EXPRESSION VECTOR AND METHOD

FIELD OF THE INVENTION

The present disclosure relates to regulatable expression vectors for systematically controlling and silencing expression of a therapeutic molecule and uses thereof, e.g. treating ocular disorders such as choroidal neovascularisation.

BACKGROUND OF THE INVENTION

Gene delivery using viral vectors can result in long-term sustained transgene expression in the eye. However, technological improvements are required in order to move current systems into clinical practice. One issue is the ability to modulate transgene expression. Control should be maintained both temporally and quantitatively to avoid aberrant production of therapeutic. Moreover, certain diseases have a narrow therapeutic window, thus treatments must not only be specific, but timely. Incorporation of cell-specific regulating elements may aid in this process; however, such elements have not been identified in all ocular cell types, and those that have been identified are generally quite large, exceeding the size constraints of viral vectors with limited cargo capacities.

New expression vectors and methods for regulated gene delivery are therefore required.

SUMMARY OF THE INVENTION

Regulating or silencing expression of therapeutic genes in the eye may be necessary if their action leads to unexpected or unwanted effects. Accordingly, the present inventors have developed regulatable expression vectors for systematically controlling and silencing expression of a therapeutic molecule.

In a first example, the present disclosure encompasses an expression vector comprising a kill switch and a regulatable element operably linked to a nucleic acid sequence encoding a therapeutic molecule, wherein activity of the regulatable element is regulated by a regulator compound, and wherein activation of the kill switch silences expression of the nucleic acid encoding the therapeutic molecule from the vector.

In one example, the the kill switch comprises a first site-specific recombination sequence and a second site-specific recombination sequence, wherein recombination between the first site-specific recombination sequence and the second site-specific recombination sequence silences expression of the therapeutic molecule.

In one example, the first site-specific recombination sequence is positioned upstream of the nucleic acid sequence encoding the therapeutic molecule and the second

site-specific recombination sequence is positioned downstream of the nucleic acid sequence encoding the therapeutic molecule.

In one example, the first site-specific recombination sequence and the second site-specific recombination sequence are loxP sites.

In one example, the expression vector further comprises a constitutive promoter operably linked to a nucleic acid sequence encoding a regulator compound-binding polypeptide which is capable of binding a regulator compound, wherein upon binding the regulator compound, the regulator compound-binding polypeptide regulates expression of the therapeutic molecule.

In one example, upon binding of the regulator compound, the regulator compound-binding polypeptide promotes expression of the therapeutic molecule.

In one example, upon binding of the regulator compound, the regulator compound-binding polypeptide represses expression of the therapeutic molecule.

In one example, the regulator compound-binding polypeptide comprises one or more of a reverse tetracycline-controlled transactivator (rtTA), a tetracycline-controlled transactivator (tTA) or a cysteine metabolism repressor (CymR).

In an example, the regulator compound-binding polypeptide comprises an amino acid sequence set forth in SEQ ID NO: 17 or SEQ ID NO: 18.

In one example, the regulator compound is tetracycline, cumate, progesterone, a glucocorticoid, estrogen, or mifepristone.

In one example, the regulatable element comprises one or more of a tetracycline responsive element (TRE), a cumate operator (CuO), an ecdysone response element (EcRE), estrogen response element (ERE), a glucocorticoid response element (GRE), a progesterone response element (PRE), a heat shock sequence element (HSE), or a light inducible promoter.

In one example, the regulatable element comprises a nucleic acid sequence set forth in SEQ ID NO: 4 or SEQ ID NO: 5.

In one example, the constitutive promoter is a composite human CMV-EF1-HTLV promoter.

In one example, the constitutive promoter comprises a nucleic acid sequence set forth in SEQ ID NO: 6.

In one example, the therapeutic molecule inhibits angiogenesis.

In one example, the therapeutic molecule inhibits inflammation.

In one example, the therapeutic molecule is a nucleic acid or polypeptide. In one example, the therapeutic molecule is a polypeptide.

In one example, the therapeutic molecule:

- comprises endostatin, angiostatin, or a fusion of endostatin and angiostatin;
- is a binding protein;
- comprises an antigen binding site of an antibody;
- is selected from the group consisting of ranibizumab, bevacizumab, and aflibercept;
- inhibits inflammation; or,
- is interleukin 10 (IL-10), interleukin 1 receptor antagonist (IL-1RA); or a fusion of IL-10 and IL-1RA.

In one example, the expression vector further comprises a transcription blocker between the constitutive promoter and the regulatable promoter.

In one example, the expression vector comprises

- a first loxP site positioned upstream of the nucleic acid sequence encoding the therapeutic molecule and a second loxP site positioned downstream of the nucleic acid sequence encoding the therapeutic molecule,
- a regulatable promoter comprising a tetracycline responsive element operably linked to the nucleic acid sequence encoding the therapeutic molecule, and
- a constitutive promoter operably linked to a nucleic acid sequence encoding a reverse tetracycline-controlled transactivator (rtTA).

In one example, the expression vector is a viral vector. In one example, the expression vector is an adeno-associated virus (AAV) vector.

The present inventors have also identified that the vectors disclosed herein appear to be particularly effective at regulating inflammatory pathways and/or angiogenesis. These findings indicate that the expression vectors of the present disclosure may be useful for treating ocular disorders. Therefore, in another example, the present disclosure encompasses a method of treating an ocular disorder in a subject, the method comprising administering an effective amount of an expression vector which comprises a kill switch and a nucleic acid encoding a therapeutic molecule, wherein activation of the kill switch silences expression of the therapeutic molecule.

In one example, the expression vector further comprises a regulatable element operably linked to the nucleic acid encoding the therapeutic molecule, wherein the activity of the regulatable element is regulated by administration of a regulator compound to the subject.

In one example, the method described herein further comprises administering a second expression vector which comprises a constitutive promoter operably linked to a nucleic acid sequence encoding a regulator compound-binding polypeptide which is capable of binding a regulator compound, wherein upon binding the regulator compound,

the regulator compound-binding polypeptide regulates expression of the therapeutic molecule from the first expression vector.

In one example, the expression vector further comprises a constitutive promoter operably linked to a nucleic acid sequence encoding a regulator compound-binding polypeptide which binds the regulator compound, wherein upon binding the regulator compound, the regulator compound-binding polypeptide regulates expression of the therapeutic molecule.

In one example, upon binding the regulator compound, the regulator compound-binding polypeptide promotes expression of the therapeutic molecule.

In one example, upon binding the regulator compound, the regulator compound-binding polypeptide represses expression of the therapeutic molecule.

In one example, activation of the kill switch excises:

- one or more promoters; and/or,
- the nucleic acid encoding the therapeutic molecule.

In one example, the kill switch comprises site-specific recombination sequences which flank:

- one or more promoters; and/or
- the nucleic acid encoding a therapeutic molecule.

In one example, the method further comprises administering an expression vector as defined herein.

In one example, the ocular disorder is diabetic retinopathy, cystoid macular oedema, clinically significant macular oedema, uveitis, iritis, giant cell arteritis, vasculitis, pars planitis, corneal transplant rejection, intraocular inflammation or lamellar corneal transplant rejection.

In one example, the ocular disorder is a cancer. In one example, the cancer is uveal melanoma.

In one example, the ocular disorder is macular degeneration, diabetic retinopathy, cystoid macular oedema, clinically significant macular oedema, central retinal vein occlusion, branch retinal vein occlusion or ocular neovascularisation.

In one example, the method described herein comprises intravitreal or subretinal administration of the expression vector.

Current treatment of various ocular disorders disclosed herein requires continual administration of therapeutic via invasive intravitreal injection. Along with being generally unpleasant, intravitreal administration of therapeutic is not without risk of infection or other side effect. One advantage of the vectors disclosed herein is their capacity for non-invasive regulation. For example, while vectors disclosed herein may

be initially administered via intravitreal or subretinal injection, they may be subsequently regulated by topical or oral administration of regulator compound(s). For example, the regulator compound may be administered as an eye drop formulation. Accordingly, in another example, the methods described herein further comprise administering the regulator compound to the eye topically.

In one example, the regulator compound is a small molecule.

In one example, the regulator compound is tetracycline or cumate.

In one example, the regulator compound is administered in eye drops.

In one example, the method described herein further comprises activating the kill switch by administering a site-specific recombinase or a nucleic acid encoding a site-specific recombinase, wherein the site-specific recombinase catalyses the recombination between a first site-specific recombination sequence and the second site-specific recombination sequence, thereby silencing expression of the therapeutic molecule.

In another example, the present disclosure encompasses a method of treating an ocular disorder in a subject, the method comprising administering an effective amount of an expression vector, the expression vector comprising a kill switch, a nucleic acid encoding a therapeutic molecule, a regulatable promoter operably linked to the nucleic acid encoding the therapeutic molecule and a constitutive promoter operably linked to a nucleic acid sequence encoding a regulator compound-binding polypeptide, wherein the activity of the regulatable promoter is regulated by the regulator compound-binding polypeptide following administration of a regulator compound to the subject and wherein, activation of the kill switch silencing expression of the therapeutic molecule.

In another example, the present disclosure encompasses an expression vector defined herein for use in treating an ocular disorder in a subject. In another example, the present disclosure encompasses use of an expression vector defined herein in the manufacture of a medicament for the treatment of an ocular disorder.

In another example, the present disclosure encompasses a pharmaceutical composition comprising a first expression vector as defined herein and a second expression vector which comprises a constitutive promoter operably linked to a nucleic acid sequence encoding a regulator compound-binding polypeptide which is capable of binding a regulator compound, wherein upon binding the regulator compound, the regulator compound-binding polypeptide regulates expression of the therapeutic molecule from the first expression vector.

In another example, the present disclosure encompasses a kit comprising an expression vector(s) defined herein and an eye drop formulation comprising a regulator compound(s) defined herein. In one example, the kit comprises a therapeutic system.

In one example, the kit further comprises a kill switch activator as defined herein.

In one example, the kit further comprises an expression vector comprising a nucleic acid sequence encoding a kill switch activator as defined herein.

In one example, the kit comprises

- an expression vector(s) defined herein,
- an eye drop formulation comprising tetracycline, and
- a Cre recombinase or an expression vector comprising a nucleic acid sequence encoding a Cre recombinase.

Any example herein shall be taken to apply *mutatis mutandis* to any other example unless specifically stated otherwise.

The present disclosure is not to be limited in scope by the specific examples described herein, which are intended for the purpose of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the disclosure, as described herein.

Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or group of compositions of matter.

The disclosure is hereinafter described by way of the following non-limiting Examples and with reference to the accompanying drawings.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS

Figure 1. Map of the backbone of a kill switch vector. It is a bi-cistronic vector encoding a repressor cloning site driven by the composite human CMV-EF1-HTLV promoter and cloning sites for a promoter and gene of interest. The promoter sites are separated by a transcription blocker. The construct is flanked by two AAV2 ITR sequences to package into AAV, and LoxP sites allowing controlled excision and killing of the vector.

Figure 2. Schematic of vector construction. Tetracycline or cumate repressor genes (SEQ ID NO: 2 and SEQ ID NO: 3 respectively) were cloned into the repressor cloning site 1 using XbaI and EcoRI restriction sites. The corresponding promoter (SEQ

ID NO: 4 and SEQ ID NO: 5 respectively) was cloned into promoter cloning site 2 using BstBI and SalI restriction sites. The conditionally regulated gene of interest was cloned into the GOI cloning site 3 using SalI and NheI restriction sites (e.g., GFP, IL-10, Endo/Angiostation fusion protein, IL1RN or IL10/IL1RN genes set forth in SEQ ID NO: 7 to 11) or the compatible XhoI and XbaI sites (Nano-Luciferase, SEQ ID NO: 12).

Figure 3. Schematic of the mechanism of the kill switch. The vector has been constructed with all expression sequences located within 2 LoxP sites (SEQ ID NO: 13). This configuration allows expression to occur. In order to permanently stop or kill expression, a vector encoding Cre is added to the cells. Cre removes all expression sequences by splicing at the two lox sites. As a result, the vector is absent of expression machinery and cannot induce transgene expression.

Figure 4. Experimental design used for testing the kill switch in the vector constructs. A human retinal pigmented epithelial cell line (aRPE-19) was transfected with vector constructs containing Tet or Cu regulatory sequences or CMV, driving the expression of the marker gene luciferase. Twenty-four hours later, the cells were treated with or without Tet or Cu and incubated for a further 48 hours before assessing the expression of luciferase. The ability of the system to kill expression of the transgene (Luciferase) was examined by transfecting a Cre vector into the cells to cut out the expression sequences between LoxP sites. The cells are washed and drugs added back daily until day 7 when media is assayed for changes in expression levels of luciferase.

Figure 5. Experimental results obtained from testing the kill switch. Figure 5A shows the expression of luciferase in cells treated with the Tet vector with (pAAV-LTetL-luc ON) or without (pAAV-LTetL-luc OFF) Tet activation. Activated cells were then treated with Cre and luciferase assayed. Figure 5B shows the expression of luciferase in cells treated with the Cu vector with or without Cu activation. Cells with activated Cu promoters were treated with Cre and luciferase expression assayed. Figure 5C and Figure 5D show the expression of luciferase following transfection with CMV vectors, with or without Cre mediated excision respectively. Significantly less luciferase expression was detected in all Cre excised cells.

Figure 6. Schematic of the controlled expression provided by the vector system. Figure 6A shows the Tet On system. The EF1-HTLV promoter constituently expresses Tet transactivator proteins that are unable to bind to Tet responsive elements (TRE) in the Tet promoter resulting in its inactivation and the lack of transgene expression. In the presence of Tetracycline, the transactivator proteins bind to the Tet Response Elements (TRE) in the promoter, activating it and inducing the expression of the transgene. Figure 6B shows the Cu On system. In this system, the Cu repressor protein binds to the Cu

operator sequence in the absence of Cu, thus inactivating the promoter resulting in no transgene transcription. With the addition of Cu, the Cu binds to the repressor protein, bound to the Cu promoter, thus releasing the repressor allowing transgene transcription.

Figure 7. Schematic of the experimental design used to test the ability of the vector constructs to regulate transgene expression. A human retinal pigmented epithelial cell line (aRPE-19) was transfected with vector constructs containing Tet or Cu regulatory sequences driving the expression of the marker gene luciferase. Twenty-four hours later, the cells were treated with or without Tet or Cu and incubated for a further 48 hours before assessing the expression of luciferase. The ability of the system to stop expression of the transgene (Luciferase) was examined by removing the Tet or Cu from the cells by washing prior to re-examining Luciferase expression. Cells without Tet or Cu were used as a control as were cells where Tet or Cu was not removed.

Figure 8. Regulated gene expression using vector constructs. Figure 8A shows the results from a luminescence assay performed on day 7 of the experiment using tetracycline as the agent to switch on transgene production. Figure 8B shows the results from a luminescence assay performed on day 7 of the experiment using cumate as the agent to switch on transgene production. In both experiments, the substrate (Tet or Cu) was able to induce significant levels of luciferase and expression could be regulated by the removal of the substrate resulting in significantly less luciferase detected.

Figure 9. Schematic of the experimental design used to examine the effects of a vector containing an endo/angiostatin fusion (EAS) flanked by LoxP sites on angiogenesis using a tube formation assay with primary human umbilical vein epithelial cells (HUVEC), with or without Cre. Early passage (p2-4) HUVECs were transfected with the cumate regulated vector producing EAS (pAAV-LCuL-EAS). Two days post transfection, cells were counted and seeded in geltrex coated wells and incubated at 370C, 5%CO2 for 14-18 hours after which the tubes formed in the geltrex were photographed and analysed using ImageJ angiogenesis analyser.

Figure 10. Results obtained from testing the effects of EAS on the formation of tubules in HUVECs. Figure 10A shows HUVEC tube formation in cells transfected with the non-EAS expressing pAAV-LCuL-EAS without Cu activation. Figure 10B shows the inhibition of tube formation when HUVECs were treated with activated pAAV-LCuL-EAS. Figure 10C shows the return of tube formation capacity when the activated pAAV-LCuL-EAS was excised by Cre. Figure 10D and 10E quantifies the total mesh area and total branch length respectively using ImageJ Angiogenesis Analyzer software. The pAAV-LCuL-EAS vector was able to reduce tube formation, which was largely reversed when the vector was excised by Cre.

Figure 11. Schematic of the experimental design used to evaluate a vector containing Cu regulated IL-10, flanked by LoxP sites, on the induction of proinflamatory signalling as determined by IL-6 expression with or without Cre. A human retinal pigmented epithelial cell line (aRPE-19) was transfected with vector constructs containing Cu regulatory sequences driving the expression of anti-inflammatory IL-10. The negative control consisted of the IL10 vector lacking Cu activation, treated with Cre. The IL-10 group consisted of the IL10 vector activated by Cu. The killed group consisted of activated IL10 vector, treated with Cre. Cells were assayed by ELISA for the expression of IL-10 and the pro-inflammatory marker IL-6.

Figure 12 Results obtained from simultaneous ELISAs of IL-10 and IL-6 following transfection with cumate regulated vectors containing IL-10 with or without the addition of Cre. In cells treated with the negative control vector (without Cu and with Cre), the level of IL-10 detected was low, correlating to high levels of the proinflamatory IL-6. Conversely, cells treated with the Cu regulated IL10 vector in the presence of Cu (IL10 vector: on) expressed high levels of IL-10 which correlated to low levels of IL-6 expression. Vectors where the IL-10 was excised by Cre (IL10 vector: killed) had IL-10 and IL-6 levels comparable to that of the negative control showing that the vector expression was successfully killed.

KEY TO SEQUENCE LISTING

SEQ ID NO: 1: Nucleic acid sequence, Kill switch vector backbone.

SEQ ID NO: 2: Nucleic acid sequence, reverse tetracycline-controlled transactivator (rtTA).

SEQ ID NO: 3: Nucleic acid sequence, Cumate Repressor (CymR).

SEQ ID NO: 4: Nucleic acid sequence, Tetracycline Promoter.

SEQ ID NO: 5: Nucleic acid sequence, Cumate Promoter.

SEQ ID NO: 6: Nucleic acid sequence, CMV-EF1-HTLV Promoter.

SEQ ID NO: 7: Nucleic acid sequence, Green fluorescent protein (GFP).

SEQ ID NO: 8: Nucleic acid sequence, Therapeutic polypeptide, Interleukin (IL)10.

SEQ ID NO: 9: Nucleic acid sequence, Therapeutic polypeptide, endo/angiostatin.

SEQ ID NO: 10: Nucleic acid sequence, Therapeutic polypeptide, Interleukin-1 Receptor Antagonist (IL-1RA).

SEQ ID NO: 11: Nucleic acid sequence, Therapeutic polypeptide, IL-10-IL-1RA.

SEQ ID NO: 12: Nucleic acid sequence, Nanoluciferase

SEQ ID NO: 13: Nucleic acid sequence, LoxP site

SEQ ID NO: 14: Nucleic acid sequence, Transcription blocker

SEQ ID NO: 15: Nucleic acid sequence, pAAV-LCuL-EAS

SEQ ID NO: 16: Nucleic acid sequence, pAAV-LCuL-IL10

SEQ ID NO: 17: Amino acid sequence, reverse tetracycline-controlled transactivator (rtTA)

SEQ ID NO: 18: Amino acid sequence, Cumate Repressor (CymR)

SEQ ID NO: 19: Amino acid sequence, Therapeutic polypeptide, Interleukin (IL)10

SEQ ID NO: 20: Amino acid sequence, Therapeutic polypeptide, endo/angiostatin

SEQ ID NO: 21: Amino acid sequence, Therapeutic polypeptide, Interleukin-1 Receptor Antagonist (IL-1RA)

SEQ ID NO: 22: Nucleic acid sequence, LoxP site - variant

SEQ ID NO: 23: Nucleic acid sequence, LoxP site - lox 511

SEQ ID NO: 24: Nucleic acid sequence, LoxP site - lox 5171

SEQ ID NO: 25: Nucleic acid sequence, LoxP site - lox 2272

SEQ ID NO: 26: Nucleic acid sequence, LoxP site - M2

SEQ ID NO: 27: Nucleic acid sequence, LoxP site - M3

SEQ ID NO: 28: Nucleic acid sequence, LoxP site - M7

SEQ ID NO: 29: Nucleic acid sequence, LoxP site - M11

SEQ ID NO: 30: Nucleic acid sequence, LoxP site - lox 71

SEQ ID NO: 31: Nucleic acid sequence, LoxP site - lox 66

SEQ ID NO: 32: Nucleic acid sequence, FRT site

SEQ ID NO: 33: Nucleic acid sequence, FRT site - variant

SEQ ID NO: 34: Nucleic acid sequence, FRT site - FL-IL-10A

SEQ ID NO: 35: Nucleic acid sequence, FRT site - FRT/FL-IL-10A

SEQ ID NO: 36: Nucleic acid sequence, rox site - WT

SEQ ID NO: 37: Nucleic acid sequence, rox site - rox7

SEQ ID NO: 38: Nucleic acid sequence, rox site - rox8

SEQ ID NO: 39: Nucleic acid sequence, rox site - rox12

SEQ ID NO: 40: Nucleic acid sequence, rox site - rox61

SEQ ID NO: 41: Nucleic acid sequence, rox site - rox85

SEQ ID NO: 42: Nucleic acid sequence, att site - attP

SEQ ID NO: 43: Nucleic acid sequence, att site - attB

SEQ ID NO: 44: Nucleic acid sequence, att site - proB

SEQ ID NO: 45: Nucleic acid sequence, att site - trpC

SEQ ID NO: 46: Nucleic acid sequence, att site - galT

SEQ ID NO: 47: Nucleic acid sequence, att site - thrA

SEQ ID NO: 48: Nucleic acid sequence, att site - rrnB

DETAILED DESCRIPTION OF THE INVENTION

Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (e.g., molecular biology, gene therapy, genetic modification, biochemistry, physiology, and clinical studies).

