Age-related Macular Degeneration treatment

Field of the invention

This invention is directed to an RNA interference (RNAi) agent and the use of that RNAi agent to treat Age-related Macular Degeneration, as well as pharmaceutical compositions containing the RNAi agents of the invention.

Background of the invention

Age related macular degeneration (AMD) is the leading cause of irreversible vision loss in the United States and many other industrialised countries. "Dry" AMD is the most common type of macular degeneration and affects 90% of the people who have the condition. The dry form is characterized by the formation of drusen within the macula, a specialized structural region of the retina which capture the light that enters the eye. Typically, drusen is formed under the retinal pigment epithelial (RPE) cells and its presence is thought to lead to atrophy of photoreceptors due to a breakdown or thinning of the RPE layer of that supports the photoreceptor cells. It is also thought that persistence of drusen within the retina leads to a persistent inflammatory reaction and results in a cascade of secondary responses that eventually can lead to wet AMD.

The "wet" form of AMD is characterized by an abnormal outgrowth of blood vessels from the vasculature situated behind the retina in a process that is often referred to as choroidal neovascularization (CNV). While not as prevalent as the dry form, it has a more rapid onset and is more severe phenotype, often leading to reduction of a substantial portion of the visual field.

The current standard of care for wet AMD is Ranibizumab (RAN), a monoclonal antibody fragment with strong affinity to the vascular endothelial growth factor-A (VEGF-A), a molecular moiety secreted from cells and known to cause the formation or growth of nascent blood vessels. RAN binds to and inhibits the biologic activity of VEGF-A, thereby preventing the interaction of VEGF-A with its receptors (VEGFR1 and VEGFR2) on the surface of endothelial cells. This results in a reduction in endothelial cell proliferation, less vascular leakage, and a reduction in new blood vessel formation characteristic of CNV.

The ocular half-life of RAN, however, is only nine days following intravitreal injection, thus therapeutic doses must be administered monthly to patients to remain effective at suppressing vascular proliferation. Although useful at stabilizing visual acuity in nearly 95% of patients, improved vision was noted in only 29%-40% of patients. RAN acts as a molecular sponge to mop-up secreted VEGF-A. Inefficiencies in this process may be one reason why vision is only stabilized, not improved in most patients. In other words, it treats the symptoms but not the cause.

The principal drawback with existing monoclonal antibody wet AMD therapies is the requirement for frequent, continuous treatment, typically involving monthly injections into the eye. Combined with a rapidly aging population and correspondingly low numbers of clinicians who are qualified to administer intravitreal injections, application of this therapy This has placed enormous strain on healthcare systems. Thus there is clearly a need for longer lasting treatments and/or treatments that can reverse the symptoms. Alternative treatments for wet AMD have been similarly unsatisfactory, also as a result of their frequency of administration, but as well as their side effects or poor efficacy.

One of the newer drugs to commence clinical trials is that of the VEGF Trap Eye (VTE) which incorporates the second binding domain of the VEGFR-1 receptor and the third domain of the VEGFR2 receptor 1. By fusing these extracellular protein sequences to the Fc segment of a human IgG backbone, developers have created a chimeric protein with a very high VEGF binding affinity (Stewart MW. Br J Ophthalmol (2012). doi:10.1136/bjophthalmol-2011-300654). As well as binding all isomers of the VEGF-A family, it also binds VEGF-B and placental growth factor.

Given the fact that the chimera protein still has a relatively short half-life, VTE however must still be regularly administered – every 2 months.

AAV2-sFLT01 is a gene therapy vector that expresses a modified soluble Flt1 receptor coupled to a human IgG1 Fc. As a high affinity VEGF binding protein, AAV2-sFLT01 functions to neutralize the pro-angiogenic activities of VEGF for treatment of wet AMD via an intravitreal injection. (Wasworth et al. Molecular Therapy vol. 19 no. 2 Feb. 2011; 326–334). The use of an AAV vector is expected to ensure long-term expression, lasting for many months or even years, from a single injection. However, in order to accommodate the sFLT01 and IgG1 Heavy Chain Fc fusion protein, single stranded

AAV must be used, which in turn requires high quantities of vector for efficient transduction and thus increases the risk of an immune response to the viral capsid proteins. Moreover, a high prevalence of the normal adult population has been exposed to serotype 2 variant of AAV, and may have pre-existing immunity against it.

The molecule PF-04523655 is a 19 nucleotide siRNA that inhibits the expression of the hypoxia-inducible gene RTP801 (Nguyen et al. Ophthalmology. 2012 Sep;119(9):1867-73). In clinical studies conducted to date, it has been found to prevent neovascularization and vessel leakage, although does so via a different pathway than VEGF. It has been demonstrated that the siRNA only persists in the eye for several weeks, meaning that like so many of the other existing and developing therapies, patients will require regular intravitreal injections for treatment. A failure to do so with many treatments has seen a continued loss of visual acuity, and a progression of degeneration.

More generally, previous siRNA-based approaches for treating and managing wet AMD have failed. Although initial pre-clinical experimental results were encouraging, it was subsequently demonstrated that mode of action of these molecules was not through a sequence specific RNAi-based mechanism, but rather through induction of a non-specific interferon response mediated by the interaction of siRNAs with Toll-like receptor TLR3 (Kleinmann *et al* 2008). Toll-like receptors are transmembrane proteins that play a key role in the innate immune system. Often positioned on either the cell surface or on intracellular vesicles such as the endosome, some family members of this family recognize double stranded RNA, not normally present in the endogenous cell, as foreign substance and triggers a cascade of molecule responses. This leads to interferon activation, which has a transitory therapeutic effect in mouse models. However interferon has a much lower efficacy in humans which explains the poor efficacy of this treatment in human clinical testing.

Retinostat is an equine infectious anaemia virus (EIAV) based lentivirus vector expressing angiostatin and endostatin, both of which are naturally occurring angiogenesis inhibitors in the ocular compartment. Endostatin blocks VEGF signalling, reduces vascular permeability, decreases cell matrix adhesion and promotes endothelial cell apoptosis. Angiostatin prevents endothelial cell proliferation and migration. The genes are delivered via a subretinal injection and inhibit the formation of

new blood vessels. Sub-retinal delivery however requires an intensive surgical procedure, which, unlike intravitreal delivery, does not lend itself to outpatient treatments or treatment at a local doctor.

Despite the large amount of development activity in the field of AMD therapeutics, and wet AMD in particular, there remains a need to create more effective therapies that are also patient friendly with respect to side effects, the mode of treatment and the frequency thereof. This invention is directed to a RNA interference (RNAi) agent and the use of that RNAi agent to manage and treat wet AMD in individuals.

The RNAi pathway is initiated by the enzyme Dicer, which cleaves double-stranded RNA (dsRNA) molecules into short fragments (commonly referred to as siRNAs) of ~20-25 nucleotides. One of the two strands of each fragment, known as the *guide strand* or *active strand*, is then incorporated into the RNA-induced silencing complex (RISC) through binding to a member of the Argonaute protein family. After integration into the RISC, the guide strand base-pairs with its target mRNA and is thought to either inhibit a target by inhibiting translation (by stalling the translational machinery) and/or inducing cleavage of the mRNA, thereby preventing it from being used as a translation template.

While the fragments produced by Dicer are double-stranded, only the guide strand, directs gene silencing. The anti-guide strand (referred to commonly as a passenger strand, carrier strand or * strand) is frequently degraded during RISC activation (Gregory R *et al.*, 2005). RISC assembly is thought to be governed by an enzyme that selects which strand of a dsRNA Dicer product is loaded into RISC. This strand is usually the one whose 5' end is less tightly paired to its complement. There also appears to be a clear bias for A, and to a lesser extent U, at the 5' position to facilitate binding to some Argonaute proteins (Schwarz DS *et al.*, 2003; Frank F *et al.*, 2010).

The present invention seeks to overcome the problems associated with other therapies as already discussed above, while overcoming the previous challenges faced by RNAi therapeutics in this field.

Reference to any prior art in the specification is not, and should not be taken as, an acknowledgment or any form of suggestion that this prior art forms part of the common general knowledge in Australia or any other jurisdiction or that this prior art could

reasonably be expected to be ascertained, understood and regarded as relevant by a person skilled in the art.

Summary of the invention

There is an unmet need for long term therapy for AMD, and in particular, wet AMD. It has been discovered by the current inventors that particular RNAi constructs have the ability to down-regulate the expression of genes associated with the development of AMD (collectively referred to as 'AMD associated genes'). This in turn can slow the progression of AMD and the accompanying vision loss, and in some instances, result in an improvement in visual acuity. By utilising RNAi technology to achieve long term suppression of those target sequences, together with the use of a vector delivery vehicle that directs the RNAi agent to the target cells in a non-invasive manner, this need can be met and it can be met in a patient convenient and friendly manner. Moreover, because RNA agents expressed from DNA directed RNAi (ddRNAi) constructs are produced in the nucleus and do not interact with Toll-like receptors on either the cell surface or within the endomal compartment, ddRNAi agents can be produced without activating an interferon response via the TLRs.

In one aspect of the invention, there is provided a DNA-directed RNA interference (ddRNAi) agent (being an RNA molecule), and an expression cassette or construct to express that agent in a cell (including *in vivo*), for inhibiting, preventing or reducing expression of one or more target sequences in an AMD associated gene, preferably a wet AMD associated gene, where the agent comprises

- an effector sequence (described further below) of at least 17 nucleotides in, and
- an effector complement sequence

wherein the effector sequence is complementary or substantially complementary to one or more target regions in a transcript of the one or more target sequences.

The target region can be selected from the group consisting of any 10 or more contiguous nucleotides within a transcript of a target sequence selected from any one or more of SEQ ID NOS: 1-39. The effector complement sequence is substantially complementary to the effector sequence such that it will tend to anneal so as to form a double stranded RNA segment.

The effector sequence is directed to a target region within a transcript of a target sequence of a target gene. Thus the effector sequence is 'directed to' a target region by being substantially complementary (as 'substantial complementarity' is defined below) in sequence to a transcript from a target gene containing the target region. An RNAi agent, such as a ddRNAi agent, having a double-stranded portion containing the effector sequence, can therefore "inhibit expression of a target gene sequence" by virtue of the target gene sequence containing the target region. Accordingly, within a cell having an AMD associated gene, the RNAi agent is capable of inhibiting expression of a target gene sequence because the sequence of the effector (as 'effector' is defined below) is substantially complementary to (at least) a region of the mRNA target sequence of the target gene. This can be illustrated by considering the following random, hypothetical short sequence:

5'GGCATTGCG3' – target region within target sequence

5'GGCAUUGCG3' – transcript of target sequence

3'GUAACG5' – effector sequence, which is substantially complementary to the target region in the transcript of the target sequence.

Typically, a target region is a region of nucleic acid sequence within the mRNA of a gene that is intended to be silenced or to have its expression (at the level of transcription or translation) reduced, inhibited or prevented.

As can be seen in the explanatory comparison above, 'substantial complementarity' between the effector sequence and the effector complement sequence can be 100% complementarity. However as more particularly explained and defined further below, substantial complementarity can be 80% to 100% complementary. So in an effector sequence having a length of, for example, 20 nucleotides, the effector sequence is substantially complementary to the effector complement sequence if 17 of the 20 nucleotides are complementary ie 85% complementarity. Moreover, usually one end of the double stranded segment will be linked by a loop sequence so as to form a 'hairpin' shaped structure referred to as shRNA. This is also known as an 'interrupted inverted repeat' structure, as the DNA encoding such an RNA sequence contains an inverted repeat of the region of the target gene that is transcribed to the effector sequence, interrupted by a stuffer or spacer sequence encoding the loop.

The concept of substantial complementary described in the paragraph above applies equally to the substantial complementarity between the effector sequence and the target sequence where substantial complementarity can be 80% to 100% complementarity. That is, if the target region within a target sequence is 20 nucleotides long, and the effector sequence is 20 nucleotides long, then the effector sequence may have, for example, 16, 17, 18 or 20 nucleotides that are complementary with the target, equating to 80%, 85%, 90% and 100% complementarity respectively.

In both situations, one will appreciate that substantial complementarity may not equate to a whole number. For example, at least 85% complementarity to a sequence of 22 nucleotides would be 18.7 nucleotides, so is effectively a requirement for 19 of 22 to be complementary.

Alternatively, substantial complementarity of 80 to 100% complementarity (both in the context of substantial complementarity between the effector and target, and effector and its complement) can be described with reference to the number of nucleotides that will not G-C/A-U base pair (except for wobble pairs as described below). There may be 1, 2, 3, 4 or 5 nucleotides within the complementary region between the 2 RNAs that are not themselves complementary with a nucleotide on the other strand when considering at least 80% complementarity across a nucleotide sequence. As to whether there can be 1, 2, 3, 4 or 5 nucleotides that do not base pair is dependent on the length of the relevant sequence. For example, if the effector sequence is 17 nucleotides long, it cannot have 5 nucleotides that will not base pair, as this would equate to only 71% complementarity. In a 17 nucleotide sequence, there must be complementarity between 14 of the 17 nucleotides for at least 80% complementarity.

In a preferred embodiment of the invention, the double stranded region formed by the effector sequence and its complement is expressed as part of a microRNA (miRNA) structure similar to the structure of endogenous miRNAs which are a natural substrate for endogenous RNAi processing pathways. Processing of double stranded RNAs expressed from ddRNAi constructs can be imprecise, and can result in toxicity. McBride *et al.* (2008) designed "artificial miRNA" constructs which expressed sequences from the base and loop of endogenous miRNAs, and suggested that more precise processing of expressed shRNAs from the miR-backbone led to reduced toxicity from the constructs. Wu *et al.* (2011) showed that mismatched duplexes (containing

mismatches in the passenger strand) sometimes showed increased silencing activity, due possibly to their greater structural resemblance to endogenous miRNAs.

In one aspect of the invention, there is provided a ddRNAi agent and an expression cassette to express that agent in a cell, for inhibiting, preventing or reducing expression of an AMD associated gene, preferably a wet AMD associated gene, where the agent comprises

an effector sequence of at least 17 nucleotides in length complementary to or substantially complementary to one or more target regions in a transcript of a target region, and an effector complement sequence

wherein the effector sequence and the effector complement sequence are expressed within a miRNA structure. The target region may be selected from the group consisting of any 10 or more contiguous nucleotides within a transcript of a sequence selected from any one or more of SEQ ID NOS: 1-39.

In some forms of the invention, the agent has more than one effector sequence. Multiple effectors may target the same region of a wet AMD associated gene (typically variants of the same region), different regions of a wet AMD associated gene, more than one wet AMD associated gene, or a combination of all of the above.

RNAi agents, such as ddRNAi agents, can contain 2 or 3 or more effector sequences. As explained above, the ddRNAi agent comprises an effector complement sequence for each effector sequence, thus forming effector – effector complement pairs (ie a first effector – first effector complement pair, a second effector – second effector complement pair, etc). These pairs may be, but need not be, contiguous to one another, as long as the RNAi agent can fold so as to permit each pair to anneal. Various other considerations suggest one order or another of the effectors and effector complements along the length of the RNAi agent. In addition, as would be understood by one skilled in the art, and as illustrated in the Figures, any particular effector sequence may be swapped in position with its complement in the agent. The important feature, as exemplified in the various embodiments below, is that the effector sequence is able to anneal with its complement to form a double stranded region. For example:

- ddRNAi agent comprising, in a 5' to 3' direction, a first effector sequence; a second effector sequence; second effector complement sequence ; and a first effector complement sequence;
- a ddRNAi agent comprising, in a 5' to 3' direction, a first effector sequence; a second effector sequence; a third effector sequence; a third effector complement sequence; a second effector complement sequence; and a first effector complement sequence;
- a ddRNAi agent comprising, in a 5' to 3' direction, a first effector; a first effector complement sequence; a second effector sequence; and a second effector complement sequence;
- a ddRNAi agent comprising, in a 5' to 3' direction, a first effector sequence; a first effector complement sequence; a second effector sequence; a second effector complement sequence; a third effector sequence; and a third effector complement sequence;
- a ddRNAi agent comprising, in a 5' to 3' direction, a first effector sequence; a second effector sequence; a loop sequence of 2 to 100 non-self-complementary nucleotides; a second effector complement sequence; and a first effector complement sequence;
- a ddRNAi agent comprising, in a 5' to 3' direction, a first effector sequence; a loop sequence of 2 to 100 non-self-complementary nucleotides; a first effector complement sequence; a sequence of 2 to 100 non-self-complementary nucleotides; a second effector sequence; a loop sequence of 2 to 100 non-self-complementary nucleotides; and a second effector complement sequence;
- a ddRNAi agent comprising, in a 5' to 3' direction, a first effector sequence; a loop sequence of 2 to 100 non-self-complementary nucleotides; a first effector complement sequence; a spacer sequence of 2 to 100 non-self-complementary nucleotides; a second effector sequence; a loop sequence of 2 to 100 non-self-complementary nucleotides; and a second effector complement sequence;
- a ddRNAi agent comprising, in a 5' to 3' direction, a first effector sequence; a first effector complement sequence; a spacer sequence of 2 to 100 non-self-

complementary nucleotides; a second effector sequence; a second effector complement sequence; a spacer sequence of 2 to 100 non-self-complementary nucleotides; a third effector sequence; and a third effector complement sequence.

The non-self-complementary nucleotides act as loop sequences when located between an effector and its complement, and as spacer sequences when located between the complement of one effector sequence, and the next effector sequence. In each of these embodiments, the effector sequence and its complement, as well as any additional sequence such as a sequence of 2 to 100 non-self-complementary nucleotides, is expressed within or part of a miRNA structure.

In particular forms of each of the embodiments described above, each effector sequence is at least 17 nucleotides in length, preferably 17 to 30 nucleotides in length, and more preferably 17 to 21 nucleotides in length, and comprises a nucleotide sequence selected from the group consisting of any 10 or more contiguous nucleotides from a sequence from any one of SEQ ID NOS: 40-78. The effector sequences may all be the same, or may all be different, or may be a combination, e.g. 2 effector sequences of at least 10 contiguous nucleotides of SEQ ID NO:47 and one effector sequence of at least 10 contiguous nucleotides of SEQ ID NO: 56.

Preferably, the effector sequence is selected from the group consisting of any contiguous 11, 12, 13, 14, 15 or 16 nucleotides within any one of SEQ ID NOS: 40-78, and preferably 17 or more contiguous nucleotides within any one of SEQ ID NOS: 40-78 and most preferably 17 to 21 contiguous nucleotides within any one of SEQ ID NOS: 40-78. Typically, the effector complement will be the same length, or about the same length (ie ± 15% nucleotide length, or 1 to 3 nucleotides depending on the total length) as its corresponding effector sequence.

In particular embodiments the effector sequence of the ddRNAi agent consists of, or consists essentially of, a nucleotide sequence selected from the group consisting of any one of SEQ ID NOS: 40-78 inclusive. In these embodiments, a ddRNAi agent SEQ ID NOS: 40-78 as well as additional nucleotides or other chemical modifications would "consist essentially of" SEQ ID NOS: 40-78 as long as it exhibits activity for inhibiting, reducing or preventing the expression of the target gene, as may be determined in accordance with the assays described below. Similarly, an RNAi agent "consists

essentially of" one of SEQ ID NOS: 40-78 where it is shorter than the corresponding SEQ ID as long as it exhibits activity for inhibiting, reducing or preventing the expression of the target gene, as may be determined in accordance with the assays described below.

In alternative embodiments, the dsRNA is comprised of 2 separate RNA strands that are annealed to form a duplex. That duplex may then be embedded in a miRNA backbone.

ddRNAi agents may be expressed from a DNA expression cassette inserted into any suitable vector or ddRNAi construct. Accordingly, in aspects of the invention there is provided a ddRNAi expression cassette comprising (in no particular order):

- one or more promoter sequences
- one or more DNA sequences that encode for one or more effector sequences, preferably being DNA sequences that encode for any 10 or more and preferably any 17 or more contiguous nucleotides within a sequence from any one of SEQ ID NOS: 40-78,
- one or more DNA sequences that encode for one or more effector complement sequences

and optionally

- one or more terminator sequences
- one or more DNA sequences that encode for spacer sequences, loop sequences, or both; and
- one or more enhancer sequences.

In some embodiments, one promoter is operably linked to multiple effector-encoding regions such that a ddRNAi agent with multiple effector sequences is produced. In alternative embodiments, where each effector-encoding region is operably linked to its own promoter, multiple ddRNAi agents are produced from a single expression cassette. In constructs where there are multiple promoters, these may be all the same or different. Preferred promoters are pol III promoters such as U6 and H1; pol II promoters such as the RPE cell specific promoter RPE-65 (Boye *et al.* 2012) and VMD2 (Zhu *et al.*

2010), and choroid endothelial-specific promoters FLT-1 or ICAM2 can also be used to drive expression of ddRNAi constructs.

In embodiments where the effector sequence and its complement, are expressed within a miRNA structure, the ddRNAi expression cassette additionally comprises sequences that encode for the miRNA structure referred to herein as miRNA encoding (ME) sequences. The ME sequences may also encode for loop sequences.

