ribonucleoprotein (RNP) complexes to cells by microinjection of Cas9 protein and gRNA complexes was described by Cho [Cho et al., "Heritable gene knockout in Caenorhabditis elegans by direct injection of Cas9-sgRNA ribonucleoproteins," *Genetics* (2013) 195:1177-1180]. Delivery of Cas9 protein/gRNA complexes via electroporation was described by Kim [Kim et al., "Highly efficient RNA-guided genome editing in human cells via delivery of purified Cas9 ribonucleoproteins" *Genome Res.* (2014) 24:1012-1019]. Delivery of Cas9 protein-associated gRNA complexes via liposomes was reported by Zuris [Zuris et al., "Cationic lipid-mediated delivery of proteins enables efficient protein-based genome editing in vitro and in vivo" *Nat Biotechnol.* (2014) doi: 10.1038/nbt.3081].

SUMMARY

In one aspect, the present disclosure refers to a method of modifying a gene encoding or processed into a RNA silencing molecule to a target RNA in a eukaryotic cell isolated from its natural environment, with the proviso that said eukaryotic cell is not a plant cell, the method comprising introducing into the eukaryotic cell isolated from its natural environment a DNA editing agent which redirects a silencing specificity of said RNA silencing molecule towards a second target RNA, said target RNA and said second target RNA being distinct, thereby modifying the gene encoding the RNA silencing molecule such that the original specificity of the RNA silencing molecule is destroyed and a new specificity towards the second target RNA is gained.

In another aspect, the present disclosure refers to a non-therapeutic method of modifying a gene encoding or processed into a RNA silencing molecule to a target RNA in a eukaryotic cell, with the proviso that said eukaryotic cell is not a plant cell, the method comprising introducing into the eukaryotic cell a DNA editing agent which redirects a silencing specificity of said RNA silencing molecule towards a second target RNA, said target RNA and said second target RNA being distinct, thereby modifying the gene encoding the RNA silencing molecule such that the original specificity of the RNA silencing molecule is destroyed and a new specificity towards the second target RNA is gained.

In another aspect, the present disclosure refers to a method of generating a eukaryotic non-human organism, with the proviso that said organism is not a plant, wherein at least some of the cells of said organism comprise a modified gene encoding or processed into a RNA silencing molecule comprising a silencing specificity towards a target RNA , the method comprising introducing into a eukaryotic non-human cell isolated from its natural environment a DNA editing agent which redirects a silencing specificity of a gene encoding or processed into a RNA

silencing molecule to an initial target RNA towards said target RNA, said initial target RNA and said target RNA being distinct, thereby modifying the gene encoding the RNA silencing molecule such that the original specificity of the RNA silencing molecule is destroyed and a new specificity towards the target RNA is gained, thereby generating the eukaryotic non-human organism.

In another aspect, the present disclosure refers to a non-therapeutic method of generating a eukaryotic non-human organism, with the proviso that said organism is not a plant, wherein at least some of the cells of said organism comprise a modified gene encoding or processed into a RNA silencing molecule comprising a silencing specificity towards a target RNA , the method comprising introducing into a eukaryotic non-human cell a DNA editing agent which redirects a silencing specificity of a gene encoding or processed into a RNA silencing molecule to an initial target RNA towards said target RNA, said initial target RNA and said target RNA being distinct, thereby modifying the gene encoding the RNA silencing molecule such that the original specificity of the RNA silencing molecule is destroyed and a new specificity towards the target RNA is gained, thereby generating the eukaryotic non-human organism.

In yet another aspect, the present disclosure refers to a use of a DNA editing agent in the manufacture of a medicament for treating an infectious disease, a monogenic recessive disorder, an autoimmune disease or a cancerous disease in a subject in need thereof, wherein the use comprises that said DNA editing agent which redirects a silencing specificity of a gene encoding or processed into a RNA silencing molecule to a target RNA towards a second target RNA is to be introduced to at least one cell of the subject, the target RNA and the second target RNA being distinct, wherein the second target RNA is associated with onset or progression of said infectious disease, said monogenic recessive disorder, said autoimmune disease or said cancerous disease, wherein the original specificity of the RNA silencing molecule is destroyed and a new specificity towards the second target RNA is gained.