Unless otherwise indicated, the molecular and statistical techniques utilized in the present disclosure are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present), Ed Harlow and David Lane (editors) Antibodies: A Laboratory Manual, Cold Spring Harbour Laboratory, (1988), J.E. Coligan et al. (editors) Current Protocols in Immunology, John Wiley & Sons (including all updates until present).

Various subjects can be administered an expression vector according to the present disclosure. In an example, the subject is a mammal. The mammal may be a companion animal such as a dog or cat, or a livestock animal such as a horse or cow. In another example, the subject is a human. Terms such as "subject", "patient" or "individual" are terms that can, in context, be used interchangeably in the present disclosure.

As used herein, terms such as "treating" or "treatment" refer to clinical intervention designed to alter the natural course of the individual or cell being treated during the course of clinical pathology. Desirable effects of treatment include decreasing the rate of disease progression, ameliorating or palliating the disease state, and remission or improved prognosis. An individual is successfully "treated", for example, if one or more symptoms associated with a disease are mitigated or eliminated. In an example, the term "treatment" refers to both therapeutic treatment and prophylactic or preventative measures for ocular disorders such as, for example, exudative macular degeneration, diabetic retinopathy, cystoid macular oedema, clinically significant macular oedema, central retinal vein occlusion, branch retinal vein occlusion and ocular neovascularisation wherein the object is to reverse, prevent or slow down (lessen) the targeted disorder.

Those in need of treatment include those already having an ocular disorder or those prone to having such disorders or those in whom such disorders are to be prevented. In an example, treatment encompasses stabilization and/or improvement of visual acuity.

An "effective amount" refers to at least an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic or prophylactic result. An effective amount can be provided in one or more administrations. In some examples of the present disclosure, the term "effective amount" is used to refer to an amount necessary to effect treatment of an ocular disorder or condition as hereinbefore described. The effective amount may vary according to the disease or condition to be treated and also according to the weight, age, racial background, sex, health and/or physical condition and other factors relevant to the mammal being treated. Typically, the effective amount will fall within a relatively broad range (e.g. a "dosage" range) that can be determined through routine trial and experimentation by a medical practitioner. The effective amount can be administered in a single dose or in a dose repeated once or several times over a treatment period.

A "therapeutically effective amount" is at least the minimum concentration required to effect a measurable improvement of a particular ocular disorder (e.g. exudative macular degeneration). A therapeutically effective amount herein may also vary according to factors such as the disease state, age, sex, and weight of the patient, and the ability of the therapeutic to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the therapeutic are outweighed by the therapeutically beneficial effects. For exudative macular degeneration therapy, efficacy *in vivo* can, for example, be measured by one or more of the following: assessing the mean change in the best corrected visual acuity (BCVA) from baseline to a desired time, assessing the proportion of subjects who lose fewer than 15 letters in visual acuity at a desired time compared with baseline, assessing the proportion of subjects who gain greater than or equal to 15 letters in visual acuity at a desired time compared with baseline, assessing the proportion of subjects with a visual-acuity Snellen equivalent of 20/2000 or worse at a desired time, assessing the NEI Visual Functioning Questionnaire, assessing the size of choroidal neovascularization (CNV) and/or amount of leakage of CNV at a desired time, as assessed by fluorescein angiography.

As used herein, the term "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences of a genomic gene, including the TATA box or initiator element, which is required for accurate transcription initiation, with or without additional regulatory elements (e.g., upstream activating sequences,

transcription factor binding sites, enhancers) that alter expression of a nucleic acid, e.g., in response to a developmental and/or external stimulus, or in a tissue specific manner. Exemplary promoters can contain additional copies of one or more specific regulatory elements to further enhance expression and/or alter the spatial expression and/or temporal expression of said nucleic acid. In an example, the promoter is a regulatable promoter described herein. Other examples of promoters encompassed by the present disclosure include “constitutive promoters”. Such promoters are constantly active (i.e. they constantly confer, activate or enhance the expression of a nucleic acid).

The term “operably linked” means positioning an expression element relative to a nucleic acid defined herein such that expression of the nucleic acid is controlled by the expression element.

As used in this specification and the appended claims, terms in the singular and the singular forms "a," "an" and "the," for example, optionally include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to "a therapeutic moleucle" optionally includes one or more therapeutic molecules.

As used herein, the term “about”, unless stated to the contrary, refers to +/- 10%, more preferably +/- 5%, more preferably +/- 1%, of the designated value.

The term “and/or”, e.g., “X and/or Y” shall be understood to mean either “X and Y” or “X or Y” and shall be taken to provide explicit support for both meanings or for either meaning.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Kill switch

Expression vectors encompassed by the present disclosure comprise a “kill switch”. The term “kill switch” refers to element(s) of vectors defined herein that can silence expression of a nucleic acid encoding a therapeutic molecule(s) from the vector. The term “silence” is used in this context to refer to complete and irreversible suppression of expression. In an example, silencing completely abolishes expression of a nucleic acid encoding a therapeutic molecule from an expression vector defined herein.

In an example, the kill switch can facilitate removal of a nucleic acid sequence encoding a therapeutic molecule(s) or part thereof from the vector. In another example, the kill switch facilitates removal of some or all of the transcriptional machinery required for expression of the therapeutic molecule(s) from the vector. In another example, the

kill switch facilitates removal of one or more promoters. In another example, the kill switch facilitates removal of a transactivator gene. In another example, the kill switch facilitates removal of the entire expression cassette from the vector (i.e. all foreign genes and regulatory elements are removed). In another example, the kill switch facilitates inversion of the sequence encoding one or more of the above elements. For example, the kill switch can facilitate inversion of some or all of the sequence(s) encoding a promoter(s), therapeutic molecule(s) or transactivator. An example of a kill switch vector backbone is provided in SEQ ID NO: 1.

In an example, the kill switch is a pair of site-specific recombination sequences. In this example, recombination between the site-specific recombination sequences silences expression of the nucleic acid encoding the therapeutic molecule. In another example, the kill switch is a pair of site-specific recombination sequences that are cleaved following contact with a recombinase. In an example, the site-specific recombination sequences "flank" the nucleic acid encoding the therapeutic molecule. Put another way, a first site-specific recombination sequence is positioned upstream of the therapeutic molecule and a second site-specific recombination sequence is positioned downstream of the therapeutic molecule. In another example, the site-specific recombination sequences flank some or all of the transcriptional machinery involved in expressing the nucleic acid encoding the therapeutic molecule. For example, the site-specific recombination sequences can flank one or more promoters disclosed herein. In another example, the site-specific recombination sequences flank all genes and regulatory elements in the vector. It will be appreciated by those skilled in the art that having site-specific recombination sequences that flank all genes and regulatory elements in the vector provides a safety mechanism, such that all foreign genes and regulatory elements can be removed from the vector in the case of, for example, an aberrant immune response or malignant transformation in the host.

One of skill in the art will appreciate that position and orientation of site-specific recombination sequences can direct inversion or excision of regions from vectors. Thus, in an example, site-specific recombination sequences are positioned to direct excision. For example, site-specific recombination sequences can be positioned in the same direction. In another example, site-specific recombination sequences can be positioned to direct inversion. For example, site-specific recombination sequences can be positioned in opposite orientations.

In an example, the kill switch can comprise a pair of LoxP sites which are cleaved following contact with Cre recombinase. Exemplary LoxP sites are described in Hoess et al. (1982) PNAS, 3398:402. For example, vectors defined herein can comprise

canonical loxP sequences ATAACTTCGTATA-[spacer]-TATACGAAGTTAT. In an example, vectors defined herein can comprise a pair of sites having a nucleotide sequence shown in SEQ ID NO: 13. Other examples or LoxP sites are shown in Table 1.

Table 1. Examples of LoxP sites

SEQ ID NO:	Name	13bp Recognition Region	Spacer Region	13bp Recognition Region
22	Variant	ATAACTTCGTATA	GCATACAT	TATACGAAGTTAT
23	lox 511	ATAACTTCGTATA	ATGTATaC	TATACGAAGTTAT
24	lox 5171	ATAACTTCGTATA	ATGTgTaC	TATACGAAGTTAT
25	lox 2272	ATAACTTCGTATA	AaGTATcC	TATACGAAGTTAT
26	M2	ATAACTTCGTATA	AgaaAcca	TATACGAAGTTAT
27	M3	ATAACTTCGTATA	taaTACCA	TATACGAAGTTAT
28	M7	ATAACTTCGTATA	AgaTAGAA	TATACGAAGTTAT
29	M11	ATAACTTCGTATA	aGATAgaa	TATACGAAGTTAT
30	lox 71	taccgTTCGTATA	NNNTANNN	TATACGAAGTTAT
31	lox 66	ATAACTTCGTATA	NNNTANNN	TATACGAAcggta

In another example, the kill switch can comprise a pair of short flippase recognition target (*FRT*) sites which are cleaved following contact with flippase (Flp). Exemplary FRT sites are described in Bolusani et al. 2006 Nucleic Acids Res. 34:5259-69. For example, vectors defined herein can comprise canonical FRT sequences GAAGTTCCTATTC-[spacer]-GtATAGGAACTTC (e.g., SEQ ID NO: 32). Other examples or FRT sites are shown in Table 2.

