et al. (1995) J. Biol. Chem. 270: 24420-24427). Furthermore, Rab27a is expressed at high levels in the retinal pigment epithelium and choriocapillaries, the two sites of earliest degeneration in choroideremia.

However, assays relying on the prenylation of Rab27a give rise to very weak signals. As a result, the sensitivity of these assays is low and they may not be suitable for reliable screening of clinical gene therapy vectors. Accordingly, a significant need exists for more reliable and sensitive assays which can be used to determine REP1 activity and test gene therapy vectors.

SUMMARY

The present inventors have surprisingly found that, despite a prevailing understanding that Rab27a provides the most suitable prenylation assay substrate, use of Rab6a as a substrate in a prenylation reaction provides a more sensitive method for determining the activity of Rab escort protein 1 (REP1). Not only does Rab6a provide for increased sensitivity in an assay detecting prenylation, it also provides for beneficial signal-to-noise ratios, better dynamic range of signal and better consistency.

Moreover, the inventors have demonstrated that the increased sensitivity of a Rab6a-based assay may be harnessed to accurately and reliably determine the activity of REP1-encoding vectors, in particular AAV gene therapy vectors, such as those suitable for use in the clinic.

Accordingly, in one aspect, the present disclosure refers to an *in vitro* method for determining the activity of Rab escort protein 1 (REP1)-encoding gene therapy vector for use in the treatment of choroideremia, the method comprising the steps:

(a) contacting a sample comprising REP1 with Rab6a, Rab geranylgeranyltransferase (Rab GGTase) and a lipid donor substrate, wherein the sample comprising REP1 is from a cell genetically engineered to express REP1 using the REP1-encoding gene therapy vector; and

(b) detecting the lipidated Rab6a product.

For example, the method of the disclosure may be for testing gene therapy vectors suitable for the delivery of REP1 to a target cell or for quality control analysis of vector stocks (e.g. medicament stocks).

Validation of gene therapy vectors is mandatory for the safe and efficacious implementation of gene therapy in the clinic. For analysis and quality control steps, comparison with control experiments or reference levels may provide a measure of the activity of the gene therapy vector or REP1 relative to a known or accepted standard (e.g. better or worse than a known

or accepted standard). This may be used to validate whether a gene therapy vector stock meets specific targets and regulations.

In one example, comparison is made to a sample of REP1 or REP1-encoding AAV vector that is defined as a primary reference standard. The method of the disclosure may be, for example, carried out in parallel on a test sample and the primary reference standard sample. Potency and/or behaviour of the test sample may be, for example, defined relative to the primary reference standard.

Put another way, the method of the disclosure may, for example, be used for quality control analysis and validation of a gene therapy vector as efficacious (e.g. for the treatment of choroideremia), preferably an AAV vector particle comprising a REP1-encoding nucleotide sequence, preferably wherein an output activity or efficacy of the vector determined by the method of the disclosure above a threshold activity or within a specified target range (e.g. by comparison to a control experiment or reference level) indicates the vector is suitable for gene therapy purposes.

Accordingly, the method of the disclosure is for quality control analysis of a Rab escort protein 1 (REP1)-encoding gene therapy vector (preferably an AAV vector).

Disclosed is a method for quality control analysis of a Rab escort protein 1 (REP1)-encoding gene therapy vector (preferably an AAV vector) comprising the steps:

(a) transducing a cell with the vector, culturing the cell under conditions suitable for the expression of the REP1 and lysing the cells to provide a sample comprising REP1;

(b) contacting the sample of step (a) with Rab6a, Rab geranylgeranyltransferase (Rab GGTase) and a lipid donor substrate; and

(c) detecting the lipidated Rab6a product.