There is also provided ddRNAi expression constructs, into which the ddRNAi expression cassettes are inserted for expression. In addition, when the vector backbone of the construct is compatible with a delivery system, the ddRNAi expression constructs are also delivery constructs. A particularly preferred delivery construct is a viral vector, such as a modified adeno-associated virus (AAV) vector (Petrs-Silva et al. 2011) that allows delivery of ddRNAi expression cassettes to appropriate cells deep in the retina following intravitreal injection. Use of a modified AAV to deliver an expression construct that produces the therapeutic ddRNAi agent from within the cell avoids an interferon response often caused by direct interactions of nucleic acids with surface-expressed toll-like receptor 3. This is hypothesised to be the reason for a number of failures of siRNA-based ocular drugs in clinical trials.

Accordingly, in this embodiment there is provided a ddRNAi expression construct comprising a ddRNAi expression cassette for expressing a ddRNAi agent for inhibiting expression of one or more target sequences in an AMD associated gene, the expression cassette comprising (in no particular order)

one or more promoter sequences

one or more DNA sequences that encode for one or more effector sequences,

one or more DNA sequences that encode for one or more effector complement sequences;

and optionally

one or more terminator sequences

one or more DNA sequences that encode for loop sequences, spacer sequences or both,

one or more enhancer sequences,

wherein the construct is a viral vector delivery vehicle.

Preferably the expression cassette further comprises ME sequence so that the ddRNAi agent is expressed as part of or within a miRNA structure.

In one embodiment, the expression cassette of the viral vector delivery construct comprises one DNA sequences that encodes a first effector sequence of any 10 or more contiguous nucleotides within 5' UAUGUGGGUGGGUGUGUCUAC 3' of the AMD-associated gene VEGF-A (SEQ ID NO:47).

In a further embodiment, the expression cassette of the viral vector delivery construct comprises two DNA sequences that encode a first effector sequence of any 10 or more contiguous nucleotides within 5' UGUAACAGAUGAGAUGCUCCA 3' of the AMD-associated gene VEGRF-2 (SEQ ID NO:56) and a second effector sequence of any 10 or more contiguous nucleotides within 5' UAUGUGGGUGGGUGUGUCUAC 3' of the AMD-associated gene VEGFA (SEQ ID NO:47).

In yet another alternative embodiment, the expression cassette of the viral vector delivery construct comprises three DNA sequences that encode a first effector sequence of any 10 or more contiguous nucleotides within 5' AAGUAGCCAGAAGAACAUGGC 3' of the AMD-associated gene VEGRF-2 (SEQ ID NO:52); a second effector sequence of any 10 or more contiguous nucleotides within 5' UUAUAGAAAACCCAAAUCCUC 3' of the AMD-associated gene CFB (SEQ ID NO:78); and a third effector sequence of any 10 or more contiguous nucleotides within 5' UAGCUGAAGCCCACGAGGUCC 3' of the AMD-associated gene PDGFR-β (SEQ ID NO:63).

The invention also provides for siRNA agents that comprise a sequence of at least 17 nucleotides in length selected from the group consisting of any 10 or more contiguous nucleotides within a sequence from any one of SEQ ID NOS: 40-78 and a sequence complement with which the sequence forms a duplex, and that are capable of inhibiting expression of a wet AMD associated gene.

In accordance with some embodiments, there is provided a method of inhibiting the expression of an mRNA or polypeptide encoded by an AMD associated gene in a subject comprising administering to the subject a composition of the invention comprising a ddRNAi agent that consists essentially of or consists of a nucleotide

sequence selected from the group consisting of any one of SEQ ID NOS: 40-78 and sequences that vary from SEQ ID NOS: 40-78 by 1, 2, 3, 4 or 5 nucleotides. A ddRNAi expression cassette or ddRNAi expression construct for expressing the ddRNAi agent may also be administered.

In another embodiment the invention provides a composition for the treatment of AMD in a subject, preferably wet AMD, or treatment of other diseases that are caused by inappropriate vascularisation within the retina, comprising as an active ingredient a ddRNAi agent, ddRNAi expression cassette or ddRNAi expression construct of the invention for inhibiting, preventing or reducing expression of one or more target sequences in an AMD associated gene.

In another embodiment the invention provides a pharmaceutical composition comprising an effective amount of a ddRNAi agent, ddRNAi expression cassette or ddRNAi expression construct of the invention as a main ingredient for inhibiting, preventing or reducing expression of one or more target sequences in an AMD associated gene.. The composition may be used for example for the treatment of AMD in a subject, preferably wet AMD, or treatment of other diseases that are caused by inappropriate vascularisation within the retina. In some embodiments, the composition further comprises a pharmaceutically acceptable carrier or diluent.

In another embodiment the invention provides a composition for the treatment of AMD in a subject, preferably wet AMD, or treatment of other diseases that are caused by inappropriate vascularisation within the retina, comprising as an active ingredient a ddRNAi agent, ddRNAi expression cassette or ddRNAi expression construct of the invention for inhibiting, preventing or reducing expression of one or more target sequences in an AMD associated gene.

In another embodiment the invention provides a composition for inhibiting, preventing or reducing expression of one or more target sequences in an AMD associated gene comprising a ddRNAi agent, ddRNAi expression cassette or ddRNAi expression construct of the invention for use in the treatment of wet AMD in a subject. In some embodiments, the composition further comprises a pharmaceutically acceptable carrier or diluent.

In another embodiment, the invention provides a ddRNAi agent, ddRNAi expression cassette or ddRNAi expression construct for inhibiting, preventing or reducing expression of one or more target sequences in an AMD associated gene in the preparation of a medicament for the treatment of AMD in a subject. Preferably the medicament is for wet AMD.

In another embodiment the invention provides an AMD treatment composition comprising an effective amount of a ddRNAi agent, ddRNAi expression cassette or ddRNAi expression construct of the invention for inhibiting, preventing or reducing expression of one or more target sequences in an AMD associated gene as a main ingredient, optionally with a pharmaceutically acceptable carrier or diluent.

The invention also provides a method for treating or delaying the progression of diseases that are caused by inappropriate vascularisation within the retina in a subject, comprising administering to the subject a ddRNAi agent, ddRNAi expression cassette or ddRNAi expression construct or composition of the invention for inhibiting, preventing or reducing expression of one or more target sequences in an AMD associated gene, thereby reducing the severity of AMD.

Yet a further aspect of the invention provides a method for reducing the progression of AMD in a subject, preferably wet AMD, comprising administering to the subject a ddRNAi agent, ddRNAi expression cassette or ddRNAi expression construct or composition of the invention for inhibiting, preventing or reducing expression of one or more target sequences in an AMD associated gene, thereby reducing the severity of AMD.

In each of the methods of the invention, the ddRNAi agent, ddRNAi expression cassette or ddRNAi expression construct or composition of the invention is preferably delivered to the subject's eye/s by intravitreal injection or subretinal injection.

In a further aspect, the present invention provides a kit of parts including (a) a ddRNAi agent, ddRNAi expression cassette or ddRNAi expression construct or composition of the invention and (b) a pharmaceutically acceptable carrier or diluent.

In certain embodiments an RNAi agent or pharmaceutical composition of the invention may be provided in the form of a device, disposable or reusable, including a receptacle for holding the RNAi agent or pharmaceutical composition. In one embodiment, the

device is a syringe, preferably a syringe suitable for intravitreal injection or subretinal injection. The RNAi agent or pharmaceutical composition may be provided in the device in a state that is ready for use or in a state requiring mixing or addition of further components.

Although the invention finds application in humans, the invention is also useful for veterinary purposes. The invention is useful for the treatment of AMD or other diseases caused by inappropriate vascularisation in domestic animals such as cattle, sheep, horses and poultry; companion animals such as cats and dogs; and zoo animals.

Brief description of the drawings / figures

Figure 1A-G illustrates some of the ddRNAi agent structures of the invention.

Fig 2: A. Map of pSilencer (Invitrogen). This expression cassette contains the human U6 promoter (black arrow) and was designed to express shRNA sequences cloned into this vector as BamH I / Hind III fragments. **B.** A generalized map showing the schematic layout of a BamH I / Hind III shRNA fragment designed to silence AMD associated genes. The positions of BamHi / Hind III restriction sites are shown; the white arrows denote sequences from the 5' stem of miR30a, a sequence derived from the loop of mir30a and the 3' stem of miR30a. The grey arrow represents the predicted passenger strand and the black arrow the predicted guide strand. The relative positioning of the predicted guide strand and the predicted passenger strand may be interchangeable. The black line denotes a pol III termination signal. **C.** DNA sequence of the miR-8 fragment, which potently silences VEGF-A, is shown and corresponds to SEQ ID NO: 98. The lowercase letters denote restriction sites, mir30a-related sequences and pol III terminator sequences. The underlined sequences are derived from the base of human miR30a pre-cursor RNA (both 5' and 3'); sequences in italics are derived from the loop sequences of miR30a. The upper case sequences indicate the predicted passenger strand sequence, the bold uppercase sequences denote the predicted effector sequence (SEQ ID NO: 47). **D.** Predicted RNA secondary structure of miR-8, determined using the M-fold program (SEQ ID NO:147); the predicted Dicer and Drosha processing sites are indicated by arrows.

Fig 3: A. Map of pGL3-VEGFA-sense reporter. The plasmid encodes firefly luciferase (Fluc+) driven by the SV40 promoter (grey arrows) and a eukaryotic transcriptional

terminator. A portion of the non-coding strand of the VEGF-A gene was inserted into the 3' UTR of FLuc+ transcriptional unit using Xba I and Fse I restriction sites present in the 3'UTR of the parent plasmid (pGL3; Promega). This plasmid was used to quantify inhibitory activity of the passenger strand of miR-2 in dual luciferase assays. **B.** Map of pGL3-VEGFa-antisense reporter, features are shown as in Figure 3A. The corresponding portion of the VEGF-A gene used in Fig 3A was inserted into the 3' UTR of FLuc+ transcriptional unit using Xba I and Fse I restriction sites, but used the coding strand of the VEGF-A gene. This plasmid was used to quantify inhibitory activity of the effector strand of miR-2 in dual luciferase assays.

Figure 4: A. The graph shows the percent inhibition of VEGF-A expression determined using dual luciferase assays; activities against both sense and antisense targets in sensor constructs are shown (n = 3 ± SD). **B.** The graph shows percent inhibition of VEGF-A mRNA levels determined by qRT PCR in HEK293T cells co-transfected with either miR-2, miR-5 and miR-8 along with a full length cDNA that expresses the full length VEGF-A protein. Percent inhibition is calculated to untransfected cells and empty vector controls (pSilencer and an empty U6 expression cassette). **C.** The graph shows levels of VEGF-A mRNA and protein in ARPE-19 cells that have been transduced with an adenovirus vector expressing miR-8. Samples of RNA and protein were collected at 24, 48, 72 and 96 hours post transduction. The triangles show intracellular levels of mature, processed miR-8 in which the loop sequences have been cleaved.

Figure 5: A. The graph shows the percent inhibition of VEGFR2 determined using firefly luciferase reporters as previously described; activities against both sense and antisense reporter constructs are shown (n = 3 ± SD). **B.** The graph shows percent inhibition of VEGFR2 mRNA levels determined by RT QPCR in HEK203T cells that were co-transfected with miR-V-2, miR-V-3, miR-V-7 or miR-V-10 and a plasmid expressing a full length cDNA to VEGFR2. Percent inhibition was calculated as mRNA remaining as compared to empty vector controls (pSilencer; Invitrogen and an unrelated plasmid). **C.** Western blot analysis of cells transfected in parallel conditions as in 5B and showing reductions in VEGFR2 (arrow). Protein extracts from HUVEC cells were run in parallel to show positioning of VEGFR2 on the gel.

Figure 6: A. The graph shows percent inhibition of PDGFR-β mRNA levels in HEK293T cells that were co-transfected with either miR-V-4 or miR-V-9 and a plasmid expressing a full length cDNA to PDGFR-β. Percent inhibition was calculated as mRNA remaining as compared to controls (pSilencer, Invitrogen and an unrelated plasmid and an empty U6 expression cassette). **B.** Western blot analysis of cells transfected in parallel conditions as in 6B and showing reductions in PDGFR-β (arrows).

Figure 7: A. The graph shows the percent inhibition of CFB expression determined using firefly luciferase reporters as previously described; activities against both sense and antisense reporter constructs are shown (n = 3 ± SD). **B.** The graph shows percent inhibition of CFB mRNA levels in HEK293T cells co-transfected with miR-C-1, miR-C-8 or miR-C-9 and a plasmid expressing a full length cDNA to CFB. Percent inhibition was calculated as compared to controls (pSilencer, Invitrogen, an unrelated plasmid and an empty U6 expression cassette). **C.** Western blot analysis performed on parallel treated wells as in 7B showed reductions in CFB (arrows).

Figure 8: A. Map of U6-miR-7. This uses the human U6 promoter (black arrow) to drive expression of miR-7 which targets VEGF-A. The miR-7 coding sequences are identical to those in Fig. 2A and are shown as a white arrow, the positions of miR-7 passenger and miR-7 effector sequences are shown as grey arrows. The sequence of the U6-miR-7 fragment is listed as SEQ ID NO: 132. **B.** Map of VMD2-miR-7. This uses the human VMD2 promoter (black arrow) to drive expression of miR-7 (white arrow), which targets VEGF-A. The sequence of the VMD2-miR-7 fragment is listed as SEQ ID NO: 133. **C.** Map of ICAM2-miR-7. This uses the human ICAM2 promoter (black arrow) to drive expression of miR-7 (white arrow), which targets VEGF-A. The sequence of the ICAM2-miR-7 fragment is listed as SEQ ID NO: 134. **D.** Map of RPE-65-miR-7. This uses the human RPE65 promoter (black arrow) to drive expression of miR-7 (white arrow), which targets VEGF-A. The sequence of the RPE65-miR-7 fragment is listed as SEQ ID NO: 135. **E.** Map of FLT-miR-7. This uses the human FLT promoter (black arrow) to drive expression of miR-7 (white arrow), which targets VEGF-A. The sequence of the FLT-miR-7 fragment is listed as SEQ ID NO: 136.

Figure 9: A. Map of U6-miR-7-miR-V-7. This uses the human U6 promoter (black arrow) to drive expression of miR-7-miR-V-7 which targets VEGF-A and VEGFR2. The miR-7-miR-V-7 coding sequences are shown as a white arrow, the positions of miR-7

passenger and miR-7 effector sequences and miR-V-7 passenger and miR-V-7 effector sequences are shown as grey arrows. The sequence of the U6-miR-7 fragment is listed as SEQ ID NO: 137. **B.** Map of VMD2-miR-7 miR-V-7. This uses the human VMD2 promoter (black arrow) to drive expression of miR-7 miR-V-7 (white arrow), which targets VEGF-A and VEGFR2. The sequence of the VMD2-miR-7 miR-V-7 fragment is listed as SEQ ID NO: 138. **C.** Map of ICAM2-miR-7 miR-V-7. This uses the human ICAM2 promoter (black arrow) to drive expression of miR-7 miR-V-7 (white arrow), which targets VEGF-A and VEGFR2. The sequence of the ICAM2-miR-7 miR-V-7 fragment is listed as SEQ ID NO: 139. **D.** Map of RPE-65-miR-7 miR-V-7. This uses the human RPE65 promoter (black arrow) to drive expression of miR-7 miR-V-7 (white arrow), which targets VEGF-A and VEGFR2. The sequence of the RPE65-miR-7 miR-V-7 fragment is listed as SEQ ID NO: 140. **E.** Map of FLT-miR-7 miR-V-7. This uses the human FLT promoter (black arrow) to drive expression of miR-7 miR-V-7 (white arrow), which targets VEGF-A and VEGFR2. The sequence of the FLT-miR-7 fragment is listed as SEQ ID NO: 141.

Figure 10: **A.** Map of U6-miR-V-7-miR-C-8-miR-P-9. This uses the human U6 promoter (black arrow) to drive expression of miR-V-7-miR-C-8-miR-P-9 which targets VEGFR2, CFB and PDGFR- β. The miR-V-7-miR-C-8-miR-P-9 coding sequences are shown as a white arrow, the positions of miR-V-7 passenger and miR-V-7 effector sequences, miR-C-8 passenger and miR-C-8 effector sequences, and miR-P-9 passenger and miR-P-9 effector sequences are shown as grey arrows. The sequence of the U6-miR-V-7-miR-C-8-miR-P-9 fragment is listed as SEQ ID NO: 142. **B.** Map of VMD2-miR-V-7-miR-C-8-miR-P-9. This uses the human VMD2 promoter (black arrow) to drive expression of miR-V-7-miR-C-8-miR-P-9 (white arrow), which targets VEGFR2, CFB and PDGFR- β. The sequence of the VMD2-miR-V-7-miR-C-8-miR-P-9 fragment is listed as SEQ ID NO: 143. **C.** Map of ICAM2-miR-V-7-miR-C-8-miR-P-9. This uses the human ICAM2 promoter (black arrow) to drive expression of miR-V-7-miR-C-8-miR-P-9 (white arrow), which targets VEGFR2, CFB and PDGFR- β. The sequence of the ICAM2 miR-V-7-miR-C-8-miR-P-9 fragment is listed as SEQ ID NO: 144. **D.** Map of RPE65-miR-V-7-miR-C-8-miR-P-9. This uses the human RPE65 promoter (black arrow) to drive expression of miR-V-7-miR-C-8-miR-P-9 (white arrow), which targets VEGFR2, CFB and PDGFR- β. The sequence of the RPE65-miR-V-7-miR-C-8-miR-P-9 fragment is listed as SEQ ID NO: 145. **E.** Map of FLT-miR-V-7-miR-C-8-miR-P-9. This uses the

human FLT promoter (black arrow) to drive expression of miR-V-7-miR-C-8-miR-P-9 (white arrow), which targets VEGFR2, CFB and PDGFR- β. The sequence of the FLT-miR-V-7-miR-C-8-miR-P-9 fragment is listed as SEQ ID NO: 146.

Figure 11: sequences referred to throughout the specification.

Detailed description of the embodiments

Reference will now be made in detail to certain embodiments of the invention. While the invention will be described in conjunction with the embodiments, it will be understood that the intention is not to limit the invention to those embodiments. On the contrary, the invention is intended to cover all alternatives, modifications, and equivalents, which may be included within the scope of the present invention as defined by the claims.

One skilled in the art will recognize many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. The present invention is in no way limited to the methods and materials described.

It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the invention.

For purposes of interpreting this specification, the following definitions will apply and whenever appropriate, terms used in the singular will also include the plural and vice versa. In the event that any definition set forth conflicts with any document incorporated herein by reference, the definition set forth below shall prevail.

Definitions

As used herein, except where the context requires otherwise, the term "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not intended to exclude further additives, components, integers or steps.

The term "RNA interference" or "RNAi" refers generally to a RNA dependent gene silencing process that is initiated by double stranded RNA (dsRNA) molecules in a cell's cytoplasm. The dsRNA reduces the expression of a target nucleic acid sequence, which may be a DNA whose RNA expression products are reduced, or an RNA, with which the dsRNA molecule shares substantial or total homology.

By "double stranded RNA" or "dsRNA" it is meant a double stranded RNA molecule that is capable of inhibiting expression of a target nucleic acid sequence with which it shares homology. In some embodiments the dsRNA is a hairpin or stem loop structure, with a duplex region optionally linked by at least 1 nucleotide, and is referred to as a "hairpin RNA" or "short hairpin RNAi agent" or "shRNA". The duplex is formed between an effector sequence and a sequence complementary to the effector sequence herein referred to as an "effector complement". Typically, the effector complement will be the same length as its corresponding effector sequence. As will be explained below, the effector sequence is complementary to the target nucleic acid sequence.

An "effector sequence" is the nucleotide sequence that, when part of the RISC complex, binds to the target nucleotide sequence, thereby targeting that sequence for destruction by the cell. It is analogous to the "guide" strand discussed in the background section. The effector sequence is 'directed to' a target region by being complementary or substantially complementary in sequence to the transcript from the target region such that an RNA agent having a double stranded portion containing the effector sequence inhibits expression of the target gene sequence.

The "effector complement", which is analogous to the passenger strand discussed in the background is of sufficient complementarity to the effector such that it anneals to the effector sequence. It is likely that the effector complement will be of a similar sequence to the target gene sequence, but does not necessarily have to be.

As already detailed in the sections above "substantially complementary", or "substantial complementarity", it is meant that the sequences are of sufficient complementarity to enable hybridisation of annealing (as later defined). Briefly, substantial complementarity as described above may be described in terms of:

- percentage identity (being 80 to 100%) between an effector and its complement, or between an effector and the target region of a target sequence; or
- number of nucleotides that are not complementary, being 1, 2, 3, 4 or 5, provided that number is consistent with the percentage identity requirement of 80 to 100%.

Substantial complementarity therefore includes 100% complementarity, but 100% complementarity may also be referred to throughout the specification as "complementary", or "being complementary". A sequence complementary to or

substantially complementary to a region of a target gene has the degree of sequence complementarity across a contiguous target sequence. Generally, a double stranded RNA region of the invention may be subjected to mutagenesis to produce single or several nucleotide substitutions, deletions or additions. It is believed that this level of difference between an effector and its complement, or between an effector and the target region of a target sequence will not negatively impact on the ability of the ddRNAi agent to be able to inhibit expression of the target sequence.