Table 2. Examples of FRT sites.

SEQ ID NO:	Name	13bp Recognition Region	Spacer Region	13bp Recognition Region
33	Variant	GAAGTTCCTATAC	tttctaga	GAATAGGAACTTC
34	FL-IL-10A	agtGaTttgATAC	ttacatga	GtAaAGGAAtTag
35	FRT/FL-IL-10A	GAAGTTCCTATAC	ttacatga	GtAaAGGAAtTag

In another example, the kill switch can comprise a pair of *rox* sites which are cleaved following contact with Dre recombinase. Exemplary *rox* sites are described in Anastassiadis et al., 2009 Dis Model Mech 2:508-515. For example, vectors defined herein can comprise a canonical *rox* sequence TAACTTTAAATAAT-[spacer]-

ATTATTTAAAGTTA. Other examples of *rox* sites are described in Chuang et al. 2016 G3 (Bethesda) 6:559-571, and include those shown in Table 3.

Table 3. Examples of *rox* sites.

SEQ ID NO:	Name	14bp Recognition Region	Spacer Region	14bp Recognition Region
36	WT	TAACTTTAAATAAT	GCCA	ATTATTTAAAGTTA
37	rox7	TAACTTTAAATAAg	GCCA	gTTATTTAAAGTTA
38	rox8	TAACTTTAAATAAc	GCCt	cTTATTTAAAGTTA
39	rox12	TAACTTTAAATAAg	GCCt	gTTATTTAAAGTTA
40	rox61	TAACTTTAAATAAg	GCCc	gTTATTTAAAGTTA
41	rox85	TAACTTTAAATAAg	GCCg	gTTATTTAAAGTTA

In another example, the kill switch can comprise a pair of *att* sites which are cleaved following contact with phiC31 integrase. Exemplary *att* sites are described in Thorpe and Smith, 1998 PNAS 95:5505-5510. For example, vectors defined herein can comprise a canonical *attP* sequence TCAGCTTTTTTATACTAAGTTGG (SEQ ID NO: 42) and a canonical *attB* sequence CCTGCTTTTTTATACTAACTTGA (SEQ ID NO: 43). Examples of secondary *att* sites include those shown in Table 4.

Table 4. Examples of secondary *att* sites.

SEQ ID NO:	Name	Sequence
44	*proB*	TGCGCTAA TTTATAC GAGGCTAC
45	*trpC*	GCGTAATG TTTATAA ATGGCGGC
46	*galT*	CGCCTTTG TTTTCAA AAACCTGC
47	*thrA*	CGGGCTTT TTTCTGT GTTTCCTG
48	*rrnB*	TTGGCTAT TTTACCA CGACTGTC

A kill switch defined herein can be activated using various mechanisms. For example, the kill switch can be contacted with a "kill switch activator". The term "kill switch activator" is used in the context of the present disclosure to refer to elements capable of activating a kill switch disclosed herein and silencing expression of a nucleic acid encoding a therapeutic polypeptide. In an example, the kill switch activator is an enzyme. For example, the enzyme can bind one or more of the above referenced site-specific recombination sequences. In an example, the enzyme is a recombinase. In an example, the enzyme is a tyrosine recombinase. In an example the enzyme is Cre recombinase (see for e.g. Sauer and Henderson (1988) PNAS 85:5166-5170; US

4959317; accession P06956). In an example, the enzyme is Dre recombinase (see for e.g. Anastassiadis et al., 2009 Dis Model Mech 2:508-515; accession AAV84949). In another example, the enzyme is a flippase. For example, the enzyme can be FLP recombinase (see for e.g. Sadowski and Zhu (1995) J Biol Chem. 270:23044:23054; accession P03870). In another example, the enzyme is a serine recombinase. In an example the enzyme is phiC31 integrase (see for e.g. Thorpe and Smith, 1998 PNAS 95:5505-5510; accession NP_047974).

Expression vector

The term "expression vector" is used in the context of the present disclosure to refer to a genetic construct which is capable of facilitating expression of a nucleic acid in a host cell. An expression vector can exist in the form of an isolated polynucleotide, for example "naked DNA", or can comprise one or more agents which enhance delivery to the host cell, such as a viral capsid and/or envelope, a lipid, or a polymer. Accordingly, examples of expression vectors encompassed by the present disclosure include, without limitation, naked DNA, phage, viruses, nanoparticles such as lipid-based nanoparticles, plasmids, linear DNA, cosmids, episomes, mini-circle DNA (for example, as described in US 2004/0214329), and bacteria. In another example, the expression vector is a transposon such as PiggyBac and PiggyBat (see Wu et al., PNAS, 103:15008-13, 2006; and WO 2010/085699).

Expression vectors encompassed by the present disclosure include DNA or RNA vectors. In an example, expression vectors are single stranded. In another example, expression vectors are double stranded.

Exemplary vector components include, but are not limited to, one or more of the following: a sequence encoding a therapeutic molecule and/or a regulator compound-binding polypeptide, a promoter such as a regulatable promoter defined herein, and a transcription termination sequence.

In an example, the expression vector is capable of transforming a host cell and effecting expression of a nucleic acid encoding a therapeutic molecule. In an example, the expression vector is also capable of replicating within the host cell. In another example, the expression vector is not capable of replicating within the host cell. In some examples, the expression vector is capable of integrating into the host cell's genome.

The selection of expression vector will depend on a variety of factors such as, for example, the host, immunogenicity of the vector, the desired duration of therapeutic molecule production, and the like. In one example, the expression vector is a viral vector. In one example, the expression vector is an adeno-associated virus (AAV) vector. In one

example, the expression vector is a retroviral vector. In one example, the expression vector is a lentiviral vector. In one example, the expression vector is an adenovirus vector.

AAV vectors typically comprise inverted terminal repeats (ITRs) of 145 nt at either end, which contain sequences necessary for DNA replication and packaging into virions for nucleic acid delivery. In one example, the ITRs of AAV serotype 2 are used. However, ITRs from other suitable serotypes may be selected. These ITRs or other AAV components may be readily isolated using techniques available to those of skill in the art from an AAV serotype (see WO 2006/110689). Various recombinant AAV vector systems have been developed for nucleic acid delivery because they are non-pathogenic and exhibit a broad range of tissue specificity. AAV vectors can be readily constructed using techniques known in the art. See, e.g., U.S. Pat. Nos. 5,173,414 and 5,139,941; International Publication Nos. WO 1992/01070 and WO 1993/03769; Lebkowski et al. Molec. Cell. Biol. 5:3988-3996, 1988; Vincent et al. (1990) Vaccines 90 (Cold Spring Harbor Laboratory Press); Carter Current Opinion in Biotechnology 5:533-539, 1992; Muzyczka. Current Topics in Microbiol, and Immunol. 158:97-129, 1992; Kotin, Human Gene Therapy 5:793-801, 1994; Shelling and Smith Gene Therapy 7:165-169, 1994; and Zhou et al. J Exp. Med. 179:1867-1875, 1994.

In another example, the AAV is a self-complementary AAV (sc-AAV) (see for example, US2012/0141422). Self-complementary AAV vectors package an inverted repeat genome that can fold into dsDNA without the requirement for DNA synthesis or base-pairing between multiple vector genomes.

A retroviral vector generally comprises cis-acting long terminal repeats (LTRs) with packaging capacity for up to 6-10 kb of foreign sequence. The minimum cis-acting LTRs are sufficient for replication and packaging of a vector, which is then used to integrate an expression construct into the target cell to provide long term expression. Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), simian immunodeficiency virus (SrV), human immunodeficiency virus (HIV), and combinations thereof (see, e.g., Buchscher et al., J Virol. 56:2731-2739 (1992); Johann et al, J. Virol. 65:1635-1640 (1992); Sommerfelt et al, Virol. 76:58-59 (1990); Wilson et al, J. Virol. 63:274-2318 (1989); Miller et al., J. Virol. 65:2220-2224 (1991); PCT/US94/05700; Miller and Rosman BioTechniques 7:980-990, 1989; Miller, A. D. Human Gene Therapy 7:5-14, 1990; Scarpa et al Virology 75:849-852, 1991; Burns et al. Proc. Natl. Acad. Sci USA 90:8033-8037, 1993).

Additional viral vectors include, for example, those derived from the pox family of viruses, such as vaccinia virus and avian poxvirus or an alphavirus or a conjugate virus vector (e.g. that described in Fisher-Hoch et al., PNAS 56:317-321, 1989).

In another example, the expression vector is a plasmid. In an example, the expression vector can be a high copy number plasmid. In an example, the high copy number plasmid can provide about 100 – 1,000 copies per cell. In another example, the high copy number plasmid can provide about 200 – 500 copies per cell. In another example, the expression vector is a low copy number plasmid. In an example, the low copy number plasmid can provide about 20 – 100 copies per cell. In another example, the low copy number plasmid can provide about 20 – 50 copies per cell.

Other suitable exemplary expression vectors also include those that function (i.e., direct expression) in mammalian, preferably human cells.

In some examples, the expression vector directs expression of the therapeutic polypeptide in a particular cell type. Thus, in some examples, the expression vector comprises a cell-type specific promoter. In one example, the expression vector comprises a retinal cell specific promoter, such as a mouse phosphoglycerate kinase 1 (PGK) promoter, elongation factor-1 (EFS) promoter, vitelliform macular dystrophy (VMD2) promoter, red/green opsin promoter, thymocyte antigen promoter or rhodopsin (Rho) promoter. In other examples, expression vectors direct expression in tumour cells. Such vectors can for example, comprise a tumour specific promoter(s) and/or a molecule that binds a tumour specific cell surface molecule to facilitate entry into tumour cells.

Expression vectors encompassed by the present disclosure may be modified to increase expression of nucleic acids encoding therapeutic molecules. Recombinant techniques useful for increasing expression include, but are not limited to, operatively linking nucleic acid sequences encoding therapeutic molecules to high-copy number plasmids, integration of the same molecule into one or more host cell chromosomes, addition of vector stability sequences, substitutions or modifications of transcription control signals (e.g., promoters, operators, enhancers), substitutions or modifications of translational control signals (e.g., ribosome binding sites, Shine-Dalgarno sequences), modification of nucleic acid sequences encoding a therapeutic molecule to correspond to the codon usage of the host cell, and the deletion of sequences that destabilise transcripts.