Accordingly, the method may comprise carrying out a plurality of experiments comprising steps (a) to (c) in which parameters relating to the sample comprising REP1 are varied, while other parameters (e.g. parameters relating to the Rab6a, Rab GGTase and lipid donor substrate) are kept constant. Such parameters may include, for example, the amino acid sequence of the relevant protein (e.g. REP1), the REP1-encoding nucleotide sequence comprised in a vector used to express the REP1 in a cell, the type of vector used to deliver a REP1-encoding nucleotide sequence to a cell (e.g. the type of viral vector, such as the type

of adeno-associated viral (AAV) vector), the concentration of REP1 and/or the multiplicity-of-infection (MOI) of a vector used to deliver a REP1-encoding nucleotide sequence to a cell. In a preferred example, the method comprises carrying out a plurality of experiments comprising steps (a) to (c) at different MOIs of a vector used to deliver a REP1-encoding nucleotide sequence to a cell (e.g. to generate a dose-response curve).

In one example, the detection of the lipidated Rab6a product comprises quantifying the amount of the lipidated Rab6a product. In a preferred example, the detection of the lipidated Rab6a product comprises quantifying the amount of the lipidated Rab6a product relative to a control or reference level. The quantification may be, for example, made relative to a sample of REP1 or REP1-encoding AAV vector that is defined as a primary reference standard. The method of the disclosure may be, for example, carried out in parallel on a test sample and the primary reference standard sample. Potency and/or behaviour of the test sample may be, for example, defined relative to the primary reference standard.

In one example, the method comprises a further step of comparing the amount of lipidated Rab6a product (e.g. prenylated, such as geranylgeranylated or biotin-geranylated, Rab6a) with an amount determined from a control experiment, such as an experiment using a known or standard sample of REP1.

In another example, the method comprises a further step of comparing the amount of lipidated Rab6a product (e.g. prenylated, such as geranylgeranylated or biotin-geranylated, Rab6a) with a reference level.

In one example, the sample comprising REP1 is from a cell genetically engineered to express the REP1. Preferably, the sample comprising REP1 is a lysate of a cell genetically engineered to express the REP1. Preferably, a cell is transfected or transduced with a vector comprising a REP1-encoding nucleotide sequence to provide the cell genetically engineered to express the REP1. Preferably, the vector is a viral vector.

In one example, the REP1 is expressed using a viral vector comprising a REP1-encoding nucleotide sequence.

In one example, the viral vector is an adeno-associated viral (AAV) vector. Preferably the viral vector is in the form of a viral vector particle.

The AAV vector may be of any serotype (e.g. comprise any AAV serotype genome and/or capsid protein). Preferably, the vector is capable of infecting or transducing cells of the eye.

In one example, the AAV vector comprises an AAV serotype 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 genome. In another example, the AAV vector comprises an AAV serotype 2, 4, 5 or 8 genome. Preferably, the AAV vector comprises an AAV serotype 2 genome.

In one example, the AAV vector particle comprises an AAV serotype 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11 capsid protein. In another example, the AAV vector particle comprises an AAV serotype 2, 4, 5 or 8 capsid protein. The AAV serotype 8 capsid protein may, for example, be a AAV8/Y733F mutant capsid protein. Preferably, the AAV vector particle comprises an AAV serotype 2 capsid protein.

In one example, the AAV vector particle comprises an AAV2 genome and AAV2 capsid proteins (AAV2/2); an AAV2 genome and AAV5 capsid proteins (AAV2/5); or an AAV2 genome and AAV8 capsid proteins (AAV2/8). Preferably, the AAV vector particle comprises an AAV2 genome and AAV2 capsid proteins (AAV2/2).

The AAV vector particle may be a chimeric, shuffled or capsid-modified derivative of one or more naturally occurring AAVs. In particular, the AAV vector particle may comprise capsid protein sequences from different serotypes, clades, clones or isolates of AAV within the same vector (i.e. a pseudotyped vector). Thus, in one example the AAV vector is in the form of a pseudotyped AAV vector particle.

In one example, the REP1 is human REP1.

In one example, the REP1 comprises an amino acid sequence that has at least 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 5, preferably wherein the amino acid sequence substantially retains the natural function of the protein represented by SEQ ID NO: 5.