When the first effector sequence does have 1, 2, 3, 4 or 5 nucleotides that will not G-C/A-U base pair with the target sequence, it is preferred that the differences are in the first or last 5 nucleotides of the first effector sequence, with only 1 or 2 nucleotide changes in the centre portion of the effector sequence.

As noted above, substantial complementarity is intended to mean that the sequences are hybridisable or annealable. The terms "hybridising" and "annealing" (and grammatical equivalents) are used interchangeably in this specification in respect of nucleotide sequences and refer to nucleotide sequences that are capable of forming Watson-Crick base pairs due to their complementarity. Preferably the substantially complementary sequences are able to hybridise under conditions of medium or high stringency:

- high stringency conditions: 0.1xSSPE (or 0.1xSSC), 0.1%SDS, 65°C
- medium stringency conditions: 0.2xSSPE (or 1.0xSSC), 0.1%SDS, 50°C

Alternatively, "substantially complementary" would also be understood by the person skilled in the art to involve non-Watson-Crick base-pairing, especially in the context of RNA sequences, such as a so-called "wobble pair" which can form between guanosine and uracil residues in RNA. "Complementary" is used herein in its usual way to indicate Watson-Crick base pairing, and "non-complementary" is used to mean non-Watson-Crick base pairing, even though such non-complementary sequences may form wobble pairs or other interactions. In the context of the present invention, reference to "non-pairing" sequences relates specifically to sequences between which Watson-Crick base pairs do not form.

The term "RNAi agent" refers to a dsRNA sequence that elicits RNAi. This term may be used interchangeably with "small interfering RNAs" (siRNA agents) and small hairpin RNA (shRNAi or hpRNAi agents), wherein a hairpin has a stem-loop structure.

The "loop" of a hairpin structure is an additional sequence wherein at least some of the nucleotides are non-complementary to either itself, the target sequence, the effector sequence or the effector complement. The loop can be a sequence of 2 to 100 nucleotides which are capable of forming a loop. Not all of the nucleotides of the loop sequence need be non-annealed. For example, in a loop sequence of **ACU**GUGAAGCAGAUG**AGU**, nucleotides ACU may be annealed with AGU, while the intervening GUGAAGCAGAUG sequence remains non-annealed.

In embodiments in which the ddRNAi agent is expressed as part of a miRNA structure, the loop sequence may be derived from the miRNA, and is encoded by the ME sequence.

A "microRNA" or "miRNA" is a naturally occurring, small non-coding RNA molecule present in organisms that functions in the post-transcriptional regulation of gene expression. miRNA transcripts are capable of forming hairpin-like structures; typically contain mismatches and bulges within or adjacent to the double stranded RNA regions. The miRNA structure in which the ddRNAi agents of the invention are preferably expressed contains mismatches and insertions, as detailed above. Wu *et al.* (2011) showed that mismatched duplexes (containing mismatches in the passenger strand) sometimes showed increased silencing activity, due possibly to their greater structural resemblance to endogenous miRNAs. Similarly Gu *et al.* (2012) showed the introduction of bulges adjacent to loop sequences in shRNA molecules can result in increased precision of Dicer processing.

In the double stranded, folded miRNA structure, at least 50% of the nucleotides on the top strand are annealed to nucleotides of the bottom strand. Of the non-annealed (ie unpaired) nucleotides, they may be insertions ie they lack a complementary nucleotide on the opposing strand, or they may be mismatches such that they do not anneal. For example, a G and an A. The double stranded, folded miRNA structure can contain 2 or more annealed nucleotides, separated by 1 or more non-annealed nucleotides, to give a double stranded RNA structure with "bubbles" or 'bulges" where the nucleotides are not annealed.

By "miRNA encoding sequence" or "ME sequence", it is meant the DNA sequence contained within a ddRNAi expression cassette (see below for definition and description) that encodes for RNA which is capable of folding in to a miRNA structure. The effector sequence and the effector complement of a ddRNAi agent is expressed within or as part of that miRNA structure. The ME sequence has a first and second part. In an expression cassette for expressing a single hairpin (having one or more effector/effector complement pairs), the first part of the ME sequence is located upstream (ie 5') of the 5' most effector or effector complement encoding sequence, and the second part is located downstream (ie 3') to the 3' most effector or effector complement encoding sequence.

In the case of an expression cassette for a multiple hairpin structure, each effector/effector complement pair has a corresponding first and second ME sequence, wherein the first ME sequence is upstream of the effector or effector complement encoding sequence and the second part is downstream of the corresponding effector or effector complement encoding sequence. In an expression cassette having the following exemplary structure, in a 5' to 3' direction:

- a promoter
- a first ME sequence;
- a first effector;
- a first effector complement sequence;
- a second ME sequence;
- a third ME sequence;
- a second effector sequence;
- a second effector complement sequence; and
- a fourth ME sequence

it will be appreciated that the second and third ME sequence can either be (using exemplary sequences to illustrate the point) consecutive, can have intervening sequence between them, or can be a single ME sequence that serves the same function as the second and third ME sequence.

i) consecutive: ggtatattgctgttgacagtgagcgaggtatattgctggggacagtgagccc
ME sequence 2 ME sequence 3

ii) intervening: ggtatattgctgttgacagtgagcgaATTGCCATGggtatattgctggggacagtgagccc
ME sequence 2 INTERVENING ME sequence 3

iii) single: ggtatattgctgttgacagtgagcgaggtatattgctggggacagtgagccc
ME sequence

The double stranded or duplex region of the RNAi agent is at least 17 base pairs long, and usually in the range of 17 to 30 base pairs. RNAi agents can be synthesized chemically or enzymatically outside of cells and subsequently delivered to cells or can be expressed *in vivo* by an appropriate vector in cells (see, e.g., U.S. Pat. No. 6,573,099, WO 2004/106517 and WO1999/49029, all of which are incorporated herein by reference).

The term "DNA-directed RNAi agent" or "ddRNAi agent" refers to an RNAi agent that is transcribed from a DNA expression cassette ("ddRNAi expression cassette"). Depending on the arrangement of terminators and promoters within the ddRNAi expression cassettes, they may express ddRNAi agents with single or multiple effector sequences, or may express multiple ddRNAi agents. A ddRNAi agent transcribed from the expression cassette may be transcribed as a single RNA that is capable of self-annealing into a single hairpin structure with a duplex region linked by at least 2 nucleotides. The single hairpin may include one effector sequence and its complement (see Figure 1B or E) or multiple effector sequences and their complements (see Figure 1A or D). Alternatively, the agent may be a single RNA with multiple shRNA domains (ie multiple hairpin structures formed by the effector sequences and their complement – see Figure 1C or F).

The ddRNAi expression cassette can be ligated into vectors referred to as ddRNAi vectors or ddRNAi constructs. The vectors may provide sequences specifying transcription of the ddRNAi expression cassette *in vivo* or *in vitro*. The vector may additionally serve as the delivery vehicle for the ddRNAi expression cassette. Viral based vectors for example will generate a ddRNAi construct that is useful for expression of the ddRNAi expression cassette as well as being compatible with viral delivery.

A cell has been "transformed", "transduced" or "transfected" by an exogenous or heterologous nucleic acid or vector when such nucleic acid has been introduced into the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a host cell chromosome or is maintained extra-chromosomally (episomally) so that the transforming DNA is inherited by daughter cells during cell replication. In non-replicating, differentiated cells the transforming DNA may persist as an episome.

"Gene expression" can be a reference to either or both transcription or translation.

"Inhibition of expression" refers to the absence or observable decrease in the level of protein and/or mRNA product from the target gene. The inhibition does not have to be absolute, but may be partial inhibition sufficient for there to a detectable or observable change as a result of the administration of a RNAi or ddRNAi agent or siRNA agent or ddRNAi expression cassette or expression construct of the invention. Inhibition may be measured by determining a decrease in the level of mRNA and/or protein product from a target nucleic acid relative to a cell lacking the ddRNAi agent or construct, and may be as little as 1%, 5% or 10%, or may be absolute ie 100% inhibition. The effects of inhibition may be determined by examination of the outward properties ie quantitative and/or qualitative phenotype of the cell or organism.

"Off-target" effects is a term used to describe unintentional side-effects of treatment with an RNAi reagent. This is frequently thought to involve unintended knockdown of a target sequence as a consequence of chance homology with the passenger or effector sequences and another target gene, although subtler effects arising from metabolic compensation of a knockdown can also occur. Processing of miRNAs by endogenous RNAi pathways frequently results in the loading of only the effector strand into RISC, and degradation of the passenger strand. One potential source of off-target effects is the unanticipated incorporation of the passenger strand into RISC such that passenger sequences can consequently silence genes which they fortuitously share homology with. There is evidence that a step in RISC loading "senses" the predicted thermodynamic stability of an RNA duplex across a potential target site in dsRNA precursors and preferentially loads the strand whose 5' end is from the less stable end of the duplex. One strategy to minimise the potential for off-target effects is to screen

ddRNAi molecules for activity of the passenger strand using Dual Luciferase assays. Loading of this strand into RISC is undesirable.

As used herein, a "vascular endothelial growth factor-A gene" or "VEGF-A gene", includes a gene that encodes a protein that stimulates angiogenesis. In one embodiment the VEGF-A gene encodes a nucleotide sequence as shown in Genbank with accession number NM_001025366 (SEQ ID NO:79) which encodes human VEGF-A. In another embodiment, a VEGF-A gene is an orthologous or paralogous gene to the VEGF-A gene, including but not limited to a nucleotide sequence as shown in Genbank with accession number NM_001025250 (*Mus musculus*, SEQ ID NO:80) or XM_001089925 (*Macaca mulatta*, SEQ ID NO:81). In another embodiment, the VEGF-A gene may be a human gene or gene from an animal as described herein and includes allelic variants.

As used herein, a "vascular endothelial growth factor receptor 2 gene" or "VEGFR2 gene" includes a gene that encodes a receptor for VEGF. In one embodiment the VEGFR2 gene encodes a nucleotide sequence as shown in Genbank with accession number NM_002253 (SEQ ID NO: 82) which encodes human VEGFR2. In another embodiment, a VEGFR2 gene is an orthologous or paralogous gene to the VEGFR2, including but not limited to a nucleotide sequence as shown in Genbank with accession number NM_010612 (*Mus musculus*, SEQ ID NO:83) or XM_001086814 (*Macaca mulatta*, SEQ ID NO:84). In another embodiment, VEGFR2 gene may be a human gene or gene from an animal as described herein and includes allelic variants.

As used herein, a "Beta-type platelet-derived growth factor receptor gene" or "PDGFR-β gene" includes a gene that encodes the PDGFR-β protein. In one embodiment the PDGFR-β gene encodes a nucleotide sequence as shown in Genbank with accession number NM_002609 (SEQ ID NO:85) which encodes human PDGFR-β. In another embodiment, a PDGFR-β gene is an orthologous or paralogous gene to the PDGFR-β, including but not limited to a nucleotide sequence as shown in Genbank with accession number NM_001142706 (*Mus musculus*, SEQ ID NO:86) or XM_00110759 (*Macaca mulatta*, SEQ ID NO:87). In another embodiment, PDGFR-β gene may be a human gene or gene from an animal as described herein and includes allelic variants.

As used herein, a "Complement Factor B gene" or "CFB gene" includes a gene that encodes the CFB protein, a component of drusen. In one embodiment the CFB gene encodes a nucleotide sequence as shown in Genbank with accession number NM_001710 (SEQ ID NO: 88) which encodes human CFB. In another embodiment, a CFB gene is an orthologous or paralogous gene to the CFB, including but not limited to a nucleotide sequence as shown in Genbank with accession number NM_00114270 (*Mus musculus*, SEQ ID NO:89) or XM_001113553 (*Macaca mulatta*, SEQ ID NO:90). In another embodiment, CFB gene may be a human gene or gene from an animal as described herein and includes allelic variants.

Sequences are "paralogous" if they are separated by a gene duplication event: if a gene in an organism is duplicated to occupy two different positions in the same genome, then the two copies are paralogous.

Sequences are "orthologous" if they are separated by a speciation event: when a species diverges into two separate species, the divergent copies of a single gene in the resulting species are said to be orthologous.

As used herein, "a quantitative phenotypic trait" refers to a trait associated with the molecular expression of a nucleic acid in a host cell and may thus include the quantity of RNA molecules transcribed or replicated, the quantity of post-transcriptionally modified RNA molecules, the quantity of translated peptides or proteins, or the activity of such peptides or proteins.

A reduction of phenotypic expression of a nucleic acid where the phenotype is a qualitative trait means that in the presence of the RNAi agent of the invention, the phenotypic trait switches to a different state when compared to a situation in which the RNAi agent is absent. A reduction of phenotypic expression of a nucleic acid may thus be measured as a reduction in steady state levels of (part of) that nucleic acid, a reduction in translation of (part of) that nucleic acid or a reduction in the effect the presence of the transcribed RNA(s) or translated polypeptide(s) have on the eukaryotic cell or the organism, and will ultimately lead to altered phenotypic traits. It is clear that the reduction in phenotypic expression of a nucleic acid of interest may be accompanied by or correlated to an observable change in phenotype. The assessment may be by way of biochemical techniques such as Northern hybridisation, quantitative real-time

PCR assays, gene expression assays, antibody binding, ELISA, RIA, western blotting and other assays and techniques known in the art.

"Target nucleic acids" may be either RNA or DNA, whose transcription products are targeted, coding or non-coding sequence, endogenous or exogenous.

A "therapeutic composition" or "pharmaceutical composition" or "composition for treating" refers to a composition including a ddRNAi agent, ddRNAi expression cassette, ddRNAi construct or siRNA agent.

The words "treat" or "treatment" refer to therapeutic treatment wherein the object is to slow down (lessen) an undesired physiological change or disorder. For purposes of this invention, beneficial or desired clinical results include, but are not limited to, alleviation of symptoms of AMD, stabilised (i.e., not worsening or progressing) AMD, and stabilised CNV.

The phrase "therapeutically effective amount" means an amount of a compound of the present invention that (i) treats the particular disease, condition, or disorder, (ii) attenuates, ameliorates, or eliminates one or more symptoms of the particular disease, condition, or disorder, (iii) prevents or delays the onset of one or more symptoms of the particular disease, condition, or disorder described herein, (iv) prevents or delays progression of the particular disease, condition or disorder, or (v) reverses damage caused prior to treatment to some extent. The reversal does not have to absolute, but any clinically relevant return of visual acuity post-treatment is considered a reversal of damage.

The current invention provides a new RNAi agent, and use of the RNAi agent for reducing the regression of visual acuity associated with AMD in affected individuals, particularly those with wet AMD. Treatment is aimed at one or more of:

i. controlling angiogensis associated with choroidal neovascularisation (CNV) by long-term knock down of VEGF-A translation and subsequent secretion from retina cells using a DNA construct containing one or more sequences aimed at silencing specific genes associated with VEGF-A expression. VEGF-A stimulates angiogenesis, and therefore the abnormal outgrowth of blood vessels from the vasculature behind the retina. A number of existing therapies only serve to "mop up" secreted VEGF-A, which may stabilise vision, but does not necessarily improve vision in all patients.

ii. Additional control of angiogenesis might be obtained by knockdown of both VEGF-A and its receptor VEGFR2, since this strategy would be expected to interfere with the process at two distinct steps.

iii. Reversal of AMD might be achieved by knockdown of three targets, namely VEGFR2, PDGFR-β and CFB. VEGFR2 knockdown would be expected to control angiogenesis, PDGFR-β knockdown would be expected to inhibit or reverse nascent blood vessel formation and CFB knockdown would be expected to inhibit or even reverse drusen deposition

iv) limiting treatment frequency, and limiting treatment to RPE cells via localised injection of the therapeutic molecules.

Identifying appropriate target sequences within target genes, and designing RNAi agents that work based on those sequences, is not routine. As will be demonstrated in the results section, target sequences that look like good candidates on paper, may not necessarily effectively silence the target, or may not do so to an effective level for therapeutic purposes. Some effector sequences work much more effectively than others to silence a target in particular incorporation of passenger strands into RISC is undesirable since this may lead to significant off-target effects and consequent toxicity. But it is not predictable which sequences are able to be silenced by mere visual inspection of the sequence itself, let alone to what extent they may be silenced, and if that would be sufficient for the purposes of the invention. Even more so when you are seeking to silence 2 or more unrelated targets.

Despite the recognition in the art that VEGF-A is a suitable target for AMD therapies, efforts to create an effective therapy to date have been plagued by the problems summarised in the background. With respect to silencing by RNAi techniques in particular, previous efforts using *in vitro* produced siRNA agents have been unsuccessful due to siRNA interaction with membrane bound TLR3 and subsequent activation of interferon. In addition, cells which secrete the majority of VEGF-A are the RPE cells, generally found buried underneath layers of specialized cells towards the back of the eye. RNAi moieties are highly charged complexes and can be difficult to traverse across multiple layers of cells because of this physical property. The new range of targets, the ddRNAi agents and the viral delivery agents utilised seek to overcome these issues.

In addition to VEGF-A these targets include one or more of:

- VEGFR2: the receptor for VEGF-A; silencing VEGFR2 is expected to have similar consequences to silencing VEGF-A
- PDGFR-β: the receptor for PDGFR-β. This molecule plays a role in recruitment and stabilisation of endothelial cells, which is critical for stabilisation of nascent blood vessels.
- CFB: This is a major component of drusen, the hallmark extracellular deposit associated with AMD. (Anderson et al 2010). Silencing CFB may inhibit the formation of drusen.

RNA interference (RNAi) is an RNA-dependent gene silencing process that is initiated by short double-stranded RNA molecules in a cell's cytoplasm. In mammals, RNAi is mediated by double-stranded RNA molecules referred to as small interfering RNAs (siRNA). The double stranded, or duplex region of the RNAi agent is at least 17 base pairs long, and usually in the range of 17 to 30 base pairs. RNAi agents can be synthesised chemically or enzymatically outside of cells and subsequently delivered to cells or can be expressed *in vivo* by an appropriate vector in cells (such as AAV, adenovirus, lentivirus, or non-viral liposome-based delivery systems).

Pre-clinical testing of RNAi agents as AMD therapeutics requires the extensive use of animal models. Mouse (*Mus muscularis*) and primate (eg macaques, *Macaca fasciularis*) models are widely used to test the efficacy of treatments, and other species such as dogs (*Canis familiaris*) are commonly used as models to determine the clinical safety of therapeutic compounds. For RNAi therapeutics it is advantageous to design reagents that target nucleotide sequences of AMD-associated genes that are highly conserved between humans and the various pre-clinical test species since a single RNAi reagent can be used at all stages of pre-clinical testing. For poorly conserved genes multiple RNAi reagents with sequences that differ slightly between the different test species must be tested in parallel to accurately determine potential toxicity.

Accordingly, the RNAi reagents described in this application are, where possible, designed to target sequences conserved between humans and the potential test species (mice, dogs and primates such as macaques), since this provides significant advantages for a drug development program.

ddRNAi agent

RNAi agents may be expressed from DNA vectors, referred to as DNA-directed RNAi, or ddRNAi. They can directly target the activity of genes with minimum off-target events. In the case of AMD, this offers a unique opportunity to address the unmet clinical treatment needs. Accordingly, in one aspect of the invention, there is provided a DNA-directed RNA interference (ddRNAi) agent for inhibiting expression of one or more target sequences in an AMD-associated gene, the ddRNAi agent comprising at least:

a first effector sequence of at least 17 nucleotides in length; and

a first effector complement sequence;

wherein the first effector sequence is complementary or substantially complementary to one or more target regions in a transcript of the one or more target sequences.

Typically, the first effector sequence forms a double stranded region with the first effector complement sequence.

The sequences of the ddRNAi agents of the invention have to have a sufficient identity to the AMD-associated gene, such as the VEGF-A, VEGFR2, CFB and PDGFR-β genes, in order to mediate target specific RNAi.

The first effector sequence is at least 17 nucleotides long, preferably 17 to 30 nucleotides and more preferably 17 to 21 nucleotides. It may be 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 nucleotides in length. When the first effector sequence is longer than 17 nucleotides, it is preferred that at least 17 contiguous nucleotides of the first effector sequence forms the double stranded region with the complementary strand. A ddRNAi agent according to this embodiment of the invention therefore has a maximum length determined by the length and number of effector sequence/s *ie* each effector sequence is not comprised within a longer sequence.

The ddRNAi agents of the invention inhibit expression of AMD-associated target genes. Preferably the AMD-associated gene is VEGF-A, or one or more of VEGFR2, CFB and PDGFR-β, and each effector sequence is selected from the group consisting of any 10 or more contiguous nucleotides within a sequence from any one of SEQ ID NOS: 40-78.

As illustrated in the table below, when the AMD-associated gene to be inhibited, prevented or reduced is VEGF-A, each effector sequence is selected from SEQ ID

NOS: 40-49. When the AMD-associated gene to be inhibited, prevented or reduced is VEGFR2 each effector sequence is selected from SEQ ID NOS: 50-59. When the AMD-associated gene to be inhibited, prevented or reduced is PDGFR-β, each effector sequence is selected from SEQ ID NOS: 60-69. When the AMD-associated gene to be inhibited, prevented or reduced is CFB, each effector sequence is selected from SEQ ID NOS: 70-78.