In yet another aspect, the present disclosure refers to a use of a donor oligonucleotide in the manufacture of a medicament for treating an infectious disease, a monogenic recessive disorder, an autoimmune disease or a cancerous disease in a subject in need thereof, wherein the use comprises that said donor oligonucleotide is to be introduced to at least one cell of the subject, together with a DNA editing agent which redirects a silencing specificity of a gene encoding or processed into a RNA silencing molecule to a target RNA towards a second target RNA, the target RNA and the second target RNA being distinct, wherein the second target RNA is associated with onset or progression of said infectious disease, said monogenic recessive disorder, said autoimmune disease

or said cancerous disease, wherein the original specificity of the RNA silencing molecule is destroyed and a new specificity towards the second target RNA is gained.

According to an example of some examples of the present disclosure there is provided a method of modifying a gene encoding or processed into a non-coding RNA molecule having no RNA silencing activity in a eukaryotic cell, with the proviso that the eukaryotic cell is not a plant cell, the method comprising introducing into the eukaryotic cell a DNA editing agent conferring a silencing specificity of the non-coding RNA molecule towards a target RNA of interest, thereby modifying the gene encoding or processed into the non-coding RNA molecule.

According to an example of some examples of the present disclosure there is provided a method of modifying a gene encoding or processed into a non-coding RNA molecule having no RNA silencing activity in a eukaryotic cell, with the proviso that the eukaryotic cell is not a plant cell, the method comprising introducing into the eukaryotic cell a DNA editing agent conferring a silencing specificity of the non-coding RNA molecule towards a target RNA of interest.

According to an example of some examples of the present disclosure there is provided a method of modifying a gene encoding or processed into a RNA silencing molecule to a target RNA in a eukaryotic cell, with the proviso that the eukaryotic cell is not a plant cell, the method comprising introducing into the eukaryotic cell a DNA editing agent which redirects a silencing specificity of the RNA silencing molecule towards a second target RNA, the target RNA and the second target RNA being distinct, thereby modifying the gene encoding the RNA silencing molecule.

According to an example of some examples of the present disclosure there is provided a method of modifying a gene encoding or processed into a RNA silencing molecule to a target RNA in a eukaryotic cell, with the proviso that the eukaryotic cell is not a plant cell, the method comprising introducing into the eukaryotic cell a DNA editing agent which redirects a silencing

specificity of the RNA silencing molecule towards a second target RNA, the target RNA and the second target RNA being distinct.

According to an example of some examples of the present disclosure there is provided a method of treating an infectious disease in a subject in need thereof, the method comprising modifying a gene encoding or processed into a non-coding RNA molecule or encoding or processed into an RNA silencing molecule according to the method of some examples of the disclosure, wherein the target RNA of interest is associated with onset or progression of the infectious disease, thereby treating the infectious disease in the subject.

According to an example of some examples of the present disclosure there is provided a method of treating a monogenic recessive disorder in a subject in need thereof, the method comprising modifying a gene encoding or processed into a non-coding RNA molecule or encoding or processed into an RNA silencing molecule according to the method of some examples of the disclosure, wherein the target RNA of interest is associated with the monogenic recessive disorder, thereby treating the monogenic recessive disorder in the subject.

According to an example of some examples of the present disclosure there is provided a method of treating an autoimmune disease in a subject in need thereof, the method comprising modifying a gene encoding or processed into a non-coding RNA molecule or encoding or processed into an RNA silencing molecule according to the method of some examples of the disclosure, wherein the target RNA of interest is associated with the autoimmune disease, thereby treating the autoimmune disease in the subject.

According to an example of some examples of the present disclosure there is provided a method of treating a cancerous disease in a subject in need thereof, the method comprising modifying a gene encoding or processed into a non-coding RNA molecule or encoding or processed into an RNA silencing molecule according to the method of some examples of the disclosure, wherein the target RNA of interest is associated with the cancerous disease, thereby treating the cancerous disease in the subject.

According to an example of some examples of the present disclosure there is provided a method of enhancing efficacy and/or specificity of a chemotherapeutic agent in a subject in need thereof, the method comprising modifying a gene encoding or processed into a non-coding RNA molecule or encoding or processed into an RNA silencing molecule according to the method of some examples of the disclosure, wherein the target RNA of interest is associated with enhancement of efficacy and/or specificity of the chemotherapeutic agent, thereby enhancing efficacy and/or specificity of a chemotherapeutic agent in the subject.