In one example, the REP1-encoding nucleotide sequence comprises a nucleotide sequence that has at least 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 6 or 7, preferably wherein the protein encoded by the nucleotide sequence substantially retains the natural function of the protein represented by SEQ ID NO: 5.

In one example, the REP1-encoding nucleotide sequence comprises a nucleotide sequence that encodes an amino acid sequence that has at least 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 5, preferably wherein the amino acid sequence substantially retains the natural function of the protein represented by SEQ ID NO: 5.

In one example, the Rab6a and/or Rab GGTase are substantially pure.

In one example, the Rab6a comprises an amino acid sequence that has at least 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 1, preferably wherein the amino acid sequence substantially retains the natural function of the protein represented by SEQ ID NO: 1.

In one example, the Rab GGTase comprises an amino acid sequence that has at least 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 3 or 8, preferably SEQ ID NO: 8, preferably wherein the amino acid sequence substantially retains the natural function of the protein represented by SEQ ID NO: 8; and/or an amino acid sequence that has at least 70%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity to SEQ ID NO: 4 or 9, preferably SEQ ID NO: 9, preferably wherein the amino acid sequence substantially retains the natural function of the protein represented by SEQ ID NO: 9.

In another example, the Rab6a:Rab GGTase molar ratio is about 1:0.25-3, 1:0.3-2.9, 1:0.35-2.8, 1:0.4-2.7, 1:0.45-2.6 or 1:0.5-2.5, preferably about 1:0.5-2.5.

In one example, the Rab6a:Rab GGTase molar ratio is about 1:2-3, 1:2.1-2.9, 1:2.2-2.8, 1:2.3-2.7 or 1:2.4-2.6, preferably about 1:2.4-2.6. In one example, the Rab6a:Rab GGTase molar ratio is about 1:2, 1:2.1, 1:2.2, 1:2.3, 1:2.4, 1:2.5, 1:2.6, 1:2.7, 1:2.8, 1:2.9 or 1:3, preferably about 1:2.5.

In another example, the Rab6a:Rab GGTase molar ratio is about 1:0.25-0.75, 1:0.3-0.7, 1:0.35-0.65, 1:0.4-0.6 or 1:0.45-0.55, preferably about 1:0.4-0.6. In one example, the Rab6a:Rab GGTase molar ratio is about 1:0.25, 1:0.3, 1:0.35, 1:0.4, 1:0.45, 1:0.5, 1:0.55, 1:0.6, 1:0.65, 1-0.7 or 1:0.75, preferably about 1:0.5.

In one example, the lipid donor substrate is geranylgeranylpyrophosphate (GGPP) or an analogue thereof. Preferably, the lipid donor substrate is labelled with a detectable marker. For example, the lipid donor substrate may be isotopically labelled (e.g. the lipid donor substrate may comprise ^{3}H), or may comprise a fluorescent group, epitope or biotin moiety.

In a preferred example, the lipid donor substrate is biotin-geranylpyrophosphate (BGPP).

In one example, the lipidated Rab6a product is detected using an enzyme-linked immunosorbent assay (ELISA), Western blot analysis or autoradiography. In a preferred example, the lipidated Rab6a product is detected using an ELISA. The ELISA may be, for example, a sandwich ELISA.

In a preferred example, a biotin-labelled lipidated Rab6a product is detected using a detection reagent specific for biotin, for example streptavidin. Preferably, the biotin-labelled lipidated Rab6a product is detected using Western blot analysis using a detection reagent specific for biotin, for example streptavidin (e.g. a streptavidin-horseradish peroxidase conjugate). More preferably, the biotin-labelled lipidated Rab6a product is detected using an ELISA using a detection reagent specific for biotin, for example streptavidin.

In one example, the method is for determining the activity of a REP1-encoding gene therapy vector for use in the treatment of choroideremia.

In another aspect, the present disclosure refers to use of Rab6a for determining *in vitro* the activity of Rab escort protein 1 (REP1)-encoding gene therapy vector for use in the treatment of choroideremia, wherein the Rab6a is contacted with REP1 from a cell genetically engineered to express the REP1 using the REP1-encoding gene therapy vector.