Table 1: VEGF-A, VEGFR2, CFB and PDGFR-β gene target sequences and their corresponding ddRNAi effector sequences

Target[a]	Target position[b]	SEQ ID NO	Target sequence in 5' to 3' direction[c]	SEQ ID NO	Corresponding effector sequence in 5' to 3' direction[d]
VEGF-A miR-1	328-348	1	AGCAAGAGCTCCAGAGAGAAG	40	CUUCUCUCUGGAGCUCUUGCU
VEGF-A miR-2	1026-1046	2	GGCCTCCGAAACCATGAACTT	41	AAGUUCAUGGUUUCGGAGGCC
VEGF-A miR-3	1203-1223	3	CGAGACCCTGGTGGACATCTT	42	AAGAUGUCCACCAGGGUCUCG
VEGF-A miR-4	1383-1403	4	GCACATAGGAGAGATGAGCTT	43	AAGCUCAUCUCUCCUAUGUGC
VEGF-A miR-5	1422-1442	5	TGAATGCAGACCAAAGAAAGA	44	UCUUUCUUUGGUCUGCAUUCA
VEGF-A miR-6	1858-1878	6	CAGAACAGTCCTTAATCCAGA	45	UCUGGAUUAAGGACUGUUCUG
VEGF-A miR-7	2055-2075	7	TCTGGGATTCCTGTAGACACA	46	UGUGUCUACAGGAAUCCCAGA
VEGF-A miR-8	2067-2087	8	GTAGACACACCCACCCACATA	47	UAUGUGGGUGGGUGUGUCUAC
VEGF-A miR-9	3480-3500	9	GGTGCTACTGTTTATCCGTAA	48	UUACGGAUAAACAGUAGCACC
VEGF-A miR-10	3554-3574	10	CGAGATATTCCGTAGTACATA	49	UAUGUACUACGGAAUAUCUCG
VEGFR2 miR-V-1	477-497	11	TTGGACTGGCTTTGGCCCAAT	50	AUUGGGCCAAAGCCAGUCCAA
VEGFR2 miR-V-2	864-884	12	CCCAGCTACATGATCAGCTAT	51	AUAGCUGAUCAUGUAGCUGGG
VEGFR2 miR-V-3	2625-2645	13	GCCATGTTCTTCTGGCTACTT	52	AAGUAGCCAGAAGAACAUGGC
VEGFR2 miR-V-4	2661-2681	14	CGGACCGTTAAGCGGGCCAAT	53	AUUGGCCCGCUUAACGGUCCG
VEGFR2 miR-V-5	3037-3057	15	TCATGGTGATTGTGGAATTCT	54	AGAAUUCCACAAUCACCAUGA
VEGFR2 miR-V-6	3299-3319	16	CCTGACCTTGGAGCATCTCAT	55	AUGAGAUGCUCCAAGGUCAGG
VEGFR2 miR-V-7	3307-3327	17	TGGAGCATCTCATCTGTTACA	56	UGUAACAGAUGAGAUGCUCCA
VEGFR2 miR-V-8	3338-3358	18	GGCTAAGGGCATGGAGTTCTT	57	AAGAACUCCAUGCCCUUAGCC

VEGFR2 miR-V-9	3698-3718	19	ACCAGAAATGTACCAGACCAT	58	AUGGUCUGGUACAUUUCUGGT
VEGFR2 miR-V-10	3928-3948	20	ACCCCAAATTCCATTATGACA	59	UGUCAUAAUGGAAUUUGGGGT
PDGFR-β miR-P-1	1093-1113	21	ACTCCAGGTGTCATCCATCAA	60	UUGAUGGAUGACACCUGGAGU
PDGFR-β miR-P-2	1098-1118	22	AGGTGTCATCCATCAACGTCT	61	AGACGUUGAUGGAUGACACCU
PDGFR-β miR-P-3	2197-2217	23	CCATGAGTACATCTACGTGGA	62	UCCACGUAGAUGUACUCAUGG
PDGFR-β miR-P-4	2872-2892	24	GGACCTCGTGGGCTTCAGCTA	63	UAGCUGAAGCCCACGAGGUCC
PDGFR-β miR-P-5	2977-2997	25	AGGCAAGCTGGTCAAGATCTG	64	CAGAUCUUGACCAGCUUGCCU
PDGFR-β miR-P-6	3085-3105	26	GGAGAGCATCTTCAACAGCCT	65	AGGCUGUUGAAGAUGCUCUCC
PDGFR-β miR-P-7	3090-3110	27	GCATCTTCAACAGCCTCTACA	66	UGUAGAGGCUGUUGAAGAUGC
PDGFR-β miR-P-8	3181-3202	28	CCCAGAGCTGCCCATGAACGA	67	UCGUUCAUGGGCAGCUCUGGG
PDGFR-β miR-P-9	3202-3222	29	GCAGTTCTACAATGCCATCAA	68	UUGAUGGCAUUGUAGAACUGC
PDGFR-β miR-P-10	3250-3270	30	CCATGCCTCCGACGAGATCTA	69	UAGAUCUCGUCGGAGGCAUGG
CFB miR-C-1	929-949	31	CTGCCAAGACTCCTTCATGTA	70	UACAUGAAGGAGUCUUGGCAG
CFB miR-C-2	1085-1105	32	GAACATCTACCTGGTGCTAGA	71	UCUAGCACCAGGUAGAUGUUC
CFB miR-C-3	1096-1116	33	TGGTGCTAGATGGATCAGACA	72	UGUCUGAUCCAUCUAGCACCA
CFB miR-C-4	1100-1120	34	GCTAGATGGATCAGACAGCAT	73	AUGCUGUCUGAUCCAUCUAGC
CFB miR-C-5	1535-555	35	GGAGGATTATCTGGATGTCTA	74	UAGACAUCCAGAUAAUCCUCC
CFB miR-C-6	1697-1717	36	GTCTCTGAGTCTCTGTGGCAT	75	AUGCCACAGAGACUCAGAGAC
CFB miR-C-7	1817-1837	37	GGCTGTGGTGTCTGAGTACTT	76	AAGUACUCAGACACCACAGCC
CFB miR-C-8	2154-2174	38	CAGGATATCAAAGCTCTGTTT	77	AAACAGAGCUUUGAUAUCCUG
CFB miR-C-9	2201-2221	39	TCGGAAGGAGGTCTACATCAA	78	UUGAUGUAGACCUCCUUCCGA

[a] Target genes are human VEGF-A (NM_0010253660), VEGFR2 (NM_002253), PDGFR-β (NM_002609) and CFB (NM_001710); designations below gene names refer to versions of ddRNAi constructs targeting the particular genes.

[b] Target positions for human sequences listed.

[c] Target sequences are the DNA sequences recognised by the effector sequence.

[d] Effector sequences are the predicted RNA sequences produced by dicer processing of the ddRNAi agents that target AMD-associated genes; T refers to constructs where effector is modified to maintain structure of the expressed RNAs.

The ddRNAi agents of the invention are preferably expressed within or as part of a miRNA structure. These miRNA structures have the sequences shown as "miR sequences" and are listed in Table 2 (SEQ ID NOS: 91-129), which were designed to express the indicated effector sequences (SEQ IDNOS: 40-78). The corresponding constructs containing the expression cassettes for expressing the miRNA structures is also shown as "miR-designations".

Table 2: miR constructs displaying strong, sequence-specific silencing of AMD-associated genes

AMD Target	miR designations[a]	SEQ ID NO: miR sequence[b]	SEQ ID NO: effector sequence[c]
VEGF-A	miR-1	91	40
	miR-2	92	41
	miR-3	93	42
	miR-4	94	43
	miR-5	95	44
	miR-6	96	45
	miR-7	97	46
	miR-8	98	47
	miR-9	99	48
	miR-10	100	49
VEGFR2	miR-V-1	101	50
	miR-V-2	102	51
	miR-V-3	103	52
	miR-V-4	104	53
	miR-V-5	105	54
	miR-V-6	106	55
	miR-V-7	107	56
	miR-V-8	108	57
	miR-V-9	109	58
	miR-V-10	110	59
PDGFR-β	miR-P-1	111	60
	miR-P-2	112	61
	miR-P-3	113	62
	miR-P-4	114	63
	miR-P-5	115	64
	miR-P-6	116	65
	miR-P-7	117	66
	miR-P-8	118	67
	miR-P-9	119	68
	miR-P-10	120	69
CFB	miR-C-1	121	70
	miR-C-2	122	71
	miR-C-3	123	72

	miR-C-4	124	73
	miR-C-5	125	74
	miR-C-6	126	75
	miR-C-7	127	76
	miR-C-8	128	77
	miR-C-9	129	78

[a] miR constructs tested for silencing activity and favourable strand specificities against the indicated human target genes (see Figs 4 – 7).

[b] SEQ ID NOS corresponding to inserts of miR constructs.

[c] SEQ ID NOS of predicted effector sequences produced by indicated miR constructs.

Any of the ddRNAi agents of the invention can be expressed within or as part of a miRNA structure. As will be explained throughout the specification, this can assist with more accurate processing of the ddRNAi agent, and lower toxicity within the cell.

In one embodiment of the invention, the ddRNAi agent of the invention inhibits expression of one or more target sequences in a VEGF-A gene. A target sequence is preferably selected from the ddRNAi VEGF-A target sequences listed in Table 1 (SEQ ID NOS: 1-10); the corresponding effector sequences that would be produced by dicer processing of a ddRNAi agent targeting those sequences is shown in SEQ ID NOS: 40-49 respectively. Note that the VEGF-A target sequences and effector sequences have been chosen to show conservation of nucleotide sequences between human and the pre-clinical test species mouse, dog and macaque.

In an alternative embodiment of the invention, the ddRNAi agent of the invention inhibits expression of one or more target sequences in a VEGFR2 gene. A target sequence is preferably selected from the ddRNAi a VEGFR2 target sequences listed in Table 2 (SEQ ID NOS: 11-20); the corresponding effector sequences are therefore selected from SEQ ID NOS: 50-59 respectively as shown in Table 2. Note that the VEGFR2 target (SEQ ID NOS: 11-20) and effector sequences (SEQ ID NOS: 50-59) are identical to, or differ by only a single nucleotide between human and the pre-clinical test species mouse and macaque.

In an alternative embodiment of the invention, the ddRNAi agent of the invention inhibits expression of one or more target sequences in a PDGFR-β gene. A target sequence is preferably selected from the ddRNAi PDGFR-β target sequences listed in Table 2 (SEQ ID NOS: 21-30); the corresponding effector sequences are therefore selected from SEQ ID NOS: 60-69 respectively as shown in Table 2. Note that the PDGFR-β target (SEQ

ID NOS: 21-30) and effector sequences (SEQ ID NOS: 60-69) are identical to, or differ by only a single nucleotide between human and the pre-clinical test species mouse and macaque.

In an alternative embodiment of the invention, the ddRNAi agent of the invention inhibits expression of one or more target sequences in a CFB gene. A target sequence is preferably selected from the ddRNAi CFB target sequences listed in Table 1 (SEQ ID NOS: 31-39); the corresponding effector sequences are therefore selected from SEQ ID NOS: 70-78 respectively as shown in Table 2. Note that the CFB target (SEQ ID NOS: 31-39) and effector sequences (SEQ ID NOS: 70-78) are identical to, or differ by only a single nucleotide between human and the pre-clinical test species mouse and macaque.

In accordance with the explanation provided earlier, the relationship between the DNA target sequence and the corresponding effector sequence of the ddRNAi agent can be shown as (using the target SEQ ID NO:2 and its corresponding effector sequence SEQ ID NO:41 from Table 1):

5' GGCCTCCGAAACCATGAACTT 3' - target sequence of VEGF-A (SEQ ID NO:2)

5' GGCCUCCGAAACCAUGAACUU 3' – mRNA transcript of SEQ ID NO:2

3' AAGUUCAUGGUUUCGGAGGCC 5' – effector sequence of ddRNAi agent (SEQ ID NO:41) to target SEQ ID NO:2, which when read in the 5' to 3' direction, can be seen to be substantially complementary to the transcript of the target sequence.

As explained in the background section, both strands of the ddRNAi agent have the potential to be the effector sequence. However there is evidence that particular features of a sequence can favour one strand to enter the RISC and the other strand to be destroyed. There is evidence that a step in RISC loading "senses" thermodynamic stability of an RNA duplex across a potential target site in dsRNA precursors and preferentially loads the strand whose 5' end is from the less stable end of the duplex. Therefore target site sequences were typically adjusted to maximise the number of AT base pairs at the 3' end of the target site, *i.e.* maximising the number of A or U bases in the 5' end of the effector strand. The list of refined target sites was then screened for conservation between likely test species, specifically mice and monkeys. Target site sequences were then screened against the human transcriptome, using BLAST, and those showing high homology to other human genes (>3 mismatches) were discarded.

Constructs based on these target sequences were prepared in a miRNA backbone and tested empirically for activity and strand selectivity as described below. These sequence preferences are reflected in preferred embodiments, and data is provided in the examples section showing the advantages of some sequences over other.

For example, in one embodiment of this aspect of the invention, there is provided a DNA-directed RNA interference (ddRNAi) agent for inhibiting expression of one or more target sequences in an AMD-associated gene, the ddRNAi agent comprising at least:

a first effector sequence of any 10 or more contiguous nucleotides within 5' UAUGUGGGUGGGUGUGUCUAC 3' (SEQ ID NO:47); and

a first effector complement sequence.

The first effector sequence is substantially complementary to a target region in a transcript of one or more target sequences in an AMD-associated gene. In this example, the target gene is VEGF-A.

Preferably the first effector sequence is at least 17 or more contiguous nucleotides within 5' UAUGUGGGUGGGUGUGUCUAC 3' (SEQ ID NO:47).

When the first effector sequence has 1, 2, 3, 4 or 5 nucleotides different to SEQ ID NO:47, the differences are preferably present in the first and/or last 5 nucleotides, and preferably at least the centre 10 nucleotides are 100% complementary to a target region in a transcript of one or more target sequences.

In alternative embodiments, the ddRNAi agent comprises a first effector sequence of any 10 or more, preferably any 17 or more, contiguous nucleotides within SEQ ID NO:40, SEQ ID NO:41, SEQ ID NO:42, SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48, SEQ ID NO:49, SEQ ID NO:50, SEQ ID NO:51, SEQ ID NO:52, SEQ ID NO:53, SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56, SEQ ID NO:57, SEQ ID NO:58, SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61, SEQ ID NO:62, SEQ ID NO:63, SEQ ID NO:64, SEQ ID NO:65, SEQ ID NO:66, SEQ ID NO:67, SEQ ID NO:68, SEQ ID NO:69, SEQ ID NO:70, SEQ ID NO:71, SEQ ID NO:72, SEQ ID NO:73, SEQ ID NO:74, SEQ ID NO:75, SEQ ID NO:76, SEQ ID NO:77 or SEQ ID NO:78.

In particularly preferred embodiments, the ddRNAi agent comprises a first effector sequence of any 10 or more, preferably any 17 or more, contiguous nucleotides within

sequences able to inhibit the expression of a target gene region by at least 70%. Preferably, in this embodiment, the first effector is selected from SEQ ID NO:47, which targets a sequence of SEQ ID NO:8.

The first effector sequence may comprise a sequence selected from any 10 or more and preferably any 17 or more contiguous nucleotides within a sequence from the group consisting of SEQ ID NOS: 40-78, or alternatively, each effector sequence may be a variant of SEQ ID NOS:40-78, having 1, 2, 3, 4 or 5 nucleotide variations. In yet a further embodiment, each effector sequence may consist of 20 nucleotides, of which 17, 18, 19, or all 20 nucleotides are contiguous nucleotides from a sequence selected from the group consisting of SEQ ID NOS: 40-78.

Multiple targeting ddRNAi agents

ddRNAi agents with multiple effector sequences have the advantage of being able to target a range of molecular targets and naturally occurring variants thereof that may exist between individuals, as well as the advantage of the additive or synergistic effects achieved with multiple effector sequences as opposed to single effector sequences. In the present invention, where in one embodiment there are 2 or 3 different target genes selected from VEGF-A, VEGFR2, CFB and PDGFR-β, it is particularly advantageous for a single construct to be utilised to target the 2 or 3 genes. This eliminates the need to deliver multiple ddRNAi agents each targeting a different gene. As would be appreciated by the person skilled in the art, it would be difficult to ensure that delivery would result in equal and sufficient concentrations of each of the agents.

In one embodiment of the invention, the ddRNAi agent comprises two or more effector sequences to enable targeting of more than one target sequence of the AMD-associated gene. The multiple target sequences may be in the same region of the one gene. For example, a 17 to 30 nucleotide region, preferably a 17 to 21 nucleotide region, within VEGF-A, VEGFR2, CFB or PDGFR-β that has natural variation in the sequence between individuals. Alternatively, the target sequences may be in different regions of the one target gene, where the target gene may be VEGF-A, VEGFR2, CFB or PDGFR-β.

As noted above the target sequences may also be in different AMD-associated genes. For example, a first effector sequence targets a sequence in VEGFR2, whereas a

second effector sequence in the same ddRNAi agent targets a sequence in a VEGF-A gene. In a preferred embodiment, there are at least 2 effector sequences, each one targeting a sequence in each of VEGF-A and VEGFR. In an alternative embodiment, there are at least 3 effector sequences, each one targeting a sequence in each of VEGFR2, CFB and PDGFR-β.

To provide greater specificity the ddRNAi agent comprises the following (in no particular order):

- a first effector sequence of at least 17 nucleotides in length;
- a second effector sequence of at least 17 nucleotides in length;
- a first effector complement sequence; and
- a second effector complement sequence.

The first and second effector sequences of a multiple targeting ddRNAi agent form a double stranded region with their respective effector complements. Preferably, the first and second effector sequences are 17 to 30 nucleotides in length. More preferably, the first and second effector sequence are both selected from any 10 or more and preferably any 17 or more contiguous nucleotides within any one of the sequences of SEQ ID NOS: 40-78 listed in Table 1 above, or are sequences having 1, 2, 3, 4 or 5 nucleotides difference from those sequences listed in Table 1.

In one embodiment, the first effector sequence is selected from any 10 or more and preferably any 17 or more contiguous nucleotides within a sequence from any one of the group consisting of SEQ ID NOS:40-78, and the second effector sequence is selected from any 10 or more and preferably any 17 or more contiguous nucleotides within a sequence from any one of the group consisting of SEQ ID NOS: 40-78. The first and second effector sequence may both be the same sequence or may alternatively be different sequences.

The first and second effector sequence may each comprise a sequence selected from any 10 or more contiguous nucleotides within a sequence from the group consisting of SEQ ID NOS: 40-78, or alternatively, each effector sequence may also be a variant of SEQ ID NOS: 40-78, having 1, 2, 3, 4 or 5 nucleotide variations. In yet a further embodiment, each effector sequence may consist of 20 nucleotides, of which 17, 18, 19, or all 20 nucleotides are contiguous nucleotides from a sequence selected from the

group consisting of SEQ ID NOS: 40-78. When there are two or more effector sequences, they may represent a combination of the 3 types described above.

In particularly preferred embodiments, the first and second effector sequence comprise any 10 or more, preferably any 17 or more, contiguous nucleotides within sequences able to inhibit the expression of a target gene region by at least 70%. Preferably in this embodiment, each effector sequence is selected from any 10 or more and preferably any 17 or more contiguous nucleotides within a sequence consisting of SEQ ID NO:47 and SEQ ID NO:56 such that there is provided a DNA-directed RNA interference (ddRNAi) agent for inhibiting expression of one or more target sequences in an AMD associated gene, the ddRNAi agent comprising, in a 5' to 3' direction

a first effector sequence of any 10 or more contiguous nucleotides within 5' UAUGUGGGUGGGUGUGUCUAC 3' (SEQ ID NO:47);

a first effector complement sequence;

a second effector sequence of any 10 or more contiguous nucleotides within 5' UGUAACAGAUGAGAUGCUCCA 3' (SEQ ID NO:56); and

a second effector complement sequence

wherein each effector sequence is substantially complementary one or more target regions in a transcript of the one or more target sequences.

Long hairpin version

When the ddRNAi agent contains more than one effector sequence, and the ddRNAi agent is expressed as a single strand of RNA, it will fold to form different structures depending on the order of the effector sequences and the sequences complementary to the effector sequences. In one embodiment, there is provided a DNA-directed RNA interference (ddRNAi) agent for inhibiting expression of one or more target sequences in an AMD-associated gene, preferably a VEGF-A gene and/or one or more of a VEGFR2, CFB and PDGFR-β gene, the ddRNAi agent comprising, in a 5' to 3' direction, at least:

a first effector sequence of at least 17 nucleotides in length;

a second effector sequence of at least 17 nucleotides in length;

a second effector complement sequence; and

a first effector complement sequence

wherein each effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences. This will result in a ddRNAi agent with a structure as shown in Figure 1A. See also WO2004/106517, incorporated herein by reference.

Alternatively, at least one effector, and preferably both effector sequences, are 100% complementary one or more target regions in a transcript of the one or more target sequences. Preferably the first and second effector sequences are both selected from the group consisting of any 10 or more and preferably any 17 or more contiguous nucleotides within any one of SEQ ID NOS: 40-78. For example, in one embodiment, there is provided a DNA-directed RNA interference (ddRNAi) agent for inhibiting expression of one or more target sequences in an AMD-associated gene, the ddRNAi agent comprising, in a 5' to 3' direction, at least:

a first effector sequence of 5' AAGUUCAUGGUUUCGGAGGCC 3' (SEQ ID NO:41);

a second effector sequence 5' UCUUUCUUUGGUCUGCAUUCA 3' (SEQ ID NO:44);

a second effector complement; and

a first effector complement

wherein the AMD-associated gene is VEGF-A.