According to an example of some examples of the present disclosure there is provided a method of inducing cell apoptosis in a subject in need thereof, the method comprising modifying a gene encoding or processed into a non-coding RNA molecule or encoding or processed into an RNA silencing molecule according to the method of some examples of the disclosure, wherein the target RNA of interest is associated with the apoptosis, thereby inducing cell apoptosis in the subject.

According to an example of some examples of the present disclosure there is provided a method of generating a eukaryotic non-human organism, with the proviso that the organism is not a plant, wherein at least some of the cells of the organism comprise a modified gene encoding or processed into a non-coding RNA molecule comprising a silencing specificity towards a target RNA of interest, the method comprising modifying a gene according to the method of some examples of the disclosure, thereby generating the eukaryotic non-human organism.

According to some examples of the disclosure, the gene encoding or processed into the non-coding RNA molecule is endogenous to the eukaryotic cell.

According to some examples of the disclosure, the gene encoding the RNA silencing molecule is endogenous to the eukaryotic cell.

According to some examples of the disclosure, modifying the gene encoding or processed into the non-coding RNA molecule comprises imparting the non-coding RNA molecule with at least 45 % complementarity towards the target RNA of interest.

According to some examples of the disclosure, modifying the gene encoding the RNA silencing molecule comprises imparting the RNA silencing molecule with at least 45 % complementarity towards the second target RNA.

According to some examples of the disclosure, the silencing specificity of the non-coding RNA molecule is determined by measuring a RNA or protein level of the target RNA of interest.

According to some examples of the disclosure, the silencing specificity of the RNA silencing molecule is determined by measuring a RNA or protein level of the second target RNA.

According to some examples of the disclosure, the silencing specificity of the non-coding RNA molecule or the RNA silencing molecule is determined phenotypically.

According to some examples of the disclosure, determined phenotypically is effected by determination of at least one phenotype selected from the group consisting of a cell size, a growth rate/inhibition, a cell shape, a cell membrane integrity, a tumor size, a tumor shape, a pigmentation of an organism, an infection parameter and an inflammation parameter.

According to some examples of the disclosure, the silencing specificity of the non-coding RNA molecule or the RNA silencing molecule is determined genotypically.

According to some examples of the disclosure, the phenotype is determined prior to a genotype.

According to some examples of the disclosure, the genotype is determined prior to a phenotype.

According to some examples of the disclosure, the non-coding RNA molecule or the RNA silencing molecule is processed from a precursor.

According to some examples of the disclosure, the non-coding RNA molecule or the RNA silencing molecule is a RNA interference (RNAi) molecule.

According to some examples of the disclosure, the RNAi molecule is selected from the group consisting of a small interfering RNA (siRNA), a short hairpin RNA (shRNA), a microRNA (miRNA), a Piwi-interacting RNA (piRNA) and trans-acting siRNA (tasiRNA).

According to some examples of the disclosure, the non-coding RNA molecule is selected from the group consisting of a small nuclear RNA (snRNA), a small nucleolar RNA (snoRNA), a long non-coding RNA (lncRNA), a ribosomal RNA (rRNA), transfer RNA (tRNA), a repeat-derived RNA, and a transposable element RNA.

According to some examples of the disclosure, the RNAi molecule is modified to preserve originality of structure and to be recognized by cellular RNAi factors.

According to some examples of the disclosure, modifying the gene is affected by a modification selected from the group consisting of a deletion, an insertion, a point mutation and a combination thereof.

According to some examples of the disclosure, the modification is in a stem region of the non-coding RNA molecule or the RNA silencing molecule.

According to some examples of the disclosure, the modification is in a loop region of the non-coding RNA molecule or the RNA silencing molecule.

According to some examples of the disclosure, the modification is in a non-structured region of the non-coding RNA molecule or the RNA silencing molecule.

According to some examples of the disclosure, the modification is in a stem region and a loop region of the non-coding RNA molecule or the RNA silencing molecule.

According to some examples of the disclosure, the modification is in a stem region and a loop region and in non-structured region of the non-coding RNA molecule or the RNA silencing molecule.

According to some examples of the disclosure, the modification is an insertion.

According to some examples of the disclosure, the modification is a deletion.

According to some examples of the disclosure, the modification is a point mutation.

According to some examples of the disclosure, the modification comprises a modification of at most 200 nucleotides.