In some examples, expression vectors can contain regulatory sequences such as transcription control sequences, translation control sequences, origins of replication, and other regulatory sequences that are compatible with subjects and that control the expression of nucleic acid sequences encoding therapeutic molecules. Transcription control sequences are sequences which control the initiation, elongation, and/or

termination of transcription. Particularly important transcription control sequences are those which control transcription initiation, such as promoter, enhancer, operator and repressor sequences. A variety of suitable transcription control sequences are known to those skilled in the art. Examples include tac, lac, trp, trc, oxy-pro, omp/lpp, rrnB, bacteriophage lambda, bacteriophage T7, T7lac, bacteriophage T3, bacteriophage SP6, bacteriophage SP01, metallothionein, alpha-mating factor, Pichia alcohol oxidase, alphavirus subgenomic promoters (such as Sindbis virus subgenomic promoters), antibiotic resistance gene, baculovirus, *Heliothis zea* insect virus, vaccinia virus, herpesvirus, raccoon poxvirus, other poxvirus, adenovirus, cytomegalovirus (such as intermediate early promoters), simian virus 40, retrovirus, actin, retroviral long terminal repeat, Rous sarcoma virus, heat shock, phosphate and nitrate transcription control sequences.

In an example, the expression vector further comprises a transcription blocker between a constitutive and a regulatable promoter. Transcription blockers can be used in order to prevent transcriptional interference between multiple promoters in close proximity. Such transcription blockers are advantageous for tightly regulated expression systems such as those utilised for gene therapy. Transcription blockers can comprise one or more polyadenylation signal sequences and transcription pause sites for reducing background expression. Suitable transcription blockers are described in, for example, Eggermont and Proudfoot, EMBO J, 12:2539-2548, 1993. In one example, the transcription blocker comprises a sequence set forth in SEQ ID NO: 14.

In another example, the expression vector comprises the nucleic acid sequence shown in SEQ ID NO: 15. In another example, the expression vector comprises the nucleic acid sequence shown in SEQ ID NO: 16.

Regulatable elements, regulator compounds and regulator compound-binding molecules

In an example, vectors defined herein comprise a regulatable element operably linked to a nucleic acid sequence encoding a therapeutic molecule. For example, the regulatable element can be operably linked to a nucleic acid sequence encoding a therapeutic molecule described below.

The term “regulatable element” is used in the context of the present disclosure to refer to a recombinant, synthetic or fusion nucleic acid(s) which confers, activates, enhances or represses the expression of a nucleic acid to which it is operably linked. For example, a regulatable element operably linked to a therapeutic molecule defined herein confers, activates, enhances or represses expression a therapeutic molecule defined herein.

In an example, the regulatable element can comprise a nucleic acid sequence which is responsive to the presence of a regulator compound. In an example, the regulatable element can respond to the presence of a regulator compound by promoting expression of an operably linked nucleic acid. In another example, the regulatable element can respond to the presence of a regulator compound by repressing expression of an operably linked nucleic acid.

The regulatable element is not particularly limited so long as it can selectively regulate expression of a therapeutic molecule from a vector defined herein. In an example, the regulatable element comprises one or more of a tetracycline responsive element (TRE), a cumate operator (CuO), an ecdysone response element (EcRE), a hormone response element (HRE), an estrogen response element (ERE), a glucocorticoid response element (GRE), a progesterone response element (PRE), a heat shock sequence element (HSE).

In an example, the regulatable element is a regulatable promoter. Examples of regulatable promoters include, without limitation, tetracycline-regulated promoters (e.g. SEQ ID NO: 4), cumate-regulated promoters (e.g. SEQ ID NO: 5), rapamycin-inducible promoters, steroid hormone-inducible promoters, metal-responsive promoters, heat shock response promoters, light-responsive promoters, interferon-responsive promoters, and mifepristone-regulated promoters. Examples of regulatable promoters are described in Goverdhana et al., Mol Ther, 12:189-211, 2005; Agha-Mohammadi and Lotze, J Clin Invest, 105:1177–1183; and Mullick et al., BMC Biotech, 6:43, 2006). Other examples of regulatable promoters include hypoxia driven promoters, which are active when ocular neovascularization or age-related macular degeneration is associated with hypoxia, IL-8 promoters, or metallothionine-inducible promoters.

In one example, the regulatable promoter is a tetracycline-regulated promoter. In another example, the regulatable promoter is a cumate-regulated promoter.

In an example, the regulatable promoter comprises a sequence set forth in SEQ ID NO: 4 or SEQ ID NO: 5. In an example, the nucleic acid sequence of the regulatable promoter consists of a sequence set forth in SEQ ID NO: 4 or SEQ ID NO: 5.

The activity (i.e. ability to confer, activate, enhance or repress the expression of a nucleic acid) of “regulatable elements” such as regulatable promoters encompassed by the present disclosure is regulated by regulator compound either alone or in conjunction with a regulator compound-binding protein.

In an example, a regulator compound can directly bind the regulatable promoter to regulate its activity. For example, binding of the regulator compound to the regulatable promoter can promote expression of the nucleic acid encoding the therapeutic polypeptide. In another example, binding of the regulator compound to the regulatable promoter represses expression of the nucleic acid encoding the therapeutic molecule.

In another example, a regulator compound must bind a regulator compound binding molecule expressed from an expression vector defined herein in order to bind and regulate activity of the regulatable promoter. In an example, the regulator compound binding molecule is operably linked to a constitutive promoter in an expression vector defined herein. In this example, the regulator compound-binding molecule is continuously expressed from an expression vector defined herein and, upon contact with a regulator compound is capable of binding and regulating activity of the regulatable promoter operably linked to the therapeutic polypeptide.

Various constitutive promoters that may be operably linked to a nucleic acid encoding a regulator compound-binding molecule are known in the art. Examples include cytomegalovirus immediate early promoter (CMV-IE), human elongation factor 1-α promoter (EF1), small nuclear RNA promoters (U1a and U1b), α-myosin heavy chain promoter, Simian virus 40 promoter (SV40), Rous sarcoma virus promoter (RSV), Adenovirus major late promoter, β-actin promoter; hybrid regulatory element comprising a CMV enhancer/β-actin promoter or an immunoglobulin promoter or active fragment thereof. For example, the constitutive promoter can be a composite human CMV-EF1-HTLV promoter.

In an example, the constitutive promoter comprises a sequence set forth in SEQ ID NO: 6. In an example, the nucleic acid sequence of the constitutive promoter consists of a sequence set forth in SEQ ID NO: 6.

In an example, the regulator compound-binding molecule and the therapeutic molecule are expressed from the same vector. In another example, the regulator compound-binding molecule and the therapeutic molecule are expressed from separate vectors.

One of skill in the art will appreciate the appropriate regulator compound will be dictated by the regulatable promoter and/or the regulator compound-binding molecule. For example, an appropriate regulator compound for a tetracycline regulatable promoter would be tetracycline or a structural analogue thereof such as doxycycline. In another example, an appropriate regulator compound for a cumate regulatable promoter would be cumate or a structural analogue thereof. In another example, an appropriate regulator

compound for a rapamycin-inducible promoter would be rapamycin or a structural analogue thereof. In another example, an appropriate regulator compound for a steroid hormone-inducible promoter would be a steroid hormone such as progesterone or a structural analogue thereof such as mifepristone.

In an example, the regulator compound is a small molecule. In one example, the regulator compound is tetracycline. In one example, the regulator compound is cumate. In one example, the regulator compound is rapamycin.

In some examples, the regulator compound is a steroid hormone, or an analogue thereof. In one example, the regulator compound is progesterone. In one example, the regulator compound is ecdysone. In one example, the regulator compound is glucocorticoid. In one example, the regulator compound is estrogen. In one example, the regulator compound is mifepristone.

In one example, the regulatable element comprises a tetracycline responsive element (TRE) and the regulator compound is tetracycline or a structural analogue thereof. In one example, the regulatable element comprises a cumate operator (CuO) and the regulator compound is cumate or a structural analogue thereof. In one example, the regulatable element comprises an ecdysone response element (EcRE) and the regulator compound is ecdysone or a structural analogue thereof. In one example, the regulatable element comprises an estrogen response element (ERE) and the regulator compound is estrogen or a structural analogue thereof. In one example, the regulatable element comprises a glucocorticoid response element (GRE) and the regulator compound is a glucocorticoid or a structural analogue thereof. In one example, the regulatable element comprises a progesterone response element (PRE) and the regulator compound is progesterone or a structural analogue thereof.

Again, one of skill in the art will appreciate the appropriate combinations of regulator compound and regulator compound-binding molecule. In an example, the regulator compound-binding molecule is a polypeptide. In an example, the regulator compound-binding molecule comprises a tetracycline-controlled transactivator (tTA), tetracycline repressor (TetR), or reverse tetracycline-controlled transactivator (rtTA) (e.g. SEQ ID NO: 2; SEQ ID NO: 17). In another example, the regulator compound-binding molecule comprises a cysteine metabolism repressor (CymR) (e.g. SEQ ID NO: 3; SEQ ID NO: 18). In another example, the regulator compound-binding molecule comprises a steroid hormone receptor, such as a progesterone receptor, an estrogen receptor (ER) or an ecdysone receptor (EcR). In some examples the regulator compound-binding molecule comprises a rapamycin binding protein. In some examples the regulator compound-binding molecule comprises a Gal4 DNA binding domain. In some examples

the regulator compound-binding molecule comprises a VP16 activation domain. In another example, the regulator compound-binding molecule is a fusion of one or more of the molecules described herein. For example, the regulator compound-binding molecule can comprise a fusion of CymR and a VP16 activation domain.

In an example, the regulator compound-binding molecule comprises an amino acid sequence set forth in SEQ ID NO: 17 or SEQ ID NO: 18. In an example, the regulator compound-binding molecule consists of an amino acid sequence set forth in SEQ ID NO: 17 or SEQ ID NO: 18.

Therapeutic molecule

Therapeutic molecules encompassed by the present disclosure are not particularly limited so long as they can be expressed from an expression vector disclosed herein or translated from a nucleic acid sequence expressed from the same.

In an example, the expressed nucleic acid sequence encodes a therapeutic polypeptide. For example, a nucleic acid sequence can be expressed from an expression vector defined herein and translated by cellular machinery to produce a therapeutic polypeptide.