Each effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences.

Alternatively, at least one effector, and preferably both effector sequences, are 100% complementary to one or more target regions in a transcript of the one or more target sequences.

In particularly preferred embodiments, the first and second effector sequence comprise any 10 or more, preferably any 17 or more, contiguous nucleotides within sequences able to inhibit the expression of a target gene region by at least 70%. Preferably, in this embodiment, each effector sequence is selected from SEQ ID NOS: 40-78, more preferably from SEQ ID NOS: 40-59 and most preferably SEQ ID NOS: 40-49.

In yet another embodiment, being an embodiment where the ddRNAi agent has 3 effector sequences, there is provided a DNA-directed RNA interference (ddRNAi) agent for inhibiting expression of one or more target sequences in the target gene, the ddRNAi agent comprising, in a 5' to 3' direction, at least:

a first effector sequence of 5' AAGUUCAUGGUUUCGGAGGCC 3' (SEQ ID NO:41);

a second effector sequence of 5' UCUUUCUUUGGUCUGCAUUCA 3' (SEQ ID NO:44);

a third effector sequence of 5' UAUGUGGGUGGGUGUGUCUAC 3' (SEQ ID NO:47);

a third effector complement sequence;

a second effector complement sequence; and

a first effector complement sequence.

Each effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences.

Alternatively, at least one effector, and optionally 2 out of the 3 or all 3 of the effectors, are 100% complementary to one or more target regions in a transcript of the one or more target sequences.

In particularly preferred embodiments, the first, second and third effector sequence comprise any 10 or more, preferably any 17 or more, contiguous nucleotides within sequences able to inhibit the expression of a target gene region by at least 70%. Preferably, in this embodiment, each effector sequence is selected from SEQ ID NOS:40-78, more preferably from SEQ ID NOS: 40-59, and most preferably from SEQ ID NOS: 40-49.

It will also be appreciated by the skilled person that the order of effector and effector complements can be altered, provided that a single, long hairpin structure is formed by annealing of the effector sequence with its effector complement to form dsRNA. For example, in a 2-effector sequence ddRNAi agent, the sequences may be arranged in the following exemplary 5' to 3' orders:

- first effector – second effector – second effector complement – first effector complement;
- second effector – first effector – first effector complement – second effector complement;
- first effector – second effector complement - second effector – first effector complement;
- first effector complement – second effector complement – second effector – first effector;
- first effector complement – second effector – second effector complement – first effector.

In a 3-effector sequence ddRNAi agent, the sequences may be arranged in the following exemplary 5' to 3' orders:

- first effector – second effector – third effector – third effector complement - second effector complement – first effector complement
- first effector – second effector complement- third effector – third effector complement - second effector – first effector complement;
- first effector – second effector – third effector complement – third effector – second effector complement – first effector complement
- first effector – third effector – second effector complement – second effector – third effector complement – first effector complement
- first effector complement – second effector complement – third effector complement – third effector – second effector – first effector complement
- first effector complement – second effector complement – third effector – third effector complement – second effector – first effector.

In yet further embodiments, the first effector sequence may be selected from any 10 or more and preferably any 17 or more contiguous nucleotides within a sequence from the group consisting of SEQ ID NOS:40-78; the second effector sequence may be selected from any 10 or more and preferably any 17 or more contiguous nucleotides within a sequence from the group consisting of SEQ ID NOS:40-78; the third effector sequence

may be selected from any 10 or more and preferably any 17 or more contiguous nucleotides within a sequence from the group consisting of SEQ ID NOS:40-78; and any further effector sequences may be selected from any 10 or more and preferably any 17 or more contiguous nucleotides within a sequence from the group consisting of SEQ ID NOS:40-78. Alternatively, each effector sequence may also be a variant of SEQ ID NOS:40-78, having 1, 2, 3, 4 or 5 nucleotide variations. Preferably, the differences are present in the first and/or last 5 nucleotides, and at least the centre 11-12 nucleotides are 100% complementary to one or more target regions in a transcript of the one or more target sequences. In each of the embodiments, wherein only VEGF-A is to be targeted, each effector sequence is selected from SEQ ID NOS: 40-49; wherein only VEGFR2 is to be targeted, each effector sequence is selected from SEQ ID NOS: 50-59; wherein only PDGFR-β is to be targeted, each effector sequence is selected from SEQ ID NOS: 60-69; and wherein only CFB is to be targeted, each effector sequence is selected from SEQ ID NOS: 70-78.

The first, second and third effector sequence may each comprise a sequence selected from any 10 or more contiguous nucleotides within a sequence from the group consisting of SEQ ID NOS: 40-78, or alternatively, each effector sequence may also be a variant of SEQ ID NOS:40-78, having 1, 2, 3, 4 or 5 nucleotide variations. In yet a further embodiment, each effector sequence may consist of 20 nucleotides, of which 17, 18, 19, or all 20 nucleotides are contiguous nucleotides from a sequence selected from the group consisting of SEQ ID NOS: 40-78. When there are multiple effector sequences, they may represent a combination of the 3 types described above.

Multiple hairpin version

In an alternative embodiment, there is provided a DNA-directed RNA interference (ddRNAi) agent for inhibiting expression of one or more target sequences in one or more AMD-associated genes, preferably a VEGF-A, VEGFR2, CFB or PDGFR-β genes, the ddRNAi agent comprising, in a 5' to 3' direction, at least:

a first effector sequence of at least 17 nucleotides in length;

a first effector complement;

a second effector sequence of at least 17 nucleotides in length; and

a second effector complement

wherein each effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences.

Alternatively, at least one effector, and preferably both effector sequences, is 100% complementary to the one or more target regions of a transcript of the one or more target sequences.

This will result in a ddRNAi agent with a structure as shown in Figure 1B or C, depending on the type of expression cassette used to express it (see later in the specification). See also WO2005/087926 and WO2006/084209, incorporated herein by reference.

In either embodiment, where there are 2 target sequences, it is preferable that the first and second effector sequences are both substantially complementary to the one or more target regions of a transcript of their respective target sequences.

Preferably the first and second effector sequences are both selected from any 10 or more and preferably any 17 or more contiguous nucleotides within a sequence from the group consisting of SEQ ID NOS: 40-78. For example, in one embodiment, there is provided a DNA-directed RNA interference (ddRNAi) agent for inhibiting expression of one or more target sequences in an AMD-associated gene, the ddRNAi agent comprising, in a 5' to 3' direction, at least:

a first effector sequence of any 10 or more contiguous nucleotides within 5' UAUGUGGGUGGGUGUGUCUAC 3' (SEQ ID NO:47);

a first effector complement sequence;

a second effector sequence any 10 or more contiguous nucleotides within 5' AAGUUCAUGGUUUCGGAGGCC 3' (SEQ ID NO:41) or 5' UCUUUCUUUGGUCUGCAUUCA 3' (SEQ ID NO:44); and

a second effector complement sequence,

wherein the AMD-associated gene is VEGF-A.

Each effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences.

Alternatively, at least one effector, and preferably both effector sequences, have 100% complementarity to one or more target regions in a transcript of the one or more target sequences.

In particularly preferred embodiments, the first and second effector sequence comprise any 10 or more, preferably any 17 or more, contiguous nucleotides within sequences able to inhibit the expression of a target region by at least 70%. Preferably, in this embodiment, each effector sequence is selected from SEQ ID NOS: 40-78, more preferably SEQ ID NOS: 40-59, and most preferably SEQ ID NOS: 40-49.

In yet another embodiment, being an embodiment where the ddRNAi agent has 3 effector sequences, there is provided a DNA-directed RNA interference (ddRNAi) agent for inhibiting expression of one or more target sequences in one or more AMD-associated genes, the ddRNAi agent comprising, in a 5' to 3' direction, at least:

a first effector sequence of any 10 or more contiguous nucleotides within 5' AAGUUCAUGGUUUCGGAGGCC 3' (SEQ ID NO:41);

a first effector complement sequence;

a second effector sequence of any 10 or more contiguous nucleotides within 5' UCUUUCUUUGGUCUGCAUUCA 3' (SEQ ID NO:44);

a second effector complement sequence;

a third effector sequence of any 10 or more contiguous nucleotides within 5' UAUGUGGGUGGGUGUGUCUAC (SEQ ID NO:47); and

a third effector complement sequence,

wherein the AMD-associated gene is VEGF-A.

In yet another embodiment, being an embodiment where the ddRNAi agent has 2 effector sequences, there is provided a DNA-directed RNA interference (ddRNAi) agent for inhibiting expression of one or more target sequences in one or more AMD-associated genes, the ddRNAi agent comprising, in a 5' to 3' direction, at least:

a first effector sequence of any 10 or more contiguous nucleotides within 5' UGUAACAGAUGAGAUGCUCCA 3' of the AMD-associated gene VEGRF-2 (SEQ ID NO:56);

a first effector complement sequence;

a second effector sequence of any 10 or more contiguous nucleotides within 5' UAUGUGGGUGGGUGUGUCUAC 3' of the AMD-associated gene VEGFA (SEQ ID NO:47); and

a second effector complement sequence.

Each effector sequence in these embodiments is substantially complementary to one or more target regions in a transcript of the one or more target sequences.

It will be appreciated by the skilled person that the VEGFA sequence can be first and the VEGFR2 sequence can be second. This is an equivalent embodiment.

In yet another embodiment, being an embodiment where the ddRNAi agent has 3 effector sequences, there is provided a DNA-directed RNA interference (ddRNAi) agent for inhibiting expression of one or more target sequences in one or more AMD-associated genes, the ddRNAi agent comprising, in a 5' to 3' direction, at least:

a first effector sequence of any 10 or more contiguous nucleotides within 5' AAGUAGCCAGAAGAACAUGGC 3' of the AMD-associated gene VEGRF-2 (SEQ ID NO:52);

a first effector complement sequence;

a second effector sequence of any 10 or more contiguous nucleotides within 5' UUAUAGAAAACCCAAAUCCUC 3' of the AMD-associated gene CFB (SEQ ID NO:78);

a second effector complement sequence;

a third effector sequence of any 10 or more contiguous nucleotides within 5' UAGCUGAAGCCCACGAGGUCC 3' of the AMD-associated gene PDGFR-β (SEQ ID NO:63); and

a third effector complement sequence.

Each effector sequence in both of these embodiments is substantially complementary to one or more target regions in a transcript of the one or more target sequences. It will be appreciated by the skilled person that the sequence can be in a different 5' to 3' order and represent equivalent embodiments. For example, PDGFR-β can be first, VEGFR2 can be second and CFB can be third. Alternatively, at least one effector, and optionally

2 out of the 3 or all 3 of the effectors, is 100% complementary to one or more target regions in a transcript of the one or more target sequences.

In particularly preferred embodiments, the first, second and third effector sequence comprise any 10 or more, preferably any 17 or more, contiguous nucleotides within sequences able to inhibit the expression of a target gene region by at least 70%. Preferably, in this embodiment, each effector sequence is selected from SEQ ID NOS: 40-78, more preferably SEQ ID NOS: 40-59, and most preferably SEQ ID NOS: 40-49.

In yet further embodiments, the first effector sequence may be any 10 or more contiguous nucleotides within a sequence selected from the group consisting of SEQ ID NOS:40-78; the second effector sequence may be any 10 or more contiguous nucleotides within a sequence selected from the group consisting of SEQ ID NOS:40-78; the third effector sequence may be any 10 or more contiguous nucleotides within a sequence selected from the group consisting SEQ ID NOS:40-78; and any further effector sequences may be any 10 or more contiguous nucleotides within a sequence selected from the group consisting of SEQ ID NOS:40-78. Preferably, each effector sequence is at least 17 contiguous nucleotides.

Each effector sequence may also be a variant of SEQ ID NOS:40-78, having 1, 2, 3, 4 or 5 nucleotide variations. Preferably, the differences are present in the first and/or last 5 nucleotides, and at least the centre 10-12 nucleotides are 100% complementary to one or more target regions in a transcript of the one or more target sequences.

The first, second and third effector sequence may each comprise a sequence selected from any 10 or more contiguous nucleotides within a sequence from the group consisting of SEQ ID NOS: 40-78, or alternatively, each effector sequence may also be a variant of SEQ ID NOS:40-78, having 1, 2, 3, 4 or 5 nucleotide variations. In yet a further embodiment, each effector sequence may consist of 20 nucleotides, of which 17, 18, 19, or all 20 nucleotides are contiguous nucleotides from a sequence selected from the group consisting of SEQ ID NOS: 40-78. When there are multiple effector sequences, they may represent a combination of the 3 types described above.

Furthermore, in the long hairpin structure or the multiple hairpin structure the ddRNAi agent may include additional effector sequences and corresponding complementary sequences according to one of the following formula:

Long hairpin:

- [effector sequence]$_{1-10}$ [effector complement sequence]$_{1-10}$

Multiple hairpin:

- [effector sequence-effector complement sequence]$_{1-10}$

Preferably, in the long hairpin formula, the number of effector sequences is equal to the number of effector complement sequences. Typically, there are 2, 3, 4 or 5 effector sequences, and accordingly, 2, 3, 4 or 5 effector complement sequences respectively.

When the ddRNAi agent does contain more than one effector sequence, the effector sequences may be the same or different. For example, if a ddRNAi agent has 3 effector sequences, 2 effector sequences may have the same sequence, while 1 is different. Alternatively, all 3 effector sequences may be different. Preferably, the effector sequences are any 10 or more and preferably any 17 or more contiguous nucleotides within a sequence selected from the group consisting of SEQ ID NOS:40-78, or variants thereof which have 1, 2, 3, 4 or 5 nucleotide variations. Preferably, the differences are present in the first and/or last 5 nucleotides, and at least the centre 10-12 nucleotides are 100% complementary to one or more target regions in a transcript of the one or more target sequences.

When targeting a single region of a target sequence that has naturally occurring variants, or single nucleotide polymorphisms, it is preferably that at least one effector sequence is chosen from any 10 or more and preferably any 17 or more contiguous nucleotides within a sequence selected from the group consisting of SEQ ID NOS:40-78, whereas other effector sequences are variants of that chosen sequence. For example, a first effector sequence may comprise 20 nucleotides of SEQ ID NO: 47; the second effector sequence should therefore be a variant of SEQ ID NO:47.

Hairpin structures

In the above embodiments, the effector sequence hybridises with its corresponding effector complement sequence to form a hairpin structure. At the end of the hairpin, two or more unbound nucleotides form the 'hinge' or 'loop'. In one embodiment, the unbound nucleotides are part of the effector sequence and the effector complement, such that only a portion of the at least 17 nucleotides of the effector sequence will form

a duplex with its corresponding complementary sequence. For example, when the effector sequence and its complement are both 20 nucleotides long, 18 of the nucleotides may base pair to form a double stranded region, leaving a total of 4 nucleotides to form a single stranded loop between and joining the effector sequence and its effector complement sequence.

In an alternative embodiment, an additional sequence that is non-complementary to itself, the target sequence, the effector sequence or the effector complement may be included in the ddRNAi in order to create a 'loop'. As such, in yet another embodiment of the invention, the ddRNAi agent further includes a sequence of 2 to 100 unpaired nucleotides capable of forming a loop, more preferably, 2 to 10 unpaired nucleotides. In a preferred embodiment the loop includes the nucleotide sequence AA, UU, UUA, UUAG, UUACAA, CAAGAGA or N_1AAN_2, where N_1 and N_2 are any of C, G, U and A and may be the same or different. Otherwise, specific loop sequences include ACUGUGAAGCAGAUGGGU. In these loops, not all of the loop sequence has to remain non-annealed. In a loop of, for example, 18 nucleotides, the first and last 3 nucleotides for example may anneal with each other, leaving the intervening 15 nucleotides non-annealed.

In embodiments in which the ddRNAi agent is expressed as part of a miRNA structure the loop sequence may be derived from the miRNA, and is encoded by the miRNA encoding (ME) sequence.

There may be one or more loops depending on the ddRNAi agent structure. When a ddRNAi agent has a structure based on formula [effector sequence]$_{1\text{-}10}$ [effector complement sequence]$_{1\text{-}10}$ additional non-self-complementary sequence to give rise to a single loop structure is contained between the last effector sequence and the effector complement sequence of that last effector sequence, as illustrated in Figure 1D. In this embodiment, there is therefore provided a DNA-directed RNA interference (ddRNAi) agent for inhibiting expression of one or more target sequences in an AMD-associated gene selected from VEGF-A, VEGFR2, CFB and PDGFR-β, the ddRNAi agent comprising, in a 5' to 3' direction, at least:

a first effector sequence of at least 17 nucleotides in length;

a second effector sequence of at least 17 nucleotides in length;

a loop sequence of 2 to 100 non-self-complementary nucleotides;

a second effector complement sequence; and

a first effector complement sequence

wherein each effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences.

When the ddRNAi agent has a multiple hairpin structure based on formula [effector sequence-effector complement sequence]$_{1-10}$ additional non-self-complementary sequence is contained between each effector sequence and its complementary sequence to give rise to a loop structure, as illustrated in Figure 1E and F (depending on the type of expression cassette used to express it – see later in the specification). In this embodiment, there is provided a DNA-directed RNA interference (ddRNAi) agent for inhibiting expression of one or more target sequences in an AMD-associated gene, the ddRNAi agent comprising, in a 5' to 3' direction, at least:

a first effector sequence of at least 17 nucleotides in length;

a loop sequence of 2 to 100 non-self-complementary nucleotides;

a first effector complement sequence;

a second effector sequence of at least 17 nucleotides in length;

a loop sequence of 2 to 100 non-self-complementary nucleotides; and

a second effector complement sequence

wherein each effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences in the AMD-associated gene, and gene is selected from one or more of VEGF-A, VEGFR2, CFB and PDGFR-β.

In this embodiment where there are more than two effector and complementary sequences, and therefore more than two hairpin structures, the length of additional non-self-complementary sequence that forms each loop structure does not have to be the same. For example, one loop structure may have 5 nucleotides, while another loop structure may have 9 nucleotides.

In addition, when there are two or more hairpin structures, there may be additional non-self-complementary sequence that acts as a spacer sequence between each loop. In

this embodiment, there is provided a DNA-directed RNA interference (ddRNAi) agent for inhibiting expression of one or more target sequences in an AMD-associated gene, the ddRNAi agent comprising, in a 5' to 3' direction, at least:

a first effector sequence of at least 17 nucleotides in length;

a loop sequence of 2 to 100 non-self-complementary nucleotides;

a first effector complement sequence;

a spacer sequence of 2 to 100 non-self-complementary nucleotides;

a second effector sequence of at least 17 nucleotides in length;

a loop sequence of 2 to 100 non-self-complementary nucleotides; and

a second effector complement sequence

wherein each effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences in the AMD-associated gene, and gene is selected from one or more of VEGF-A, VEGFR2, CFB and PDGFR-β.

2 strand ddRNAi agents

As will be appreciated by one skilled in the art, it is not necessary that the entire ddRNAi agent is expressed as one sequence. For example, in one embodiment of the invention, the first effector sequence may be generated (e.g., transcribed by one DNA sequence), and the first effector complement sequence may be generated (e.g., transcribed from a separate DNA sequence). Optionally, a loop sequence may be attached to either transcript or part of the loop attached to the 3 'end of one transcript and the 5' end of the other transcript, and that loop sequence may be derived from a miRNA when the effector or effector complement sequence is expressed as part of a miRNA structure. Within the cell, the two transcripts then form the ddRNAi agent by hybridising through annealing between the first effector sequences and its complement.

in vitro expressed ddRNAi agents of chemically synthesised siRNA

While it is envisaged that effective treatment of wet AMD will require ddRNAi agents to be expressed *in vivo* from ddRNAi constructs (as will be outlined below), there may be circumstances where it is desirable to administer ddRNAi agents that are expressed *in vitro* or to administer siRNAs that are chemically synthesised, thereby functioning as therapy with transient duration of effect. Screening the patient for their reaction to the

treatment for example may benefit from a short term treatment with siRNAs that do not integrate and replicate in the cells before commencing long term therapy with *in vivo* expressed ddRNAi agents.

The ddRNAi agents of the invention may therefore be expressed *in vitro* and then delivered to target cells. Alternatively, siRNAs may be chemically synthesised and then delivered to the target cells. In light of this, in another aspect of the invention, there is provided a small interfering RNAi agent (siRNA agent) for inhibiting expression of one or more target sequences in an AMD-associated gene, the siRNA comprising

a first effector sequence of at least 17 nucleotides in length; and

a first effector complement sequence;

wherein the effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences.

Similarly to the ddRNAi agents described above, the siRNA agent may also include more than one effector sequence for multiple targeting, be that multiple targets in a single gene such as VEGF-A, or targets in more than one gene, such as VEGFR2, CFB and PDGFR-β. The effector sequences are preferably selected from any 10 or more and preferably any 17 or more contiguous nucleotides within a sequence from the group consisting of SEQ ID NOS: 40-78.