According to some examples of the disclosure, the method further comprises introducing into the eukaryotic cell donor oligonucleotides.

According to some examples of the disclosure, the DNA editing agent comprises at least one gRNA operatively linked to a plant expressible promoter.

According to some examples of the disclosure, the DNA editing agent does not comprise an endonuclease.

According to some examples of the disclosure, the DNA editing agent comprises an endonuclease.

According to some examples of the disclosure, the DNA editing agent comprises a DNA editing system selected from the group consisting of a meganuclease, a zinc finger nucleases (ZFN), a transcription-activator like effector nuclease (TALEN) and CRISPR.

According to some examples of the disclosure, the endonuclease comprises Cas9.

According to some examples of the disclosure, the DNA editing agent is applied to the cell as DNA, RNA or RNP.

According to some examples of the disclosure, the DNA editing agent is linked to a reporter for monitoring expression in a eukaryotic cell.

According to some examples of the disclosure, the reporter is a fluorescent protein.

According to some examples of the disclosure, the target RNA of interest or the second target RNA is endogenous to the eukaryotic cell.

According to some examples of the disclosure, the target RNA of interest or the second target RNA is associated with a cancer.

According to some examples of the disclosure, the target RNA of interest or the second target RNA is exogenous to the eukaryotic cell.

According to some examples of the disclosure, the target RNA of interest or the second target RNA is associated with an infectious disease.

According to some examples of the disclosure, the eukaryotic cell is obtained from a eukaryotic organism selected from the group consisting of a mammal, an insect, a nematode, a bird, a reptile, a fish, a crustacean, a fungi and an algae.

According to some examples of the disclosure, the eukaryotic cell is a mammalian cell.

According to some examples of the disclosure, the mammalian cell comprises a human cell.

According to some examples of the disclosure, the eukaryotic cell is a totipotent stem cell.

Unless otherwise defined, all technical and/or scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosure pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of examples of the disclosure, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIG. 1 is a flow chart of an embodiment computational pipeline to generate Genome Editing Induced Gene Silencing (GEiGS) templates. The computational GEiGS pipeline applies biological metadata and enables an automatic generation of GEiGS DNA templates that are used to minimally edit miRNA genes, leading to a new gain of function, i.e. redirection of their silencing capacity to target sequence of interest.

FIG. 2 is an embodiment flowchart of GEiGS replacement of miRNA with siRNA targeting Green Fluorescent Protein (GFP), generating silencing of the stably expressed GFP gene in human cell lines.

FIGs. 3A-B are photographs illustrating knock down of GFP expression levels in human cells. Control cells (Figure 3A) stably express GFP at high levels as compared to cells stably expressing siGFP in which GFP expression is silenced (Figure 3B).

FIG. 4 is an embodiment flowchart of GEiGS cells stably expressing siGFP. All positive transfection events are red fluorescent proteins (RFP) + GFP. However, since GEiGS cells stably express siGFP, positive transfected cells show only red fluorescent expression.

WHAT IS CLAIMED IS:

1. A method of modifying a gene encoding or processed into a RNA silencing molecule to a target RNA in a eukaryotic cell isolated from its natural environment, with the proviso that said eukaryotic cell is not a plant cell, the method comprising introducing into the eukaryotic cell isolated from its natural environment a DNA editing agent which redirects a silencing specificity of said RNA silencing molecule towards a second target RNA, said target RNA and said second target RNA being distinct, thereby modifying the gene encoding the RNA silencing molecule such that the original specificity of the RNA silencing molecule is destroyed and a new specificity towards the second target RNA is gained.

2. A non-therapeutic method of modifying a gene encoding or processed into a RNA silencing molecule to a target RNA in a eukaryotic cell, with the proviso that said eukaryotic cell is not a plant cell, the method comprising introducing into the eukaryotic cell a DNA editing agent which redirects a silencing specificity of said RNA silencing molecule towards a second target RNA, said target RNA and said second target RNA being distinct, thereby modifying the gene encoding the RNA silencing molecule such that the original specificity of the RNA silencing molecule is destroyed and a new specificity towards the second target RNA is gained.

3. The method of claim 1 or claim 2, wherein the gene encoding the RNA silencing molecule is endogenous to the eukaryotic cell.

4. The method of any one of claims 1-3, wherein said modifying said gene encoding said RNA silencing molecule comprises imparting said RNA silencing molecule with at least 45 % complementarity towards said second target RNA.