The method of determining the activity of REP1, the Rab6a, Rab GGTase, lipid donor substrate and the REP1 may be as described herein.

Disclosed is a method for determining the efficacy of a vector comprising a Rab escort protein 1 (REP1) encoding nucleotide sequence, wherein the method comprises the steps:

(a) providing a sample comprising REP1, wherein the REP1 is expressed using the vector comprising a REP1-encoding nucleotide sequence;

(b) contacting the sample of step (a) with Rab6a, Rab geranylgeranyltransferase (Rab GGTase) and a lipid donor substrate; and

(c) detecting the lipidated Rab6a product.

Disclosed is the use of Rab6a for determining the efficacy of a vector comprising a Rab escort protein 1 (REP1) encoding nucleotide sequence.

Preferably, the method and use are for determining the efficacy of a vector for use in the treatment of choroideremia.

The vector, REP1, Rab6a, Rab GGTase, lipid donor substrate, lipidated Rab6a product, method of detection and other features of the method and use may be as described herein.

DESCRIPTION OF THE DRAWINGS

Figure 1

CLAIMS

1. An *in vitro* method for determining the activity of Rab escort protein 1 (REP1)-encoding gene therapy vector for use in the treatment of choroideremia, the method comprising the steps:

    (a) contacting a sample comprising REP1 with Rab6a, Rab geranylgeranyltransferase (Rab GGTase) and a lipid donor substrate, wherein the sample comprising REP1 is from a cell genetically engineered to express REP1 using the REP1-encoding gene therapy vector; and

    (b) detecting the lipidated Rab6a product.

2. The method of claim 1, wherein the REP1 encoding gene therapy vector is a viral vector comprising a REP1-encoding nucleotide sequence.

3. The method of claim 2, wherein the viral vector is an adeno-associated viral (AAV) vector, preferably, wherein the adeno-associated viral vector is AAV serotype 2 (AAV2).

4. The method of any preceding claim, wherein the Rab6a and/or Rab GGTase are pure.

5. The method of any preceding claim, wherein the Rab6a:Rab GGTase molar ratio is 1:2-3, preferably 1:2.5.

6. The method of any preceding claim, wherein the lipid donor substrate is geranylgeranylpyrophosphate (GGPP) or an analogue thereof, preferably biotin-geranylpyrophosphate (BGPP).

7. The method of any preceding claim, wherein the lipidated Rab6a product is detected using an enzyme-linked immunosorbent assay (ELISA), Western blot analysis or autoradiography.

8. The method of any preceding claim, wherein the method is for quality control analysis of a REP1-encoding gene therapy vector for use in the treatment of choroideremia.

9. The method of any preceding claim, wherein the detection of the lipidated Rab6a product comprises quantifying the amount of the lipidated Rab6a product, preferably quantifying the amount relative to a control or reference level.

10. Use of Rab6a for determining *in vitro* the activity of Rab escort protein 1 (REP1)-encoding gene therapy vector for use in the treatment of choroideremia, wherein the Rab6a is contacted with REP1 from a cell genetically engineered to express the REP1 using the REP1-encoding gene therapy vector.

11. The use of claim 10, wherein the REP1 is in a lysate from the cell genetically engineered to express the REP1.

12. The use of claim 12 or 13, wherein the REP1 is expressed using a viral vector comprising a REP1-encoding nucleotide sequence.

13. The use of claim 14, wherein the viral vector is an adeno-associated viral (AAV) vector.

14. The use of any one of claims 12-17, wherein the Rab6a is pure.

15. The method of any one of claims 1-9 or the use of any one of claims 10-14, wherein the REP-1 encoding nucleotide sequence of the REP1-encoding gene therapy vector comprises a nucleotide sequence that encodes an amino acid sequence that has at least 80% identity to SEQ ID NO: 5, preferably wherein the amino acid sequence substantially retains the natural function of the protein represented by SEQ ID NO: 5.