Considerable flexibility is possible in the design of siRNAs. Typically siRNAs consist of dsRNA molecules with 5'-phosphate and 3'-hydroxyl residues, strand lengths can vary from 20-29 nucleotides and may optionally be designed to include 2 nucleotide 3' overhangs. In some embodiments each strand can be synthesised as $N_{19-27}TT$ (where TT can be deoxyribonucleotides). siRNAs can be readily designed based on regions of SEQ ID NOS: 40-78 as described above and can be used therapeutically as single sequences or in any combinations. Alternatively siRNA agents can consist of single RNA molecules containing effector and effector complement sequences similar or identical to those expressed from ddRNAi expression cassettes. These sequences can be based on SEQ ID NOS: 40-78 and can be used therapeutically as single sequences or in any combination with one another. The siRNAs can be chemically synthesized with appropriately protected ribonucleoside phosphoramidates and a conventional synthesiser and thus are widely available commercially and able to be designed and

synthesised according to routine methods in the art. In preferred embodiments, the siRNAs have the sequences of any 10 or more contiguous nucleotides within a sequence from one or more of SEQ ID NOS: 40-78.

Expression cassettes and miRNA backbones

The ddRNAi agents of the invention are expressed from DNA expression cassettes. The expression cassettes comprise the regulatory sequences required for expression, such as the promoter, together with the DNA sequence that encodes the ddRNAi agent itself. In embodiments in which the ddRNAi agent is expressed as part of a miRNA structure, the expression cassette also includes the DNA sequence that encodes for that miRNA structure.

The ddRNAi expression cassettes comprise (in no particular order):

- one of more promoter sequences
- one or more DNA sequences that encode for one or more effector sequences
- one or more DNA sequences that encode for one or more effector complement sequences;

and optionally

- one or more terminator sequences
- one or more DNA sequences that encode for loop sequences, spacer sequences or both
- one or more enhancer sequences.

The first promoter sequence and last terminator sequence may be derived from the vector in to which the expression cassette is cloned.

In one embodiment, there is provided a DNA-directed RNA interference (ddRNAi) expression cassette for expressing a ddRNAi agent, wherein the ddRNAi agent inhibits expression of one or more target sequences in an AMD-associated gene, the ddRNAi constructs comprising, in a 5' to 3' direction:

a promoter sequence

a DNA sequence that encodes for a first effector sequence

a DNA sequence that encodes for a first effector complement sequence; and

a terminator sequence.

The DNA sequence that encodes for the first effector sequence is preferably a DNA that encodes for 10 or more, preferably 17 or more, contiguous nucleotides within a sequence from any one of SEQ ID NOS: 40-78. In a particularly preferred embodiment, the first effector sequence comprise any 10 or more, preferably any 17 or more, contiguous nucleotides within sequences able to inhibit the expression of a target gene region by at least 70%. Preferably, in this embodiment, the first effector sequence is selected from SEQ ID NOS: 40-78, more preferably SEQ ID NOS: 40-59, and most preferably SEQ ID NOS: 40-49.

Alternatively, as outlined above in relation to the ddRNAi agent itself, the sequence that encodes for the effector sequence may encode an effector sequence that varies by 1, 2, 3, 4 or 5 nucleotides from SEQ ID NOS: 40-78 without effecting the ability of the sequence encoded to base pair with the transcript of the target sequence and inhibit expression of the target sequence.

The skilled person would appreciate that a DNA sequence encoding any given RNA sequence is the same sequence as the RNA but having thymine (T) bases instead of uracil (U) bases. The ddRNAi expression cassettes encoding ddRNAi agents having more than one effector sequence in a long hairpin structure comprise, in a 5' to 3' direction:

a promoter sequence;

a DNA sequence that encodes for a first effector sequence;

a DNA sequence that encodes for a second effector sequence;

optionally a sequence that encodes for sequence capable of forming a loop;

a DNA sequence that encodes for a second effector complement sequence;

a DNA sequence that encodes for a first effector complement sequence; and

optionally a terminator sequence.

Preferably the DNA sequences encode first and second effector sequence selected from any 10 or more and preferably any 17 or more contiguous nucleotides within a

sequence from the group consisting of SEQ ID NOS: 40-78. Preferably, the first and second effector sequence is selected from SEQ ID NOS: 40-59. Alternatively, the DNA sequences encode for an effector sequence that varies from SEQ ID NOS: 40-78 by 1, 2, 3, 4 or 5 nucleotides without affecting the ability of the effector sequence encoded to base pair with a transcript of the target sequence and inhibit expression of the target sequence.

When the ddRNAi agent has more than one effector sequence and a multiple hairpin structure based on formula [effector sequence-effector complement sequence]$_{1-10}$ expression of each [effector sequence-effector complement sequence] pair may be controlled by a single promoter, or alternatively by a separate promoter. When separate promoters are contemplated, the ddRNAi expression cassette comprises, in a 5' to 3' direction:

a promoter sequence

a DNA sequence that encodes for a first effector sequence

a DNA sequence that encodes for a first effector complement sequence;

optionally a terminator sequence;

a promoter sequence;

a DNA sequence that encodes for a second effector sequence;

a DNA sequence that encodes for a second effector complement sequence; and

optionally a terminator sequence.

In this embodiment, multiple ddRNAi agents are produced from the one expression cassette, as each effector/effector complement is expressed as a single hairpin structure.

When a single promoter is contemplated, the ddRNAi expression cassette comprises, in a 5' to 3' direction:

a promoter sequence

a DNA sequence that encodes for a first effector sequence

a DNA sequence that encodes for a first effector complement sequence;

a DNA sequence that encodes for a second effector sequence;

a DNA sequence that encodes for a second effector complement sequence; and

optionally a terminator sequence.

Similarly to the above embodiments, the DNA sequences preferably encode first and second effector sequence selected from any 10 or more and preferably any 17 or more contiguous nucleotides within a sequence from the group consisting of SEQ ID NOS:40-78, or, effector sequences that vary in sequence from SEQ ID NOS: 40-78 by 1, 2, 3, 4 or 5 nucleotides. Preferably, the first and second effector sequence is selected from SEQ ID NOS: 40-78, more preferably SEQ ID NOS: 40-59, and most preferably SEQ ID NOS: 40-49.

Any of the abovementioned ddRNAi agents are preferably expressed in a miRNA structure from an expression cassette.

Processing of shRNAs expressed from ddRNAi constructs can be imprecise. The expression of the ddRNAi within or as part of an RNA structure like a miRNA, which is a natural substrate for RNAi processing pathways, is one way to minimise this. McBride *et al.* (2008) designed "artificial miRNA" constructs which expressed sequences from the base and loop of endogenous miRNAs; these showed reduced toxicity suggesting more precise processing of expressed shRNAs. Wu *et al.* (2011) showed that mismatched duplexes (containing mismatches in the passenger strand) sometimes showed increased silencing activity, due possibly to their greater structural resemblance to endogenous miRNAs. Similarly Gu *et al.* (2012) showed the introduction of bulges adjacent to loop sequences in shRNA molecules can result in increased precision of dicer processing.

In embodiments where the effector and effector complement are expressed as a miRNA structure, the ddRNAi expression cassette further includes sequence that encodes for the miRNA structure referred to herein as "miRNA encoding sequence" or "ME sequence". This is the DNA sequence contained within a ddRNAi expression cassette that encodes for RNA which, once expressed, folds in to a miRNA structure. The effector sequence and the effector complement therefore are expressed as part of or within that miRNA structure. As will be appreciated from the Figures illustrating a

ddRNAi agent expressed in a miRNA structure, and as detailed earlier in the specification, the ME sequences will be located upstream and downstream of the effector sequence and the effector complement sequence as required. Using an expression cassette that expresses a ddRNAi agent with a single effector-effector complement pair as an example, there is provided a DNA-directed RNA interference (ddRNAi) expression cassette for expressing a ddRNAi agent, wherein the ddRNAi agent inhibits expression of one or more target sequences in an AMD-associated gene, the ddRNAi cassette comprising, in a 5' to 3' direction:

a promoter sequence

a first ME sequence

a DNA sequence that encodes for a first effector sequence

optionally a sequence that encodes for sequence capable of forming a loop

a DNA sequence that encodes for a first effector complement sequence;

a second ME sequence; and

optionally a terminator sequence,

wherein the sequence encoded by the first and second ME sequences is capable of forming a miRNA structure. The effector sequence and the effector complement therefore are expressed as part of or within that miRNA structure.

The optional sequence that encodes for sequence capable of forming a loop may also be ME sequence. For example, if a particular miRNA structure is being utilised as the structure in which the ddRNAi agent is expressed within or as part of, the loop sequence of the ddRNAi agent may come from the same miRNA. In alternative embodiments, the loop sequence may come from a different miRNA than the miRNA structure encoded by the ME sequences, but nonetheless, is still miRNA derived or originating sequence.

The ddRNAi expression cassette may alternatively be described by reference to the total length of the ddRNAi agent expressed, which is a product of the total length of sequence between the promoter and terminator. For example, when the length of the effector sequence in a single effector ddRNAi consists of 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides, the ddRNAi expression cassette will have a length

of 34 to 60 nucleotides between the promoter and terminator. This length may further include 2 to 100 nucleotides of "loop" or "hinge" sequence, giving a length of between 36 to 160 nucleotides. For ddRNAi agents having multiple effector sequences, where each effector sequence consists of 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides, the overall length is increased proportionally.

The presence of ME sequence for encoding the miRNA structure/s will also add to overall length.

One useful way of designing ddRNAi expression cassettes of the invention is to assume Dicer cuts every 22 nucleotides (also referred to as '22nt phasing'), and effector sequences can therefore be designed to encode any 10 or more, and preferably any 17 or more contiguous nucleotides within a sequence from the group consisting of SEQ ID NOS:40-78, together with appropriate spacers and other sequence requirements for the appropriate promoter.

Agents targeting different sites of mRNA are suitable for shRNA construction, because they can avoid the influence of secondary structures of mRNA, and thus perform their functions independently.

When a U6 promoter is used, it is preferable but not essential that the DNA sequence operably linked to the promoter starts with a guanine (G) base; when a H1 promoter is used, it is preferable but not essential that the DNA sequence operably linked to the promoter starts with an adenine (A) base. The effector encoding sequence can therefore be modified accordingly.

The use of miRNA-derived sequences to drive expression of shRNAs is particularly advantageous when using pol II promoters. Transcriptional initiation sites for pol II promoters are frequently imprecise. Since dicer processing of an shRNA is largely dependent on the structure of the shRNA, processing will not be greatly affected by slight variations in transcriptional start sites in most instances. The use of miRNA derived sequences therefore permits greater flexibility in designing ddRNAi constructs that utilise pol II promoters.

In some instances it may be advantageous to increase the length of shRNAs. One way to accomplish this is to extend the length of the effector sequence in an shRNA to maximise its complementarity to the target sequence, in either a 5' or 3' direction, and

also extend the length of the effector complement to maximise base pairing within the stem of the shRNA. For example an shRNA based on SEQ ID NO: 47 could be readily extended in a 5' or 3' direction to target additional sequences adjacent to those in SEQ ID NO:8 to produce an shRNA with a 30 nucleotide stem. The effector sequence could share substantial homology to the target as defined elsewhere in this specification.

In some instances it may be desirable to avoid the DNA sequence TTTT within effector, effector complement or loop sequences since these can act as transcriptional terminators in expression constructs which use Pol III promoters such as U6 or H1. shRNA design should also take in to account that U6 termination is expected to add a one to five U residues to the 3' end to the shRNA. When designing long hairpin RNAs, it is sometimes advantageous to modify the precise choice of effector sequences (either using sequences from, or adjacent to SEQ ID NOS: 40-78) to maximise the likelihood that Dicer processed effector sequences will include a 5'U or A, thereby encouraging incorporation into AGO2.

The choice of whether to control expression of each [effector sequence-effector complement sequence] pair with individual promoters or a single promoter depends on a number of factors. A single promoter may be utilised to minimise interference between promoters. A ddRNAi construct with only a single promoter is also smaller in size, which can be important in some cases for the stability of the construct, both during production (e.g. replication in *E.coli*) and delivery. In addition, the use of a single promoter avoids the possibility of any homologous recombination between promoters.

In circumstances where a degree of regulation of expression of each effector sequence or complement is required though, it is advantageous to design a ddRNAi construct having multiple promoters, whereby expression of each [effector sequence – effector complement sequence] pair is controlled by a separate promoter. In circumstances where the effector sequences are of a different sequence, the nature of the sequence may mean one sequence is expressed to higher expression levels. When it is desired to ensure more equal expression levels of each effector sequence, the more highly expressed effector sequence can be paired with a weaker promoter and vice versa. Moreover, more efficient expression may be achieved as the length of any one sequence to be transcribed is shorter particularly for pol III promoters. When multiple promoters are used, it is preferable that not all of the promoters are the same to

minimise the risk of any homologous recombination between them in the expression cassette. In the case of 2 promoters, each is preferably different. In the case of 3 promoters, at least 2 and optionally all 3 are different from one another.

The DNA sequence encoding the effector sequence is operably linked to the promoter sequence. A sequence is "operably linked" to another nucleotide sequence when it is placed in a functional relationship with another nucleotide sequence. For example, if a coding sequence is operably linked to a promoter sequence, this generally means that the promoter may promote transcription of the coding sequence. Operably linked means that the DNA sequences being linked are typically contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame. However, since enhancers may function when separated from the promoter by several kilobases and intronic sequences may be of variable length, some nucleotide sequences may be operably linked but not contiguous.

A "promoter" or "promoter sequence" or "promoter element" is generally a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a polynucleotide or polypeptide coding sequence such as mRNA or any kind of RNA transcribed by any class of any RNA polymerase. The promoter and terminator may be taken from different genes, but are typically matched to each other; that is, the promoter and terminator elements are taken from the same gene in which they occur naturally. Promoters also may or may not be modified using molecular techniques, or otherwise, e.g., through modification of regulatory elements, to attain weaker or stronger levels of transcription.

The term "constitutive" when made in reference to a promoter means that the promoter is capable of directing transcription of an operably linked nucleic acid sequence in the absence of a specific stimulus (e.g., heat shock, chemicals, light, etc.). Typically, constitutive promoters are capable of directing expression of a coding sequence in substantially any cell and any tissue. The promoters used to transcribe the ddRNAi agents preferably are constitutive promoters, such as the promoters for ubiquitin, CMV, β-actin, histone H4, EF-1alfa or pgk genes controlled by RNA polymerase II, or promoter elements controlled by RNA polymerase I. In other embodiments, a Pol II promoter such as CMV, SV40, U1, hAAT, β-actin or a hybrid Pol II promoter is employed. In other embodiments, promoter elements controlled by RNA polymerase III

are used, such as the U6 promoters (e.g. U6-1 , U6-8, U6-9), H1 promoter, 7SL promoter, the human Y promoters (hY1 , hY3, hY4 (see Maraia *et al.*, (1994)) and hY5 (see Maraia *et al.*, (1994)), the human MRP-7-2 promoter, Adenovirus VA1 promoter, human tRNA promoters, the 5S ribosomal RNA promoters, as well as functional hybrids and combinations of any of these promoters. Variants of all of these promoters may also be utilised, wherein the promoter is modified to decrease or increase its activity. For example, if a strong promoter causes too much expression of the sequence operably linked to it, it can be modified to decrease its activity.

When a U6 promoter is used, it is preferable that the DNA sequence operably linked to the promoter starts with a guanine (G) base; when a H1 promoter is used, it is preferable that the DNA sequence operably linked to the promoter starts with an adenine (A) base. The sequences of the nucleic acids may therefore favour the use of one promoter over another.

Alternatively in some embodiments it may be optimal to select promoters that allow for inducible expression of the multiple ddRNAi agents expressed from the ddRNAi construct. A number of systems for inducible expression using such promoters are known in the art, including but not limited to the tetracycline responsive system and the lac operator-repressor system (see WO 03/022052 A1 Publication; and U.S. Patent Publication 2002/0162126 A1), the ecdyson regulated system, or promoters regulated by glucocorticoids, progestins, estrogen, RU-486, steroids, thyroid hormones, cyclic AMP, cytokines, the calciferol family of regulators, or the metallothionein promoter (regulated by inorganic metals such as zinc or cadmium).

Promoters useful in some embodiments of the present invention may be tissue-specific or cell-specific. The term "tissue-specific" as it applies to a promoter refers to a promoter that is capable of directing selective expression of a nucleotide sequence of interest to a specific type of tissue in the relative absence of expression of the same nucleotide sequence of interest in a different type of tissue (e.g., brain). The term "cell-specific" as applied to a promoter refers to a promoter which is capable of directing selective expression of a nucleotide sequence of interest in a specific type of cell in the relative absence of expression of the same nucleotide sequence of interest in a different type of cell within the same tissue The term "cell-specific" when applied to a promoter also means a promoter capable of promoting selective expression of a nucleotide

sequence of interest in a region within a single tissue. Alternatively, promoters may be constitutive or regulatable. Additionally, promoters may be modified so as to possess different specificities.

Examples of cell specific promoters particularly useful in this invention include the RPE cell specific promoter RPE-65 and VMD2, and the choroid endothelial-specific promoters FLT-1 or ICAM2.

As noted above, enhancer elements are optionally included in the ddRNAi constructs of the invention.

When the ddRNAi expression cassette or construct contains more than one terminator sequence or element, the terminator sequences or elements may be the same, or different, or there may be a combination of termination elements represented only once and termination elements represented two times or more within any cassette. Whatever terminator sequences or elements are used they should be selected to ensure that they work appropriately with the liver-specific promoter used. In instances where Pol I, Pol II or Pol III promoters are used, appropriate terminator sequences should be employed. Termination elements useful in the present invention include the U1 termination sequence (U1 box), the synthetic polyA terminator, and the so called minimal PolyA terminator. Transcriptional pause sites, such as MAZ1 and MAZ2, (See Ashfield et al EMBO J 1994 Vol13 No 23 5656 pp and Yonaha and Proudfoot EMBO J. 2000 Jul. 17;19(14):3770-7) may be inserted upstream of the polyA terminators to assist in coupling of transcription termination and polyadenylation. For Pol III promoters, the sequences TTTT, TTTTT or TTTTTT are commonly used as terminators. In these instances transcripts are typically terminated by the sequence UU.

ddRNAi agent expression constructs

ddRNAi agents may be expressed from a DNA expression cassette inserted into any suitable vector or ddRNAi construct, referred to herein as 'ddRNAi constructs'. A challenge in the past to developing therapeutics for AMD has been efficient and uniform transduction of the correct cells to ensure long term expression without the need for recurring administrations.

When the vector backbone of the construct is compatible with a delivery system, the ddRNAi expression constructs are also delivery constructs. A particularly preferred

delivery construct is a viral vector. Use of a viral vector, like an adeno-associated virus (AAV), adenovirus (Ad) or lentivirus (LV) to deliver an expression construct that produces the therapeutic ddRNAi agent from within the cell, avoids an interferon response often caused by direct interactions of nucleic acids with surface-expressed toll-like receptor 3. This is a primary reason for a number of failures of siRNA-based ocular drugs in clinical trials.

In the case of the current invention, the ddRNAi agent of the invention is required to reach the retina pigment epithelial (RPE) cells or other cells deep within the retinal layers. To this effect, the invention utilizes a modified adeno-associated virus (AAV) vector, shown in murine models to be able to penetrate the RPE layer following intravitreal injection. Wildtype, unmodified AAV serotypes have limited ability to transduce more than the adjoining layer of cells when introduced into the eye through this route. For this reason, it is preferred that a modified AAV vector is utilised in the invention.

For example, site directed mutagenesis of AAV strains has been used to substitute tyrosine residues, leading to increased transduction (Li Zhong, Baozheng Li, Cathryn S. Mah, (2008) Proc Natl Acad Sci U S A. 105(22): 7827–7832). Similar modifications to AAV vectors have produced vectors that can transduce across all layers of the retina following intravitral injection (Hilda Petrs-Silva, Astra Dinculescu, Qiuhong Li et al. (2009) Mol Ther. 17(3): 463–471). Likewise, specific serine, threonine or lysine residues in AAV vectors have been modified to avoid the host cellular kinase/ubiquitination/proteasomal machinery and significantly increase transduction efficiency (Gabriel N, Hareendran S, Sen D et al. (2013) Hum Gene Ther Methods. 2013 (2):80-93). Methods that generate libraries of AAV capsid mutants can be screened to isolate variants with the desired properties of increased tissue specificity for a specific target tissue or reduced immunogenicity. Recently, Schaffer et al have been able to show broad transretinal delivery following intravitreal injection of an AAV mutated vector in which a 7mer peptide had been inserted into the capsid sequence (Dalkara, D., L.C. Byrne, R.R. Klimczak et al. (2013) Science Translational Medicine, 5:189ra76)

Typically, the genome of AAV contains only two genes. The "rep" gene codes for at least four separate proteins utilized in DNA replication. The "cap" gene product is

spliced differentially to generate the three proteins that comprise the capsid of the virus. When packaging the genome into nascent virus, only the Inverted Terminal Repeats (ITRs) are obligate sequences; rep and cap can be deleted from the genome and be replaced with heterologous sequences of choice. However, in order to produce the proteins needed to replicate and package the AAV- based heterologous construct into nascent virions, the rep and cap proteins must be provided in trans. The helper functions normally provided by co-infection with the helper virus, such as adenovirus or herpesvirus, can also be provided in trans in the form of one or more DNA expression plasmids. Since the genome normally encodes only two genes it is not surprising that, as a delivery vehicle, AAV is limited by a packaging capacity of 4.5 single stranded kilobases (kb). However, although this size restriction may limit the genes that can be delivered for replacement gene therapies, it does not adversely affect the packaging and expression of shorter sequences such as ddRNAi vectors.