Exemplary therapeutic polypeptides include binding proteins such as immunoglobulin, antibodies and antigenic binding fragments. For example, therapeutic polypeptides include single chain Fv fragment (scFv), dimeric scFv (di-scFv), $(scFv)_n$, scFv or di-scFv linked to a constant region of an antibody, Fc or a heavy chain constant domain (CH)2 and/or CH3, diabodies, triabodies, tetrabodies, Fab, $F(ab')_2$ and antibodies. In an example, the therapeutic polypeptide is an antibody or TRAP molecule. Examples of such therapeutic molecules include ranibizumab, bevacizumab and aflibercept.

In another example, the therapeutic polypeptide comprises an antigen binding site of an antibody.

In another example, the therapeutic molecule can be an inhibitory oligonucleotide. Exemplary inhibitory oligonucleotides include isolated or synthetic antisense RNA or DNA, siRNA or siDNA, miRNA, miRNA mimics, shRNA or DNA and Chimeric Antisense DNA or RNA. The term "antisense" as used herein means a sequence of nucleotides complementary to and therefore capable of binding to a coding sequence, which may be either that of the strand of a DNA double helix that undergoes transcription, or that of a messenger RNA molecule. The terms "short hairpin RNA" or "shRNA" refer to an RNA structure having a duplex region and a loop region. The term small interfering RNA (siRNA), sometimes known as short interfering RNA or silencing

RNA, is a class of double-stranded RNA molecules, 20-25 base pairs in length. A siRNA that inhibits or prevents translation to a particular protein is indicated by the protein name coupled with the term siRNA. Thus a siRNA that interferes with the translation of VEGF is indicated by the expression "VEGF siRNA". The term “microRNA” (abbreviated miRNA) is a small non-coding RNA molecule (containing about 22 nucleotides) found in plants, animals and some viruses, that functions in RNA silencing and post-transcriptional regulation of gene expression. The prefix "miR" is followed by a dash and a number, the latter often indicating order of naming. Different miRNAs with nearly identical sequences except for one or two nucleotides are annotated with an additional lower case letter. Numerous miRNAs are known in the art (miRBase V.21 nomenclature; Kozomara et al. 2013; Griffiths-Jones, S. 2004). In another example, an inhibitory oligonucleotide encompassed by the present disclosure inhibits the activity of one or more miRNAs. Various species are suitable for this purpose. Examples include antagomirs, interfering RNA, ribozymes, miRNA sponges and miR-masks. The term “antagomir” is used in the context of the present disclosure to refer to chemically modified antisense oligonucleotides that bind to a target miRNA and inhibit miRNA function by preventing binding of the miRNA to its cognate gene target.

In an example, the therapeutic molecule is an “anti-angiogenic molecule”. The term “anti-angiogenic molecule” is used in the context of the present disclosure to refer to molecules expressed from an expression vector defined herein which inhibit the development of blood vessels, e.g., inhibit angiogenesis, endothelial cell growth, stability of blood vessels, and/or vasculogenesis. Anti-angiogenic molecules encompassed by the present disclosure include polynucleotide(s), polypeptide(s), antibod(ies) or conjugates or fusion proteins thereof. Exemplary anti-angiogenic molecules include inhibitors of VEGF and members of the VEGF family, PlGF, PDGF family, fibroblast growth factor family (FGFs), TIE ligands (Angiopoietins), ephrins, ANGPTL3 and ANGPTL4. In other examples, anti-angiogenic molecules inhibit growth hormones such as insulin-like growth factor-I (IGF-I), VIGF, epidermal growth factor (EGF), CTGF and members of the CTGF family, TGF-α and TGF-β. Other examples of anti-angiogenic molecules include antibodies to VEGF, antibodies to VEGF receptors and angiogenesis inhibitors such as angiostatin, endostatin and fusions thereof. Accordingly, in an example, the therapeutic molecule comprises endostatin and/or angiostatin (e.g. SEQ ID NO: 9; SEQ ID NO: 20). In another example, the therapeutic molecule is a fusion of one or more of the molecules described herein. For example, the therapeutic molecule can comprise a fusion of endostatin and angiostatin.

In an example, the therapeutic molecule is an anti-inflammatory molecule. In an example, the anti-inflammatory molecule is an interleukin such as IL-10, IL-4, IL-6, IL-11 or IL-13. For example, the anti-inflammatory molecule can be IL-10 (e.g. SEQ ID NO: 8; SEQ ID NO: 19). In another example, the anti-inflammatory molecule is interleukin IL-1 receptor antagonist (IL-1RA) (e.g. SEQ ID NO: 10; SEQ ID NO: 21), or a fusion of IL-10 and IL-1RA (e.g. SEQ ID NO: 11). In other examples, the anti-inflammatory molecule inhibits a pro-inflammatory cytokine such as IL-1, tumour necrosis factor alpha (TNF-α) or IL-18. In other examples, anti-inflammatory molecules increase the production or number of CD14+CD16+ cells in a subject, reduce IL-6 levels, reduce TNF-alpha levels and/or increase IL-10 levels.

In another example, the therapeutic molecule is a neurotrophic factor. Neurotrophic factors are thought to be responsible for the maturation of developing neurons and for maintaining adult neurons. In this regard, neurotrophic factors can be used to inhibit or reverse neural cell degeneration and death. Examples of neurotrophic factors include, for example, brain-derived neurotrophic factor, nerve growth factor, transforming growth factors, glial cell-line derived neurotrophic factor, neurotrophin 3, neurotrophin 4/5, and interleukin 1-B.

In another example, the therapeutic molecule is cytotoxic to cancer cells. In other examples, the therapeutic molecule inhibits one or more of Nuclear factor-kappa B (NFκB), C-kit (CD117, stem cell-factor receptor), Heat shock protein 90 (Hsp90), Ras–Raf mitogen-activated protein kinase (MEK) pathway, Bcl-2, IL-2.

In an example, the therapeutic molecule comprises an amino acid sequence set forth in SEQ ID NOs: 19 to 21. In an example, the regulator compound-binding molecule consists of an amino acid sequence set forth in SEQ ID NOs: 19 to 21.

Treatment and Prevention

The methods of the present disclosure are directed towards the treatment and/or prevention of an ocular disorder. The term "ocular disorder" is used in the context of the present disclosure to refer to disorders and anomalies that affect the human eye and visual system. For example, ocular disorders include, but are not limited congenital, developmental, inflammatory, infectious, vascular, occlusive, angiogenic, degenerative, neoplastic, pre-neoplastic, iatrogenic, traumatic, glaucomatous, post-transplant complications, cataractous, and idiopathic diseases of the eye, retina, choroid or macula, or the systemic predispositions, associations or complications of these diseases (such as metastatic uveal melanoma or multisystem effects in an inherited gene dystrophy).

Other exemplary ocular disorders include diabetic retinopathy, cystoid macular oedema, clinically significant macular oedema, uveitis, iritis, giant cell arteritis, vasculitis, pars planitis, corneal transplant rejection, intraocular inflammation and lamellar corneal transplant rejection. Other examples of ocular disorders encompassed by the present disclosure include macular degeneration, diabetic retinopathy, cystoid macular oedema, clinically significant macular oedema, central retinal vein occlusion, branch retinal vein occlusion or ocular neovascularisation. For example, the ocular disorder can be an "intraocular neovascular disease". The term, "intraocular neovascular disease" is used in the context of the present disclosure to refer to a disease characterized by ocular neovascularization. Examples of intraocular neovascular diseases include, but are not limited to, e.g., proliferative retinopathies, choroidal neovascularization (CNV), dry age-related macular degeneration (AMD), wet age-related macular degeneration (AMD), diabetic and other ischemia-related retinopathies, diabetic macular edema, pathological myopia, von Hippel-Lindau disease, histoplasmosis of the eye, Central Retinal Vein Occlusion (CRVO), corneal neovascularization and retinal neovascularization. For example, the methods of the present disclosure encompass treating wet AMD. In another example, the methods of the present disclosure encompass treating CNV.

In an example, the methods of the present disclosure encompass inhibition of endothelial cell growth in the retina. For example, the methods of the present disclosure encompass inhibition of endothelial cell growth in the sub-retinal pigment epithelium and/or sub retinal space.

In another example, the ocular disorder is an infection.

In another example, the ocular disorder is a cancer. In one example, the cancer is uveal melanoma, ciliary body melanoma, iris melanoma, choroidal melanoma, intraocular lymphoma, retinoblastoma, or medulloepithelioma, choroidal hemangioma, choroidal metastasis, conjunctival Kaposi's sarcoma, malignant conjunctival tumor, orbital or lacrimal gland lymphoma, lymphoma of the conjunctiva, conjunctival melanoma or primary acquired melanosis with atypia, malignant tumour of the orbit, malignant tumour of the lacrimal gland, pigmented conjunctival tumour, squamous carcinoma of the conjunctiva, intraepithelial neoplasia of the conjunctiva or ocular surface squamous neoplasia. In one example, the cancer is uveal melanoma.

In another example, the ocular disorder is choroidal nevus, choroidal osteoma, nevus of Ota, conjunctival naevus, epibulbar dermoid, benign tumors of the lacrimal gland, benign tumours of the orbit, manifestations of thyroid ophthalmopathy, pingueculum, or pterygium.

In another example, the ocular disorder is Leber congenital amaurosis with RPE65 or an ocular disorder caused by RPE65 mutations. In another example, the ocular disorder is retinitis pigmentosa caused by a mutation in MERTK or RPGR or PDE6B or RLBP1 or other genes, or another disease caused by a mutation in MERTK or RPGR or PDE6B or RLBP1. In another example, the ocular disorder is choroideraemia or an ocular disorder caused by mutations in CHM. In another example, the ocular disorderis Achromatopsia or an ocular disorder caused by mutations in the CNGA3, CNGB3, GNAT2, PDE6C, PDE6H and ATF6 genes. In another example, it is X-linked retinoschisis or an ocular disorder caused by mutations in RS1. In another example, the ocular disorder is Leber's Hereditary Optic Neuropathy. In another example, the ocular disorder is a complication of transplant.

In an example, the above referenced methods comprise administering an expression vector described herein. For example, the above referenced methods can comprise administering an expression vector which comprises a kill switch and a nucleic acid encoding a therapeutic molecule, wherein activation of the kill switch silences expression of the nucleic acid encoding the therapeutic molecule. In an example, the vector further comprises a regulatable element operably linked to the nucleic acid encoding the therapeutic molecule, wherein activity of the regulatable promoter is regulated by administration of a regulator compound to the subject. In an example, the expression vector further comprises a constitutive promoter operably linked to a regulator compound-binding molecule which binds the regulator compound, wherein upon binding the regulator compound, the regulator binding-polypeptide regulates expression of the therapeutic molecule.

