression was observed in every neuronal cell. This was a surprising result given that NeuN is normally in every ganglion cell and only a subset of amacrine cells. Because these MG-derived neurons appeared to resemble amacrine/ganglion cells, single-cell RNA-sequencing was performed to determine if the cells express additional ganglion cell genes. Indeed, the sequencing results revealed that ganglion cell genes Gad2, Gap43, Nefl, Nefm, Stmn2, Stmn3, Tfap2b, Tfap2a, and Tubb3 were expressed. Taken together, these results provide the basis for therapeutic paradigms, particularly single-injection gene therapies, for reprogramming and generating new retinal neurons from MG. Moreover, this work underpins the possibility of inducing retinal regeneration to functional neurons from MG without inducing injury and/or without the need for adding a reprogramming potentiator such as an HDACi, a Jak/STATi, and/or RNAi-based Ascl activators.

[0136] REFERENCES:

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[0145] Throughout this application various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to describe more fully the state of the art to which this invention pertains. Also incorporated by reference herein in its entirety is provisional application number 62/840,264, filed April 29, 2019, to which this application claims priority.

[0146] From the foregoing it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

What is claimed is:

1. A nucleic acid molecule comprising a nucleic acid sequence encoding two or more proneural basic helix-loop-helix (bHLH) transcription factors.

2. The nucleic acid molecule of claim 1, wherein the two or more proneural bHLH transcription factors is selected from the group consisting 1) Ascl1 + Atoh1, 2) Ascl1 + Atoh7, 3) Atoh1 + Atoh7, and 4) Ascl1 + Atoh1 + Atoh7, and 5) a combination thereof.

3. The nucleic acid molecule of claim 1, wherein the two or more proneural bHLH transcription factors are Ascl1 and Atoh1.

4. The nucleic acid molecule of claim 1, wherein the nucleic acid sequence comprises an IRES or a 2A self-cleaving site.

5. The nucleic acid molecule of claim 1, wherein the nucleic acid sequence comprises a promoter sequence.

6. The nucleic acid molecule of claim 5, wherein the promoter sequence is a retinal or Müller glial (MG)-specific promoter.

7. The nucleic acid molecule of claim 6, wherein the MG-specific promoter is Rlbp1.

8. A method for inducing retinal regeneration in a subject comprising: administering to a retina of the subject a nucleic acid molecule comprising a nucleic acid sequence encoding two or more proneural bHLH transcription factors, wherein expression of the proneural bHLH transcription factors stimulates regeneration of retinal interneurons from retinal Müller glia (MG) and reprograms the MG into bipolar, amacrine, horizontal, and/or ganglion cells.

9. The method of claim 8, wherein the two or more proneural bHLH transcription factors is selected from the group consisting 1) Ascl1 + Atoh1, 2) Ascl1 + Atoh7, 3) Atoh1 + Atoh7, and 4) Ascl1 + Atoh1 + Atoh7, and 5) a combination thereof.

10. The method of claim 9, wherein the number of the MG-derived functional neurons is increased.

11. The method of claim 8, wherein the subject is treated for retinal disease, damage or degeneration in the retina.

12. The method of claim 8, wherein the subject is an adult.

13. The method of claim 8, wherein a vector comprises the nucleic acid molecule.

14. The method of claim 13, wherein the vector is a non-viral vector or a viral vector.

15. The method of claim 14, wherein the viral vector is an adeno-associated viral (AAV) vector or a lentiviral vector.

16. The method of claim 8, wherein the nucleic acid molecule further comprises a promoter sequence in operable linkage with the nucleic acid sequence encoding the two or more proneural bHLH transcription factors.