The invention provides a ddRNAi expression construct comprising a ddRNAi expression cassette according for expressing a ddRNAi agent for inhibiting expression of one or more target sequences in an AMD associated gene, the expression cassette comprising (in no particular order)

one or more promoter sequences

one or more DNA sequences that encode for one or more effector sequences,

one or more DNA sequences that encode for one or more effector complement sequences;

and optionally

one or more terminator sequences

one or more DNA sequences that encode for loop sequences, spacer sequences, or both

one or more enhancer sequences,

wherein the construct is a viral delivery construct; preferably the viral delivery construct is an AAV modified vector.

Preferably the expression cassette further comprises ME sequence so that the ddRNAi agent is expressed as part of or within a miRNA structure.

In a preferred embodiment, the expression cassette of the viral delivery construct comprises two DNA sequences that encode a first effector sequence of any 10 or more contiguous nucleotides within 5' UGUAACAGAUGAGAUGCUCCA 3' of the AMD-associated gene VEGRF-2 (SEQ ID NO:56) and a second effector sequence of any 10 or more contiguous nucleotides within 5' UAUGUGGGUGGGUGUGUCUAC 3' of the AMD-associated gene VEGF-A (SEQ ID NO:47).

The expression of the ddRNAi agents of the invention following viral delivery will be durable, potentially up to the life of a patient, from a single administration of the drug. Accordingly, in another aspect of the invention, there is provided a ddRNAi therapeutic comprising a viral vector into which a ddRNAi expression cassette according to the invention is inserted. Preferably the expression cassette encodes for multiple ddRNAi agents, as either long hairpin structures or multiple hairpin structures selected from the combinations and embodiments described throughout the specification. In a preferred embodiment, the effector sequences and the effector complement sequences of the ddRNAi agents are expressed within a miRNA structure.

Typically, in the production of viral vectors, the normal endogenous genes of a virus can be deleted from the genome and be replaced with heterologous sequences of choice. However, in order to produce the proteins needed to replicate and package the virus-based heterologous construct into nascent virion, the viral proteins stripped from the genome must be provided in *trans*. Generation of the construct can be accomplished using any suitable genetic engineering techniques well known in the art, including without limitation, the standard techniques of PCR, oligonucleotide synthesis, DNA synthesis, restriction endonuclease digestion, ligation, transformation, plasmid purification, and DNA sequencing. The viral construct also may contain genes that allow for replication and propagation of virus, though in preferred embodiments such genes will be supplied in *trans*. Additionally, the ddRNAi construct may contain genes or genetic sequences from the genome of any known organism incorporated in native form or modified. For example, the preferred viral construct comprises sequences useful for replication of the construct in bacteria.

After generation of the viral based ddRNAi construct, the construct is packaged into viral particles. Any method known in the art may be used to produce infectious viral particles whose genome comprises a copy of the viral ddRNAi construct. One method utilizes

packaging cells that stably express in *trans* the viral proteins that are required for the incorporation of the viral ddRNAi construct into viral particles, as well as other sequences necessary or preferred for a particular viral delivery system (for example, sequences needed for replication, structural proteins and viral assembly) and either viral-derived or artificial ligands for tissue entry. Following transfection of the viral ddRNAi construct into packaging cells, the packaging cells then replicate viral sequences, express viral proteins and package the ddRNAi expression constructs into infectious viral particles. The packaging cell line may be any cell line that is capable of expressing viral proteins, including but not limited to 293, HeLa, A549, PerC6, D17, MDCK, BHK, bing cherry, phoenix, Cf2Th, or any other line known to or developed by those skilled in the art. One packaging cell line is described, for example, in U.S. Pat. No. 6,218,181.

Alternatively, a cell line that does not stably express necessary viral proteins may be co-transfected with one or more constructs to achieve efficient production of functional particles. One of the constructs is the viral based ddRNAi construct; the other construct comprises nucleic acids encoding the proteins necessary to allow the cells to produce functional virus as well as other helper functions.

The packaging cell line or replication and packaging construct may not express envelope gene products. In these embodiments, the gene encoding the envelope gene can be provided on a separate construct that is co-transfected with the viral based ddRNAi construct. As the envelope protein is responsible, in part, for the host range of the viral particles, the viruses may be pseudotyped. As described *supra*, a "pseudotyped" virus is a viral particle having an envelope protein that is from a virus other than the virus from which the genome is derived. One with skill in the art can choose an appropriate pseudotype for the viral delivery system used and cell to be targeted.

In addition to conferring a specific host range, a chosen pseudotype may permit the virus to be concentrated to a very high titer. Viruses alternatively can be pseudotyped with ecotropic envelope proteins that limit infection to a specific species (e.g., ecotropic envelopes allow infection of, e.g., murine cells only, where amphotropic envelopes allow infection of, e.g., both human and murine cells). In addition, genetically-modified ligands can be used for cell- specific targeting.

After production in a packaging cell line, the viral particles containing the ddRNAi expression cassettes are purified and quantified (titred). Purification strategies include density gradient centrifugation, or, preferably, column chromatographic methods.

Methods

Administration of ddRNAi agents, ddRNAi constructs of siRNA agents of the invention inhibits expression of genes expressed in cells within the retina. Accordingly, in another aspect of the invention, there is provided a method of treating AMD in an individual comprising the administration of a therapeutically effective amount of a ddRNAi construct to a patient in need of treatment, wherein the ddRNAi agent inhibits expression of one or more target sequences in an AMD-associated gene, preferably a VEGF-A gene. Preferably, the AMD to be treated is wet AMD.

The ddRNAi agent to be administered to the patient may be one or more of:

- ddRNAi agent comprising a first effector sequence; and a first effector complement sequence; wherein the effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences
- ddRNAi agent comprising, in a 5' to 3' direction, a first effector sequence; a second effector sequence; a second effector complement sequence; and a first effector complement sequence, wherein each effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences
- a ddRNAi agent comprising, in a 5' to 3' direction, a first effector sequence; a second effector sequence; a third effector sequence; a third effector complement sequence; a second effector complement sequence; and a first effector complement sequence wherein each effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences
- a ddRNAi agent comprising, in a 5' to 3' direction, a first effector sequence; a first effector complement sequence; a second effector sequence; and a second effector complement sequence wherein each effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences
- a ddRNAi agent comprising, in a 5' to 3' direction, a first effector sequence; a first effector complement sequence; a second effector sequence; a second effector

complement sequence; a third effector sequence; and a third effector complement sequence; wherein each effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences

- a ddRNAi agent comprising, in a 5' to 3' direction, a first effector sequence; a second effector sequence; a loop sequence of 2 to 100 non-self-complementary nucleotides; a second effector complement sequence; and a first effector complement sequence wherein each effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences

- a ddRNAi agent comprising, in a 5' to 3' direction, a first effector sequence; a loop sequence of 2 to 100 non-self-complementary nucleotides; a first effector complement sequence; a second effector sequence; a loop sequence of 2 to 100 non-self-complementary nucleotides; and a second effector complement sequence wherein each effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences

- a ddRNAi agent comprising, in a 5' to 3' direction, a first effector sequence; a loop sequence of 2 to 100 non-self-complementary nucleotides; a first effector complement sequence; a spacer sequence of 2 to 100 non-self-complementary nucleotides; a second effector sequence; a loop sequence of 2 to 100 non-self-complementary nucleotides; and a second effector complement sequence wherein each effector sequence is substantially complementary one or more target regions in a transcript of the one or more target sequences

- a ddRNAi agent comprising, in a 5' to 3' direction, a first effector sequence; a first effector complement sequence; a spacer sequence of 2 to 100 non-self-complementary nucleotides; a second effector sequence; a second effector complement sequence; a spacer sequence of 2 to 100 non-self-complementary nucleotides; a third effector sequence; and a third effector complement sequence

- any of the above mentioned ddRNAi agents expressed within or as part of an miRNA structure.

As would be understood by one skilled in the art, and as illustrated in the Figures, any particular effector sequence may be swapped in position with its complement in the ddRNAi agent. In particular forms of each of the embodiments described above, each

effector sequence is at least 17 nucleotides in length selected from the group consisting of any 10 or more and preferably any 17 or more contiguous nucleotides within a sequence from any one of SEQ ID NOS: 40-78. The effector sequences may all be the same, or may all be different, or may be a combination e.g. 2 effector sequences of at least 10 contiguous nucleotides of SEQ ID NO:47 and 1 effector sequence of at least 10 contiguous nucleotides of (for example) SEQ ID NO: 56.

Preferably, the effector sequence is selected from the group consisting of any contiguous 11, 12, 13, 14, 15 or 16 nucleotides within any one of SEQ ID NOS: 40-78, and most preferably 17 or more contiguous nucleotides within any one of SEQ ID NOS: 40-78. Typically, the effector complement will be the same length, or about the same length (ie ± 15% nucleotide length, or 1 to 3 nucleotides different depending on the overall length) as its corresponding effector sequence.

Each of these ddRNAi agents may be administered via a ddRNAi expression cassette in a ddRNAi construct, as described in the earlier sections of the specification. Preferably the ddRNAi construct is the AAV based construct to enable targeting of the construct to the RPE cells in the back of the eye. Multiple targeting may be achieved by delivering two or more ddRNAi expression cassettes or constructs each capable of expressing a single ddRNAi agent, or alternatively, and most preferably, by delivering one ddRNAi expression cassettes or constructs capable of expressing more than one ddRNAi agent.

In alternative embodiments, each of the effector sequences may be 100% complementary to one or more target regions in a transcript of the one or more target sequences, or may only vary by 1, 2, 3, 4 or 5 nucleotides.

The method of treating AMD can optionally include a preliminary step of identifying an individual having symptoms of AMD and requiring treatment. That identification step can include differentially diagnosing the subject as having wet AMD or dry AMD.

For longer term or stable provision of the ddRNAi agents of the invention, the ddRNAi agent is provided via a ddRNAi construct of the invention ie *in vivo* expression of the ddRNAi agent from a ddRNAi expression cassette inserted into a suitable vector delivered to the cell. The ddRNAi expression cassette comprises:

one or more promoter sequences

one or more DNA sequences selected from the group consisting of sequences that encode for any 10 or more contiguous nucleotides within a sequence from SEQ ID NOS: 40-78;

one or more DNA sequences that encode for one or more effector complement sequences;

and optionally

one or more terminator sequences

one or more DNA sequences that encode for loop sequences, spacer sequences or both

one or more enhancer sequences.

As outlined earlier in the specification, these components of the ddRNAi expression cassette may have different 5' to 3' arrangements, all of which are suitable for use in the methods of the invention. The expression cassette preferably also includes DNA sequences that encode sequence capable of forming a miRNA structure.

Preferably, the target AMD-associated gene in the methods of the invention is VEGF-A. Accordingly, in one embodiment of the invention, the ddRNAi agent inhibits expression of one or more target sequences in the VEGF-A gene. The DNA sequence that encodes for the first effector sequence is preferably selected from the ddRNAi effector encoding sequences of any 10 or more contiguous nucleotides within a sequence from SEQ ID NOS: 40-49 listed in Table 1. Alternatively, as detailed earlier, the sequence that encodes for the effector sequence may vary from SEQ ID NOS: 40-49 by 1, 2, 3, 4 or 5 nucleotides without effecting the ability of the sequence encoded to base pair with the target sequence and inhibit expression of the VEGF-A target sequence.

Typically, each effector sequence forms a double stranded region with the corresponding effector complement sequence.

In an alternative embodiment, the target AMD-associated gene in the methods of the invention is one or more of VEGFR2, CFB and PDGFR-β.

In an alternative embodiment, the method of treating AMD in an individual comprises the administration of a therapeutically effective amount of a ddRNAi construct that

encodes a ddRNAi agent having more than one effector sequence, such as those listed above as SEQ ID NOS: 40-78, for inhibiting, preventing or reducing expression of one or more target sequences in an AMD associated gene.

In any of the treatment methods of the invention, the patient may also be receiving other treatments, such that the ddRNAi construct administered is an adjunct therapy.

AMD, and wet AMD in particular, is characterised by an abnormal outgrowth of blood vessels from the vasculature situated behind the retina in a process that is often referred to as choroidal neovascularization (CNV). Controlling CNV therefore has a positive effect on patients suffering from wet AMD. Accordingly, another aspect of the invention is a method of treating choroidal neovascularization in an individual comprising the administration of a therapeutically effective amount of a ddRNAi agent, expression cassette or construct of the invention to a patient in need of treatment, wherein the ddRNAi agent inhibits expression of one or more target sequences in one or more of VEGF-A, VEGFR2, CFB and PDGFR-β. Each of these genes is a target of interest by virtue of their role in angiogenesis, neovascularisation or the VEGF pathway.

Another important factor in the pathogenesis of AMD is the formation of extracellular deposits at the base of the eye called drusen. These deposits contribute to distortion of the macular and may also play a role in neovascularisation. CFB is a component of drusen. As such, targeting the CFB gene to inhibit expression of its protein product can reduce the amount of drusen being deposited, therefore having a positive effect on patients suffering from AMD. There is therefore provided a method of reducing drusen deposits in an individual comprising the administration of a therapeutically effective amount of a ddRNAi agent, expression cassette or construct of the invention to a patient in need of treatment, wherein the ddRNAi agent inhibits expression of one or more target sequences in CFB.

Seeking to minimise angiogenesis and therefore CNV, together with seeking to inhibit drusen deposition by way of targeting combinations of VEGF-A, VEGFR2, CFB and PDGFR-β therefore provides a multi-pronged attack strategy for AMD, particularly wet AMD, that has not been previously contemplated in the art, and seeks to not only stop progression of AMD, but to restore visual acuity.

In some instances, it may be preferred to rely on the transient presence of a ddRNAi agent or siRNA agent as opposed to long term expression of ddRNAi agents from integrated or stably maintained ddRNAi constructs. For example, where the patient's tolerance to the treatment is to be determined first. In this instance, a ddRNAi agent or siRNA agent of the invention produced *in vitro* may be administered.

In a further aspect of the invention there is provided a composition comprising ddRNAi constructs, ddRNAi agents or siRNA agents as an active ingredient for inhibiting, preventing or reducing expression of one or more target sequences in an AMD associated gene, to treat AMD, treat CNV, minimise drusen deposition or alleviate the symptoms of AMD.

In a further aspect of the invention there is provided use of a ddRNAi construct, ddRNAi agent or siRNA agent for inhibiting, preventing or reducing expression of one or more target sequences in an AMD associated gene, to treat AMD, treat CNV, minimise drusen deposition or alleviate the symptoms of AMD. Similarly, there is provided use a ddRNAi construct, ddRNAi agent or siRNA agent in the preparation of a medicament for inhibiting, preventing or reducing expression of one or more target sequences in an AMD associated gene, to treat AMD, treat CNV, minimise drusen deposition or alleviate the symptoms of AMD.

Preferably the AMD is wet AMD.

The one or more effector sequences of the ddRNAi constructs, ddRNAi agents or siRNA agents used in the methods of the invention comprise any 10 or more, preferably any 17 or more, contiguous nucleotides within sequences able to inhibit the expression of the AMD-associated target gene region by at least 70%. Preferably the one or more effector sequence is selected from SEQ ID NOS: 40-78, more preferably SEQ ID NOS: 40-59, and most preferably SEQ ID NOS: 40-49.

In each of the methods of the invention, the ddRNAi agent, ddRNAi expression cassette or ddRNAi expression construct of the invention is preferably delivered to the subject's eye/s by intravitreal injection, although subretinal injection may also be utilised.

Pharmaceutical compositions

The ddRNAi agents, the siRNA agents or the vectors comprising ddRNAi expression cassettes of the invention can be formulated into pharmaceutical compositions by

combination with appropriate, pharmaceutically acceptable carriers or diluents. Accordingly, there is provided a pharmaceutical composition comprising a ddRNAi agent, a ddRNAi expression cassette, a ddRNAi construct or a siRNA agent of the invention for inhibiting, preventing or reducing expression of one or more target sequences in an AMD associated gene, and a pharmaceutically acceptable carrier or diluent.

In another embodiment the invention provides an AMD treatment composition comprising an effective amount of a ddRNAi agent, ddRNAi expression cassette or ddRNAi expression construct of the invention as a main ingredient for inhibiting, preventing or reducing expression of one or more target sequences in an AMD associated gene, optionally with a pharmaceutically acceptable carrier or diluent.

In pharmaceutical dosage forms, the agents or the vectors comprising the ddRNAi expression cassettes may be administered alone or in association or combination with other pharmaceutically active compounds. Those with skill in the art will appreciate readily that dose levels for agents or vectors comprising the ddRNAi expression cassettes will vary as a function of the nature of the delivery vehicle, the relative ease of transduction of the target cells, the expression level of the RNAi agents in the target cells and the like.

The ddRNAi agents, the siRNA agents or the vectors comprising ddRNAi expression cassettes of the invention can be formulated into preparations for injection or administration by dissolving, suspending or emulsifying them in an aqueous or non-aqueous solvent, such as oils, synthetic aliphatic acid glycerides, esters of higher aliphatic acids or propylene glycol; and if desired, with conventional additives such as solubilisers, isotonic agents, suspending agents, emulsifying agents, stabilizers and preservatives.

The most preferred mode of administration of the pharmaceutical composition of the invention to the subject's eye/s is by intravitreal injection. An alternative method of administration is subretinal injection.

Pharmaceutically acceptable carriers or diluents contemplated by the invention include any diluents, carriers, excipients, and stabilizers that are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate,

and other organic acids; antioxidants including ascorbic acid and methionine; preservatives (such as octadecyldimethylbenzyl ammonium chloride; hexamethonium chloride; benzalkonium chloride, benzethonium chloride; phenol, butyl or benzyl alcohol; alkyl parabens such as methyl or propyl paraben; catechol; resorcinol; cyclohexanol; 3-pentanol; and m-cresol); low molecular weight (less than about 10 residues) polypeptides; proteins, such as plasma albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, histidine, arginine, or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugars such as sucrose, mannitol, trehalose or sorbitol; salt-forming counter-ions such as sodium; metal complexes (e.g. Zn-protein complexes); and/or non-ionic surfactants such as TWEEN™, PLURONICS™ or polyethylene glycol (PEG).

In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and if necessary, shaping the product. Formulation may be conducted by mixing at ambient temperature at the appropriate pH, and at the desired degree of purity, with physiologically acceptable carriers, i.e., carriers that are non-toxic to recipients at the dosages and concentrations employed.

The one or more effector sequences of the ddRNAi constructs, ddRNAi agents or siRNA agents used in the compositions of the invention comprise any 10 or more, preferably any 17 or more, contiguous nucleotides within sequences able to inhibit the expression of the AMD-associated target gene region by at least 70%. Preferably the one or more effector sequence is selected from SEQ ID NOS: 40-78, more preferably SEQ ID NOS: 40-59, and most preferably SEQ ID NOS: 40-49.

In another embodiment there is provided a kit or article of manufacture including an RNAi agent or pharmaceutical composition as described above.

In other embodiments there is provided a kit for use in a therapeutic application mentioned above, the kit including:

- a container holding a RNAi agent or pharmaceutical composition;
- a label or package insert with instructions for use.

In certain embodiments the kit may contain one or more further active principles or ingredients for treatment of AMD or for treating an AMD-related condition as described above.

The kit or "article of manufacture" may comprise a container and a label or package insert on or associated with the container. Suitable containers include, for example, bottles, vials, syringes, blister pack, etc. The containers may be formed from a variety of materials such as glass or plastic. The container holds an RNAi agent or pharmaceutical composition which is effective for treating the condition and may have a sterile access port (for example the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The label or package insert indicates that the RNAi agent or pharmaceutical composition is used for treating the condition of choice. In one embodiment, the label or package insert includes instructions for use and indicates that the RNAi agent or pharmaceutical composition can be used to treat AMD or for treating a AMD-related condition as described above.

The kit may comprise (a) an RNAi agent or pharmaceutical composition; and (b) a second container with a second active principle or ingredient contained therein. The kit in this embodiment of the invention may further comprise a package insert indicating that the RNAi agent or pharmaceutical composition and other active principle can be used to treat AMD or for treating an AMD-related condition as described above. Alternatively, or additionally, the kit may further comprise a second (or third) container comprising a pharmaceutically-acceptable buffer, such as bacteriostatic water for injection (BWFI), phosphate-buffered saline, Ringer's solution and dextrose solution. It may further include other materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, and syringes.

In certain embodiments an RNAi agent or pharmaceutical composition may be provided in the form of a device, disposable or reusable, including a receptacle for holding the RNAi agent or pharmaceutical composition. In one embodiment, the device is a syringe, preferably a syringe suitable for intravitreal injection or subretinal injection. The device may hold 1-2 mL of the RNAi agent or pharmaceutical composition. The RNAi agent or pharmaceutical composition may be provided in the device in a state that is ready for use or in a state requiring mixing or addition of further components.