In another example, the regulator compound-binding molecule and therapeutic molecule are expressed from seperate expression vectors.

Formulations

Expression vectors, regulator compounds and other molecules described herein may be formulated as a pharmaceutical composition suitable for administration to a subject. Exemplary pharmaceutical compositions can comprise a pharmaceutically acceptable carrier, diluent or excipient. Depending upon the particular route of administration, a variety of acceptable carriers, known in the art may be used, as for example described in Remington's Pharmaceutical Sciences (Mack Publishing Co. N.J. USA, 1991).

Exemplary pharmaceutical compositions may also comprise pharmaceutically acceptable sterile aqueous or nonaqueous solutions, dispersions, suspensions, or

emulsions, as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, and injectable organic esters such as ethyl oleate. Compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents, and dispersing agents or antibacterial and antifungal agents.

In another example, expression vectors can be incorporated into slow release or targeted delivery systems. Exemplary slow release systems include polymer matrices, liposomes, and microspheres. Liposomes may be biodegradable and amphiphilic drug delivery systems, which may be formulated using phospholipids and cholesterol. Microspheres may be formulated using biodegradable and biocompatible polymers.

In an example, regulator compounds are provided in a topical formulation. For example, regulator compounds can be provided as an eye drop formulation. Suitable exemplary eye drop formulations include solutions, suspensions, ointments, gels or foams. In an example, the eye drop formulation comprises a regulator compound defined herein and a suitable carrier. Exemplary carriers include saline solution, water polyethers such as polyethylene glycol, polyvinyls such as polyvinyl alcohol and povidone, cellulose derivatives such as methylcellulose and hydroxypropyl methylcellulose, petroleum derivatives such as mineral oil and white petrolatum, animal fats such as lanolin, polymers of acrylic acid such as carboxypolymethylene gel, vegetable fats such as peanut oil and polysaccharides such as dextrans, and glycosaminoglycans such as sodium hyaluronate and salts such as sodium chloride and potassium chloride.

In an example the expression vector is present in or on a device that allows controlled or sustained release of the expression vector, such as an ocular sponge, meshwork, mechanical reservoir, or mechanical implant. Implants (see, e.g., U.S. 5,443,505, 4,853,224 and 4,997,652), devices (see, e.g., U.S. 5,554,187, 4,863,457, 5,098,443 and 5,725,493), such as an implantable device, e.g., a mechanical reservoir, an intraocular device or an extraocular device with an intraocular conduit, or an implant or a device comprised of a polymeric composition are particularly useful for ocular administration of the expression vector.

In an example, the expression vector is formulated to enhance transduction efficiency, i.e., to enhance transduction of the vector into a host cell. Suitable compositions are further described in U.S. Patents 6,225,289 and 6,514,943.

Administration and dosing

In an example, an expression vector defined herein is administered to a subject. Expression of a therapeutic molecule from the expression vector is subsequently regulated via administration of a regulator compound to the subject. Expression of the therapeutic molecule is subsequently silenced by administering a kill switch activator to the subject or by administering a vector comprising a nucleic acid sequence encoding a kill switch activator. Each of the vector, regulator compound and kill switch activator can be administered to a subject as a pharmaceutical composition. The selection of administration route will depend on a variety of factors such as, for example, the host, immunogenicity of the vector, the desired duration of therapeutic molecule production, and the like.

The pharmaceutical composition can, for example, be administered topically. For example, the composition can be administered by way of eye drops. In this example, the composition or part thereof can diffuse into the intraocular environment through the hydrophobic cornea. In another example, the composition is administered via intravitreal injection. In another example, the composition is administered via subretinal injection. In one example, a vitrectomy is performed prior to subretinal injection. In another example, the composition is administered via subcutaneous injection. In another example, the composition is administered via intramuscular injection. In another example, the composition is administered via intravenous injection. In another example, the composition is administered as a food or drink composition. In other examples involving use of heat or light as regulator compounds, such compounds can be applied to the eye of a subject as required.

In an example a pharmaceutical composition comprising a vector is administered via an ophthalmologic instrument for delivery to a specific region of an eye. Use of a specialized ophthalmologic instrument ensures precise administration of the expression vector while minimizing damage to adjacent ocular tissue. Delivery of the expression vector to a specific region of the eye also limits exposure of unaffected cells to the therapeutic molecule, thereby reducing the risk of side effects. One example of such an ophthalmologic instrument is a combination of forceps and subretinal needle or sharp bent cannula.

In one example, a pharmaceutical composition comprising an expression vector is administered by intravitreal or subretinal injection, a pharmaceutical composition comprising a regulator compound(s) is subsequently administered topically by way of eye drops and a kill switch activator is subsequently administered topically and/or by intravitreal or subretinal injection.

In another example, a pharmaceutical composition comprising an expression vector is administered by intravitreal or subretinal injection, a food or drink composition comprising a regulator compound(s) is subsequently administered and a kill switch activator is subsequently administered topically and/or by intravitreal or subretinal injection.

In an example, a kill switch activator can be expressed from a vector described herein. In an example, two vectors can be administered to a subject, the first comprising the kill switch and the second comprising a nucleic acid sequence encoding a kill switch activator. Thus, the first vector can be administered to express the therapeutic molecule and then subsequently, when expression of the therapeutic molecule is no longer required or desired, the second vector can be administered to express the kill switch activator thereby silencing expression of the therapeutic molecule. In some examples, the nucleic acid sequence encoding the kill switch activator is operably linked to a regulatable promoter.

In another example, vectors comprising a kill switch disclosed herein can further comprise a kill switch activator operably linked to a regulatable promoter. Thus, in some examples, nucleic acid sequences encoding both the therapeutic molecule and the kill switch activator can be present in the same vector. In this example, a regulator compound is administered to the subject to activate the regulatable promoter, promote expression of the kill switch activator and thus activate the kill switch to silence expression of therapeutic molecule(s) from the vector. Likewise, in another example, a regulator compound-binding molecule can be expressed from another vector. For example, two vectors can be administered to a subject, the first comprising the regulatable element and the second comprising a constitutive promoter operably linked to a nucleic acid encoding a regulator compound-binding molecule. In this example, a regulator compound is administered to the subject to bind the regulator compound-binding molecule and regulate expression of the regulatable promoter.

One of skill in the art will appreciate that dosage and routes of administration can be selected to minimize loss of expression vector due to a host's immune system. For example, for contacting ocular cells *in vivo*, it can be advantageous to administer to a host a null expression vector (i.e., an expression vector not comprising the nucleic acid sequence encoding the therapeutic molecule) prior to performing the methods described herein. Prior administration of null expression vectors can serve to create an immunity (e.g., tolerance) in the host to the expression vector, thereby decreasing the amount of vector cleared by the host's immune system.

Compositions disclosed herein can also be administered systemically, such as, for example, by intravenous or intraperitoneal administration. In another example, compositions can be administered orally. In another example, compositions can be administered intranasally.

In an example a first dose is administered to a subject followed by one or more subsequent doses. In an example, the first dose comprises a vector disclosed herein. In one example, one or more subsequent doses comprising the vector are administered. A regulator compound may be administered simultaneously or as one or more subsequent doses to regulate expression of a therapeutic molecule from the vector. For example, a first dose comprising a vector disclosed herein can be administered to a subject and a series of subsequent doses comprising a regulator compound can be administered to the subject. In this example, at least two, three, four, five, six, seven, eight, nine, ten, 15, 20, 30, 50, 100 or more subsequent doses can be administered to the subject. In this example, the subsequent dose of regulator compound can be provided simultaneously or sequentially with a subsequent dose of an expression vector disclosed herein. In an example, dosing is daily, every other day, every other week, every other month. In an example, a daily dose comprises a single application or multiple applications per administration. For example, at least two, three, four or more applications can be provided per day of administration.

Kits

Compositions according to the present disclosure can be provided in a kit or pack. For example, compositions disclosed herein may be packaged in a suitable container with written instructions for treating an ocular disorder. In an example, compositions may be provided in single dose containers such as an eye dropper or pre-filled syringe.

Kits of the present disclosure may comprise a therapeutic system. Such therapeutic systems can provide pre-programmed, unattended delivery of a composition at a rate, and for a time period, established to meet a specific therapeutic need. The system can be designed to minimize the patient's intervention and to optimize compliance with the prescribed regimen, for example.

In one example, the kit comprises an expression vector(s) defined herein and an eye drop formulation comprising a regulator compound(s) defined herein for use in methods of treating an ocular disorder.

In one example, the kit further comprises a kill switch activator as defined herein.

In one example, the kit further comprises an expression vector comprising a nucleic acid sequence encoding a kill switch activator as defined herein.

In one example, the kit comprises

- an expression vector(s) defined herein,
- an eye drop formulation comprising tetracycline, and
- Cre recombinase or an expression vector comprising a nucleic acid sequence encoding a Cre recombinase.

EXAMPLES

EXAMPLE 1 – Methods

Construction of vectors

The pAAV.LL Kill switch vector backbone (Figure 1) was synthetically constructed and ligated into pAAV-mcs containing AAV ITRs. Each of the vector inserts were also synthetically synthesized with restriction sites to allow cloning into the pAAV.LL backbone. The tetracycline or cumate repressor genes, flanked by XbaI and EcoRI sites were cloned into the XbaI-EcoRI site on the backbone. The tetracycline or cumate responsive promoters and CMV promoter flanked by BstBI and SalI sites were cloned into the unique BstBI-SalI site in the backbone. Finally, the gene of interest (GFP, IL-10, Endo/angiostatin, IL-1RA and IL-10-IL-1RA) flanked by SalI and NheI was cloned into the SalI-NheI site. Nano-luciferase was cloned into the SalI-Nhe site on the kill switch vector with the compatible XhoI and XbaI sites (Figure 2).