17. The method of claim 16, wherein the promoter is a retinal or MG-specific promoter.

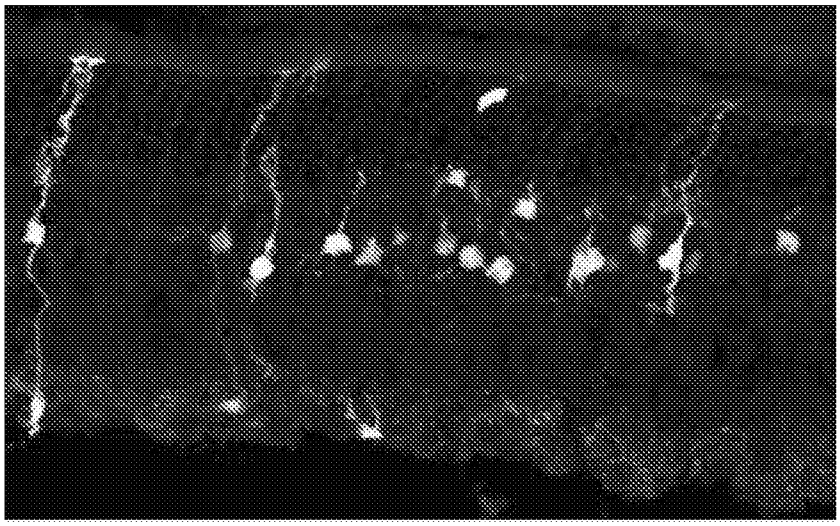
18. The method of claim 17, wherein the MG-specific promoter is Rlbp1.

19. The method of claim 8, wherein the administering to the retina is intravitreal or subretinal injection.

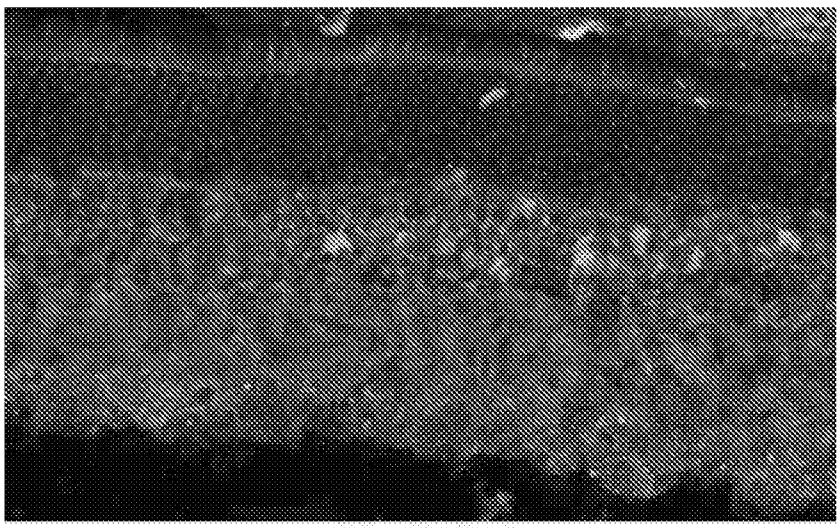
20. The method of claim 8, wherein the nucleic acid sequence comprises an IRES or 2A self-cleaving site.

21. The method of claim 8, wherein the two or more proneural bHLH transcription factors are expressed as a fusion protein.

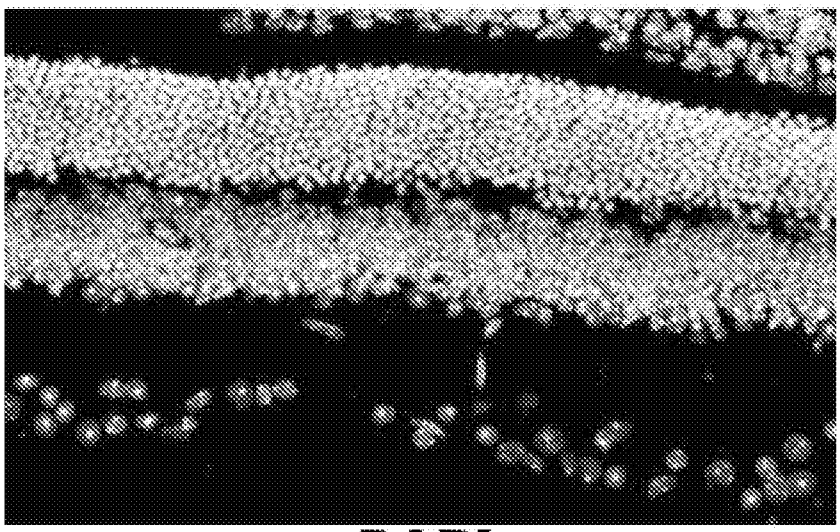
Figure 1



GFP



ATOH1



DAPI

Figure 2