The invention is now described with reference to the following non-limiting examples.

EXAMPLES

1. Design and preparation of constructs to silence VEGF-A

ddRNAi constructs expressing shRNAs targeting VEGF-A were designed, to recognise RNAi target sequences in the VEGF-A mRNA that are well conserved between human and the pre-clinical test species mouse and macaque. 10 ddRNAi constructs (miR-1, miR-2, miR-3, miR-4, miR-5, miR-6, miR-7, miR-8, miR-9 and miR-10) were generated to express the effector sequences listed in Table 2. Oligonucleotides were synthesised (Sigma Aldrich) and assembled to produce BamHI / Hind III fragments that was cloned into the BamHI / Hind III sites of pSilencer 2.1-U6 hygro according to the manufacture's protocol (Invitrogen). These constructs used the human U6 promoter to drive expression of shRNAs. Maps of the vector and an insert for one such construct are shown In Figures 2A and 2B. The sequence of the insert and predicted secondary structure of the expressed shRNA for miR-8 are shown in Figures 2C and 2D. Sequences of the BamHI / Hind III fragments used to prepare miRs-1 to 10 are listed as SEQ ID NOS: 91-100.

2. Activity and strand-specificity of constructs targeting VEGF-A

Dual luciferase assays were used to determine the activity of miR constructs. Because firefly luciferase has a relatively short half-life of approximately four hours, measurement of firefly luciferase activity provides a surrogate marker for assessing RNAi inhibitory activity. For these experiments, sensor constructs containing regions of VEGF-A cDNA were cloned into the 3' UTR of a firefly luciferase expression construct pGL3 (Promega). Regions of a VEGF-A cDNA clone, obtained from Open Biosystems (a Thermo Scientific company), were amplified by PCR using methods well known in the art, to prepare fragments flanked by XbaI and FseI restriction sites; these amplified fragments were then cloned into the XbaI / FseI sites in the 3' UTR of pGL3. Five separate regions of VEGF-A were amplified in this way to prepare reporter constructs that could be used to assay miR 1–10, as shown in Table 3. These five regions (A to E, Table 3) were cloned in both orientations which allowed the strand preference of RISC loading to be determined. "Sense" reporter constructs assayed activity of passenger strands, while reporters termed "antisense" assayed activity of effector strands. ddRNAi constructs with strong effector activity and weak passenger activity are strongly favoured for

therapeutic use since, as discussed above, since these are likely to produce less off target effects.

To assay the activity and strand-preference of each of the miR constructs targeting VEGF-A, dual luciferase assays were performed according to manufacturer's (Promega) protocol. Briefly a specific ddRNAi construct was co-transfected along with the appropriate VEGF-A sensor and a *Renilla* luciferase expressing plasmid (pRL: Promega), the latter of which was to normalize for transfection efficiency between wells, into HEK293T cells using Fugene according to manufacturer's (Roche Applied Sciences) protocol. Cells were cultured for 48 hrs and Dual Luciferase assays performed according to manufacturer's (Promega) protocol using a Turner Biosystems Veritas luminometer.

Results of typical experiments are shown in Figure 4A. These data showed that all 10 ddRNAi constructs showed significant silencing of the antisense target, but differed significantly in activity against the sense target, reflecting marked differences in RISC loading of passenger strands between the different ddRNAi constructs. Based on these data miRs-2, 5 and 8 were chosen for further analyses.

To confirm the activity of these constructs against native VEGF-A, HEK293T cells were co-transfected with an expression plasmid expressing VEGF-A protein along with expression constructs for miR-2, 5 and 8 using Fugene according to manufacturer's (Roche Applied Sciences) protocol. After 48 hrs RNA were isolated using a modified Trizol Protocol (Invitrogen). VEGF-A mRNA levels were determined using RT QPCR Assay on Demand according to manufacturer's protocol (Applied Biosystems Inc). These data (Figure 4B) show that VEGF-A shMiRs 2, 5 and 8 significantly reduce steady state VEGF-A mRNA levels in the transfected cells.

To further validate the activity of shMiR-8 against endogenously expressed VEGF-A, a spontaneously arising retinal pigment epithelia (RPE) cell line termed ARPE-19 were transduced with an adenovirus construct (MOI=200) that expresses miR-8. RNA and protein were isolated from cells at 24, 48, 72 and 96 hrs. VEGF-A mRNA levels were determined using RTQPCR as described above. Protein levels were determined using an ELISA assay performed with the Human VEGF Quantikine ELISA Kit according to manufacturer's (R&D Systems) protocol. These data (Figure 4C) showed that miR-8

potently silences VEGF-A expression at both the protein and mRNA level, with levels of knockdown increasing over time.

To quantify levels of shRNA expression from miR-8, a custom RT-QPCR assay was developed. A synthetic RNA standard (Sigma Aldrich) and forward DNA primers (Sigma Aldrich) were used to develop this assay in order to quantify the levels of effector RNA processed from the expressed shRNA of miR-8. The sequences of the synthetic RNA standard and DNA primers were:

Synthetic RNA standard: UGGGUUAUGUGGGUGGGUGUGUCUACCGCCU (SEQ ID NO: 130)

Forward primer (DNA):TATGTGGGTGGGTGTGTCTAC (SEQ ID NO: 131)

Reverse primer (DNA): miScript Universal Primer from kit (Qiagen)

RNAs were reverse transcribed using the miSCRIPT Reverse Transcription Kit according to manufacturer's protocol (Qiagen). Components of this kit polyadenylate RNAs and synthesise cDNA copies via the actions of reverse transcriptase and a clamped oligo dT primer which acts as a primer for cDNA synthesis. cDNAs were amplified and quantified using SYBR green QRT PCR assays, using protocols well known to those familiar with the art. Known amounts of the synthetic RNA standard were reverse transcribed and QPCR amplified to prepare a standard curve. RNAs were isolated from the aforementioned ARPE-19 cells and levels of expressed shRNA were quantified using this assay. Figure 4B shows that the levels of processed shRNA expressed form miR-8 increased over time and correlated with levels of VEGF-A knockdown at the protein and RNA level. Take together these data showed that miR-8 potently silenced VEGF-A. Note that the VEGF-A target sequences and effector sequences of miRs-2, 5 and 8 show absolute conservation of nucleotide sequences between human and the pre-clinical test species mouse and macaque.

3. Design and preparation of constructs to silence VEGFR2

ddRNAi constructs expressing shRNAs targeting VEGFR2 were designed, using the criteria described above, to recognise target sequences in VEGFR2 mRNA that are conserved between human and the pre-clinical test species mouse and macaque. In most cases, it was difficult to find sequences that were absolutely conserved between human, mouse and monkey species. 10 ddRNAi constructs (miR-V-1, miR-V-2, miR-V-

3, miR-V-4, miR-V-5, miR-V-6, miR-V-7, miR-V-8, miR-V-9 and miR-V-10) were constructed. Sequences of the BamHI / HindIII fragments used to prepare these are listed as SEQ ID NOS: 101-110 as summarised in Table 2. Inserts were cloned into pSilencer 2.1-U6 hygro as described in Example 1.

4. Activity and strand-specificity of constructs targeting VEGFR2

Dual luciferase assays were performed as described above to determine the activity and strand-preference of miR-V-1 through miR-V-10 using the protocol described in Example 2 and the reporter constructs listed in Table 3.

Table 3: Reporter constructs used to assay activity and strand specificity of miR constructs.

Target gene	GB Accession	Reporter code[a]	Positions[b]	miR[c]
VEGF-A	NM_001025366 (SEQ ID NO:79)	A-sense	727-1221	miR-2
		B-sense	1077-1829	miR-3, 4 & 5
		C-sense	1715-2357	miR-6, 7 & 8
		D-sense	3149-3614	miR-9 & 10
		E-sense	299-366	miR-1
		A-antisense	727-1221	miR-2
		B-antisense	1077-1829	miR-3, 4 & 5
		C-antisense	1715-2357	miR-6, 7 & 8
		D-antisense	3149-3608	miR-9 & 10
		E-antisense	299-366	miR-1
PDGFR-B	NM_002609 (SEQ ID NO:85)	A-sense	843-1340	miR-P-1, 2
		B-sense	1920-2435	miR-P-3
		C-sense	2672-3212	miR-P-4, 5, 6, 7, 8 & 9
		D-sense	2872-3421	miR-P-4, 5, 6, 7, 8, 9 & 10
		A-antisense	843-1340	miR-P-1, 2
		B-antisense	1920-2435	miR-P-3
		C-antisense	2672-3212	miR-P-4, 5, 6, 7, 8 & 9
		D-antisense	2872-3421	miR-P-4, 5, 6, 7, 8, 9 & 10
VEGFR-2	NM_002253 (SEQ ID NO:82)	A-sense	382-1098	miR-V1, 2
		B-sense	2519-3098	miR-V-3, 4, & 5
		C-sense	3078-3567	mir-V-6, 7 & 8
		D-sense	3549-4108	mir-V-9 & 10
		A-antisense	382-1098	miR-V1, 2
		B-antisense	2520-3098	miR-V-3, 4, & 5
		C-antisense	3078-3567	mir-V-6, 7 & 8
		D-antisense	3549-4108	mir-V-9 & 10
CFB	NM_001710 (SEQ ID NO:88)	A-sense	739-1261	miR-C-1, 2, 3 & 4
		B-sense	1361-1901	miR-C-, 5, 6 & 7
		C-sense	2008-2568	miR-C-8 & 9
		A-antisense	739-1261	miR-C-1, 2, 3 & 4

		B-antisense	1361-1901	miR-C-5, 6 & 7
		C-antisense	2008-2568	miR-C-8 & 9

[a] Code describing the particular luc fusion construct used in dual luciferase assays to assay the activity and strand specificity of miR constructs.

[b] Sequences included in luc fusion constructs.

[c] miR constructs assayed with individual reporters.

Results of these experiments are shown in Figure 5A. These data showed that all 10 constructs could achieve significant silencing of the antisense reporter construct but differed significantly in activity against the sense reporter construct, reflecting marked differences in RISC loading of passenger strands between the different ddRNAi constructs and the resulting consequent propensities for off-target effects. Based on these data, miR-V-2, -3, -7 and -10 were chosen for subsequent analyses.

To confirm the activity of these constructs against native VEGFR2 mRNA, HEK 293T cells were co-transfected with plasmids expressing VEGFR2 miRs-V-2, 3, 7 and 10 and an expression plasmid expressing the full length cDNA for VEGFR2 using Fugene according to manufacturer's (Roche Applied Sciences) protocol. After 72 hours RNA were isolated as described above. VEGFR2 mRNA levels were determined using a RT QPCR "Assay on Demand" according to manufacturer's protocol. These data (Figure 5B) showed that miRs-V-2, 3, 7 and 10 significantly reduced steady state VEGFR2 mRNA levels compared to controls. Figure 5C shows that miRs-V-2, 3, 7 and 10 also strongly reduced VEGFR2 protein levels in parallel wells of transfected cells as assessed by Western blot analysis. Take together these data showed that miRs-V-2, 3, 7 and 10 potently silenced VEGFR-2.

5. Design and preparation of constructs to silence PDGFR-β

ddRNAi constructs expressing shRNAs targeting PDGFR-B were designed, using the criteria described above, to recognise target sequences in PDGFR-B mRNA that are well conserved between human and the pre-clinical test species mouse and macaque. In most cases, there is a single nucleotide mismatch between the human sequence and the corresponding sequences in both the mouse and monkey models. 10 ddRNAi constructs (miR-P-1, miR-P-2, miR-P-3, miR-P-4, miR-P-5, miR-P-6, miR-P-7, miR-P-8, miR-P-9 and miR-P-10) were made. Sequences of the BamHI / HindIII fragments used to prepare these are listed as SEQ ID NOS: 111-120 as summarised in Table 2. Inserts were cloned into pSilencer 2.1-U6 hygro as described in Example 1.

6. Activity and strand-specificity of constructs targeting PDGFR-β

Dual luciferase assays were performed as described above and used to determine the activity and strand preference of miR-P-1 through miR-10 using the protocol described in Example 2 with the reporter constructs listed in Table 3. These data showed that all 10 constructs showed significant silencing of the antisense reporter construct. Based on these data miR-P-4 and miR-P-9 were chosen for subsequent analyses.

To confirm the activity of these constructs against native PDGFR-β mRNA, HEK 293T cells were co-transfected with plasmids expressing either PDGFR-β miRs-P-4 or miR-P-9 and a plasmid expressing a full length cDNA of PDGFR-β using Fugene according to manufacturer's (Roche Applied Sciences). After 48 hours RNA was isolated as described above. PDGFR-β mRNA levels were determined using a RT QPCR "Assay on Demand" according to manufacturer's protocol. These data (Figure 6A) showed that miRs-P-4 and miR-P-9 significantly reduced steady state PDGFR-B mRNA levels compared to controls. Figure 6B shows that miR-P-4 and miR-P-9 also strongly reduced PDGFR-β protein levels in parallel transfected wells of cells as compared to controls. Take together these data showed that miR-P-4 and miR-P-9 potently silenced PDGFR-β.

7. Design and preparation of constructs to silence CFB

ddRNAi constructs expressing shRNAs that target CFB were designed, using the criteria described above, to recognise target sequences in CFB mRNA that are conserved between human and the pre-clinical test species mouse and macaque. In most cases, there is either a single nucleotide mismatch or multiple mismatches between the human sequence and the corresponding sequences in both the mouse and monkey models. 9 ddRNAi constructs (miR-C-1, miR-C-2, miR-C-3, miR-C-4, miR-C-5, miR-C-6, miR-C-7, miR-C-8 and miR-C-9) were made. Sequences of the BamHI / HindIII fragments used to prepare these are listed as SEQ ID NOS: 121-129 as summarised in Table 2. Inserts were cloned into pSilencer 2.1-U6 hygro as described in Example 1.

8. Activity and strand-specificity of constructs targeting CFB

Dual luciferase assays were used as described above to determine the activity and strand preference of miRs-C-1 to 9 using the protocol described in Example 2 with the reporter constructs listed in Table 3. Results of these experiments are shown in Figure

7A. These data showed that most of the constructs showed significant silencing of the antisense reporter construct, but differed significantly in activity against the sense reporter construct, reflecting marked differences in RISC loading of passenger strands between the different ddRNAi constructs and consequent potential for off-target effects. Based on these data miR-C-1, miR-C-8 and miR-C-9 were chosen for subsequent analyses.

To confirm the activity of these constructs against native CFB mRNA, HEK 293T cells were co-transfected with plasmids expressing either miRs-C-1, miR-C-8 or miR-C-9 and a plasmid expressing the full length cDNA for CFB using the above mentioned methods. After 48 hours RNA was harvested and CFB mRNA levels were determined using a RT QPCR "Assay on Demand". These data (Figure 7B) showed that miR-C-1, miR-C-8 and miR-C-9 significantly reduced steady state CFB mRNA levels compared to control treated cells. Figure 7C shows that miRs-C-1, miR-C-8 or miR-C-9 also strongly reduced CFB protein levels parallel transfected cells as compared to controls. As determined by western blot analysis. Taken together these data showed that miRs-C-1, miR-C-8 and miR-C-9 potently silenced CFB.

9. Constructs targeting VEGF-A

Constructs designed to express therapeutic miR-based shRNAs targeting VEGF-A were prepared. These used either the U6 promoter which would express shRNAs in all cells, or one of four tissue-specific promoters which would express therapeutic shRNAs in appropriate cells. The four tissues-specific promoters were human VDM2 promoter, human ICAM2 promoter, human RPE65 promoter and human FLT promoter.

DNA fragments were synthesised (Blue Herron) that consisted of promoter sequences fused to the miR-7 sequences described in Figure 2C. These fragments contained flanking restriction sites to allow cloning into AAV vectors; for constructs using pol II promoters the AAV vectors contained a minimal polyadenylation site to ensure appropriate transcriptional termination. Maps of these fragments are shown in Figure 9 and are listed as SEQ ID NOS: 132 through to SEQ ID NOS: 136.

10. Constructs targeting VEGF-A and VEGFR2

Constructs designed to express therapeutic miR-based shRNAs targeting VEGFF-A and VEGFR2 were prepared. These used either the U6 promoter which would express shRNAs in all cells, or one of the four tissue-specific promoters in Example 9.

DNA fragments were synthesised (Blue Herron) that consisted of promoter sequences fused to the miR-7-miR-V-7 sequences and contained flanking restriction sites to allow cloning into AAV vectors as described in Example 9. Maps of these fragments are shown in Figure 10 and are listed as SEQ ID NOS: 137 through to SEQ ID NOS: 141.

11. Constructs targeting VEGFR2, PDGFR-β and CFB

Constructs designed to express therapeutic miR-based shRNAs targeting VEGFR2, PDGFR-β and CFB were prepared. These used either the U6 promoter which would express shRNAs in all cells, or one of the four tissue-specific promoters in Example 9. Each of the hairpins from single constructs miR-V-7, miR-C-8 and miR-P-9 sequences were subcloned into a single vector (in the same order) using a series of restriction enzymes that were engineered into the single vectors. The resultant expression construct also contained flanking restriction sites to allow cloning into AAV vectors as described in Example 9. Promoters reduced to practice used for the expression of these constructs included the human U6 promoter, the FLT promoter, and the ICAM2 promoter which were independently synthesized at Blue Heron. Each of the constructs produced were sequence verified prior to use. Maps of these fragments are shown in Figure 11 and are listed as SEQ ID NOS: 142 through to SEQ ID NOS: 146.

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The claims defining the invention are as follows:

1. A DNA-directed RNA interference (ddRNAi) agent for inhibiting expression of one or more target sequences in an AMD associated gene, the ddRNAi agent comprising, in a 5' to 3' direction

a first effector sequence of at least 17 nucleotides in length;

a first effector complement sequence,

wherein the effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences.

2. A ddRNAi agent according to claim 1, comprising a second effector sequence and second effector complement sequence.

3. A ddRNAi agent according to claim 2 comprising, in a 5' to 3' direction

a first effector sequence of at least 17 nucleotides in length;

a second effector sequence of at least 17 nucleotides in length

a second effector complement sequence; and

a first effector complement sequence,

wherein each effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences.

4. A ddRNAi agent according to claim 2 comprising, in a 5' to 3' direction

a first effector sequence of at least 17 nucleotides in length;

a first effector complement sequence;

a second effector sequence of at least 17 nucleotides in length; and

a second effector complement sequence

wherein each effector sequence is substantially complementary to one or more target regions in a transcript of the one or more target sequences.

5. A ddRNAi agent according to any one of claims 1 to 4, wherein the AMD associated gene is selected from one or more of VEGF-A, VEGFR2, PDGFR-β and CFB.

6. A ddRNAi agent according to any one of claims 1 to 4, the target sequences being selected from the group consisting of any 10 or more contiguous nucleotides within a sequence from any one of SEQ ID NOS: 1-39.

7. A ddRNAi agent according to any one of claims 1 to 4, wherein the AMD associated gene is VEGF-A and each effector sequence is selected from SEQ ID NOS: 40-49.

8. A ddRNAi agent according to any one of claims 1 to 4, wherein the AMD associated gene is VEGFR2, and each effector sequence is selected from SEQ ID NOS: 50-59.

9. A ddRNAi agent according to any one of claims 1 to 4, wherein the AMD associated gene is PDGFR-β and each effector sequence is selected from SEQ ID NOS: 60-69.

10. A ddRNAi agent according to any one of claims 1 to 4, wherein the AMD associated gene is CFB and each effector sequence is selected from SEQ ID NOS: 70-78.

11. A ddRNAi agent according to any one of claims 1 to 10 wherein the agent is expressed within a miRNA structure.

12. A ddRNAi agent according to any one of claims 1 to 11 wherein the AMD is wet AMD.

13. A ddRNAi expression cassette for expressing a ddRNAi agent according to any one of claims 1 to 12, the expression cassette comprising (in no particular order)

one or more promoter sequences

one or more DNA sequences that encode for one or more effector sequences,

one or more DNA sequences that encode for one or more effector complement sequences;

and optionally

one or more terminator sequences

one or more DNA sequences that encode for loop sequences, spacer sequences, or both,

one or more enhancer sequences.

14. A ddRNAi expression cassette further comprising miRNA encoding (ME) sequences.

15. A ddRNAi expression construct comprising a ddRNAi expression cassette according to claim 13 or 14.

16. A ddRNAi expression construct according to claim 15, wherein the construct is a viral delivery construct.

17. A method of treating AMD in a subject comprising administering a therapeutically effective amount of a ddRNAi expression construct of claim 15 or 16.

18. A method according to claim 17 wherein the AMD is wet AMD.

19. A method of treating choroidal neovascularisation in a subject comprising administering a therapeutically effective amount of a ddRNAi expression construct of claim 15 or 16.

20. A method of reducing drusen deposits in a subject comprising administering a therapeutically effective amount of a ddRNAi expression construct of claim 15 or 16.

21 A method according to any one of claims 17-20 wherein the ddRNAi expression construct is administered to the subject's eye/s by intravitreal injection.

22. A pharmaceutical composition comprising a ddRNAi expression construct of claim 15 or 16, and a pharmaceutically acceptable carrier or diluent.