Evaluating the effects of the kill switch in vector constructs containing the marker gene, luciferase

ARPE-19 cells were cultured in T-75 tissue culture flasks with DMEM/F12 media supplemented with 10% Fetal Calf Serum (FCS) and 100 I.U/ml penicillin/streptomycin (P/S) until about 80% confluence. Cells were trypsinized with 0.05% Trypsin-EDTA and seeded at a concentration of 20,000 cells/well in a 96-well tissue culture treated plate. After 12-24 hours, cells were transfected using lipofectamine 3000 (Life technologies; MA, USA) with pAAV-LTetL-luc, pAAV-LCuL-luc and pAAV-LCMVL-luc all containing the marker gene, nano-luciferase.

24 hours post transfection, the cells were washed gently and media replaced with a subset of cells activated with doxycycline (structural tetracycline analogue) and cumate and a corresponding subset of cells with no activation (Figure 4). On day 4, luminescence was measured using the Nano-Glo Luciferase assay (Promega; WI, USA) to ensure activation, following which, a subset of activated cells were transfected with a vector containing Cre. The cells were washed daily and luminescence was measured on day 7. Briefly, the Nano-Glo Luciferase Assay was performed by adding 10 ul of media from

each treatment well to a black, clear bottom, 384 well plate. 10 ul of the promega substrate was subsequently added to these wells, mixed and left for 3 minutes at room temperature. Luminescence was read on a Luminescence plate reader. The schematic for this experiment is shown in Figure 4 and the results of vector killing is shown in Figure 5.

Evaluating activation and deactivation of vector constructs containing the marker gene, luciferase

ARPE-19 cells were seeded as described above. 12-24 hours later, cells were transfected with pAAV-LTetL-luc and pAAV-LCuL-luc. Media was replaced the next day and doxycycline or cumate was added to a subset of cells (Figure 7). The media from the cells were assayed for the expression of marker gene, luciferase, using the Nano-Glo Luciferase assay on day 4 after having a media change on day 3. The Nano-Glow luciferase assay was performed as described above.

Following the luciferase assay, media was changed in all the wells. A subset of cells activated with doxycycline and cumate were washed to deactivate the vector. After 3 days of washes, Nano-Glow luciferase assay was again performed and results shown in Figure 8.

Evaluating the effects of vector constructs containing endo/angiostatin on angiogenesis

The effects of the vector on angiogenesis were evaluated using a standard endothelial cell tube formation assay as per manufacturer's protocol (Life Technologies; Angiogenesis Starter Kit: A14609-01). Briefly, primary Human Umbilical Vein Endothelial Cells (HUVECs) were transfected with the cumate regulated vector containing endo/angiostatin (pAAV-LCuL-EAS) with or without Cre (as described above). The cells were washed within 24 hours of transfection, seeded on geltrex (basement membrane matrix) after 48 hours transfection. After 14-18 hours, the tubes were photographed and analysed with ImageJ Angiogenesis Analyser software (NIH; MD, USA). The schematic for the experiment is shown in Figure 9 and the results are shown in Figure 10.

Evaluating the regulation of inflammatory markers in vector constructs containing IL-10

ARPE-19 cells were seeded and transfected (as described above) with the vector containing Cu regulated IL-10, pAAV-LCuL-IL-10. This vector, containing the anti-inflammatory cytokine IL-10, was used to test the induction of proinflammatory signalling by measuring the expression of IL-6. Cre was used to activate the internal kill

switch and silence the activated as well as the inactivated vector. After transfection of ARPE-19, the cells were washed for 2 days and the media collected on day 4 was used in enzyme linked immusorbent assay (ELISA) to determine the concentration of IL-6 and IL-10, as per manufacturer's directions (elisakit.com-0012, elisakit.com-0031). The schematic for the experiment is shown in Figure 11 and the results are shown in Figure 12.

Statistical analysis

All statistical analysis was performed using Sigma Plot 13.0 (Systat Software; CA, USA). All differences in gene regulation of vectors were assessed by t-test with a p-value <0.05 considered statistically significant.

EXAMPLE 2 – Expression vectors

Tetracycline or Cumate repressor genes (SEQ ID NOs: 2 and 3 respectively) were cloned into repressor cloning site 1 of the vector backbone (Figure 1) using XbaI and EcoRI restriction sites. The corresponding promoter was cloned into the promoter cloning site 2 using BstBI and SalI restriction sites (SEQ ID NOs: 4 and 5 respectively). The conditionally regulated polypeptide was cloned into the GOI cloning site 3 using SalI and NheI restriction sites (GFP, IL-10, Endo/Angiostation fusion protein, IL-1RA or IL-10/IL-1RA genes; SEQ ID NOs: 7 to 11) or the compatible XhoI and XbaI sites (Nano-Luciferase; SEQ ID NO: 12). Figure 2 shows a schematic of how the expression vectors were constructed.

EXAMPLE 3 – Kill switch activation

The expression vector was constructed with all expression sequences located within 2 LoxP sites (Figure 1 and Figure 2). In order to permanently silence expression, the cells were transfected with a vector expressing Cre. Cre removes all expression sequences by site-specific recombination at the two loxP sites (SEQ ID NO: 13). The resulting vector is absent of expression machinery and cannot induce transgene expression.

A human retinal pigmented epithelial cell line (aRPE-19) was transfected with vector constructs containing Tetracycline or Cumate regulatory elements or a CMV promoter, driving the expression of a marker gene, luciferase. Twenty-four hours later, the cells were treated with or without Tetracycline (Tet) or Cumate (Cu) and incubated for a further 48 hours before assessing the expression of luciferase. The ability of the system to kill expression of the transgene (luciferase) was examined by transfecting a

Cre vector into the cells to cut out the expression sequences between LoxP sites. The cells were washed and regulator compounds (Tet and Cu) were added back daily until day 7 when media was assayed for changes in expression levels of luciferase.

Expression of luciferase in cells treated with the Tet vector with (pAAV-LTetL-luc ON) or without (pAAV-LTetL-luc OFF) Tet activation is shown in Figure 5a. Expression of luciferase in cells treated with the Cu vector with or without Cu activation is shown in Figure 5b. Expression of luciferase following transfection with CMV vectors, with or without Cre mediated excision is shown in Figure 5c. Luciferase expression was effectively silenced in all Cre excised cells.

EXAMPLE 4 – Therapeutic regulation

The Tet On system that was used is summarised in Figure 6a. The EF1-HTLV promoter constituently expresses a Tet transactivator protein that is unable to bind to Tet responsive elements (TRE) in the Tet promoter resulting in its inactivation and the lack of transgene expression. In the presence of Tet, the transactivator proteins bind to the Tet Response Elements (TRE) in the promoter, activating it and inducing expression of the transgene. The Cu On system that was used is summarised in Figure 6b. In this system, the Cu repressor protein binds to the Cu operator sequence, in the absence of Cu, thus inactivating the promoter resulting in no transgene transcription. With the addition of Cu, the Cu binds to the repressor protein, bound to the Cu promoter, thus releasing the repressor allowing transgene transcription.

A human retinal pigmented epithelial cell line (aRPE-19) was transfected with vector constructs containing Tet or Cu regulatory sequences driving the expression of the marker gene luciferase. Twenty-four hours later, the cells were treated with or without Tet or Cu and incubated for a further 48 hours before assessing the expression of luciferase. The ability of the system to stop expression of the transgene (Luciferase) was examined by removing the Tet or Cu from the cells by washing prior to re-examining Luciferase expression. Cells without Tet or Cu were used as a control as were cells where Tet or Cu was not removed.

Figure 8a shows the results from the luminescence assay performed on day 7 of the experiment using tetracycline as the agent to switch on transgene production. Figure 8b shows the results from the luminescence assay performed on day 7 of the experiment using cumate as the agent to switch on transgene production. In both experiments, the substrate (Tet or Cu) was able to induce significant levels of luciferase expression and was regulated by the removal of the substrate resulting in significantly less luciferase being detected.

EXAMPLE 5 – Tube formation assay

Effects of a vector containing endo/angiostatin (EAS) flanked by LoxP sites on angiogenesis was assessed using a tube formation assay in primary human umbilical vein epithelial cells (HUVEC), with or without Cre (Figure 9). Early passage (p2-4) HUVECs were transfected with the cumate regulated vector producing EAS (pAAV-LCuL-EAS). Two days post transfection, cells were counted and seeded in geltrex coated wells and incubated at 37°C, 5% CO_2 for 14-18 hours after which the tubes formed in the geltrex were photographed and analysed using ImageJ angiogenesis analyser.

Figure 10A shows HUVEC tube formation in cells transfected with the non-EAS expressing pAAV-LCuL-EAS without Cu activation. Figure 10B shows the inhibition of tube formation when HUVECs were treated with activated pAAV-LCuL-EAS. Figure 10C shows the return of tube formation capacity when the activated pAAV-LCuL-EAS was excised by Cre. Figure 10D and 10E quantifies the total mesh area and total branch length respectively using ImageJ Angiogenesis Analyzer software. The pAAV-LCuL-EAS vector was able to reduce tube formation, which was largely reversed when the vector was excised by Cre.

EXAMPLE 6 – Pro-inflammatory signalling

Effects of a vector containing Cu regulated IL-10, flanked by LoxP sites on the induction of pro-inflamatory signalling was determined by measuring IL-6 expression with or without Cre (Figure 11). A human retinal pigmented epithelial cell line (aRPE-19) was transfected with vector constructs containing Cu regulatory sequences driving the expression of anti-inflammatory IL-10. The negative control consisted of the IL-10 vector lacking Cu activation, treated with Cre. The IL-10 group consisted of the IL-10 vector activated by Cu. The killed group consisted of activated IL-10 vector, treated with Cre. Cells were assayed by ELISA for the expression of IL-10 and the pro-inflammatory marker IL-6.

Figure 12 shows the results obtained from simultaneous ELISAs of IL-10 and IL-6 following transfection with cumate regulated vectors containing IL-10 with or without the addition of Cre. In cells treated with the negative control vector (without Cu and with Cre), the level of IL-10 detected was low, correlating to high levels of the proinflamatory IL-6. Conversely, cells treated with the Cu regulated IL-10 vector in the presence of Cu (IL-10 vector: on) expressed high levels of IL-10 which correlated to low levels of IL-6 expression. Vectors where the IL-10 was excised by Cre (IL-10 vector:

killed) had IL-10 and IL-6 levels comparable to that of the negative control showing that expression of IL-10 was effectively silenced.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the disclosure as shown in the specific embodiments without departing from the spirit or scope of the disclosure as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

All publications discussed and/or referenced herein are incorporated herein in their entirety.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

The present application claims priority from AU 2018900206 filed 23 January 2018, the entire contents of which are incorporated herein by reference.