5. The method of any one of claims 1-4, wherein said silencing specificity of said RNA silencing molecule is determined by measuring a RNA or protein level of said second target RNA, and/or is determined phenotypically and/or is determined genotypically.

6. The method of any one of claims 1-5, wherein said RNA silencing molecule or said

non-coding RNA molecule is processed from a precursor,

optionally wherein said non-coding RNA molecule or said RNA silencing molecule is a RNA interference (RNAi) molecule,

optionally wherein said RNAi molecule is selected from the group consisting of a small interfering RNA (siRNA), a short hairpin RNA (shRNA), a microRNA (miRNA), a Piwi-interacting RNA (piRNA) and trans-acting siRNA (tasiRNA);

optionally wherein said RNAi molecule is modified to preserve secondary RNA structure and to be recognized by cellular RNAi factors.

7. The method of any one of claims 1-6, wherein said modifying said gene is affected by a modification selected from the group consisting of a deletion, an insertion, a point mutation and a combination thereof,

optionally wherein said modification is in a stem region of said non-coding RNA molecule or said RNA silencing molecule, is in a loop region of said RNA silencing molecule, is in a non-structured region of said RNA silencing molecule, is in a stem region and a loop region of said RNA silencing molecule, or is in a stem region and a loop region and in non-structured region of said RNA silencing molecule,

optionally wherein said modification comprises a modification of at most 200 nucleotides.

8. The method of claim 7, wherein said method further comprises introducing into said eukaryotic cell a donor oligonucleotide sequence.

9. The method of any one of claims 1-8, wherein said DNA editing agent comprises an endonuclease,

optionally wherein the DNA editing agent comprises an endonuclease and a DNA targeting molecule,

optionally wherein the DNA targeting molecule is gRNA,

optionally wherein said endonuclease comprises Cas9.

10. The method of any one of claims 1-8, wherein said DNA editing agent is applied to the cell as DNA, RNA or RNP, wherein the DNA editing agent is introduced into the eukaryotic cell using DNA delivery methods or DNA-free methods,

optionally wherein the DNA editing agent is introduced into the eukaryotic cell using expression vectors,

optionally wherein said DNA editing agent comprises at least one gRNA operatively linked to a cis-acting regulatory element active in eukaryotic cells.

11. The method of any one of claims 1-8, wherein said DNA editing agent is of a DNA editing system selected from the group consisting of a meganuclease, a zinc finger nucleases (ZFN), a transcription-activator like effector nuclease (TALEN) and CRISPR.

12. The method of any one of claims 1-11, wherein said second target RNA is endogenous or exogenous to said eukaryotic cell.

13. The method of claim 12, wherein said second target RNA is a product of a gene associated with a cancer or with an infectious disease.

14. The method of any one of claims 1-13, wherein said eukaryotic cell was obtained from a eukaryotic organism selected from the group consisting of a mammal, such as a cattle or a pig, an insect, a nematode, a bird, a reptile, a fish, a crustacean, such as a shrimp, a fungi and an algae.

15. The method of any one of claims 1-14, wherein said eukaryotic cell is a mammalian cell, optionally a non-human mammalian cell.

16. A method of generating a eukaryotic non-human organism, with the proviso that said organism is not a plant, wherein at least some of the cells of said organism comprise a modified gene encoding or processed into a RNA silencing molecule comprising a silencing specificity towards a target RNA , the method comprising introducing into a eukaryotic non-human cell isolated from its natural environment a DNA editing agent which redirects a silencing

specificity of a gene encoding or processed into a RNA silencing molecule to an initial target RNA towards said target RNA, said initial target RNA and said target RNA being distinct, thereby modifying the gene encoding the RNA silencing molecule such that the original specificity of the RNA silencing molecule is destroyed and a new specificity towards the target RNA is gained, thereby generating the eukaryotic non-human organism.

17. A non-therapeutic method of generating a eukaryotic non-human organism, with the proviso that said organism is not a plant, wherein at least some of the cells of said organism comprise a modified gene encoding or processed into a RNA silencing molecule comprising a silencing specificity towards a target RNA , the method comprising introducing into a eukaryotic non-human cell a DNA editing agent which redirects a silencing specificity of a gene encoding or processed into a RNA silencing molecule to an initial target RNA towards said target RNA, said initial target RNA and said target RNA being distinct, thereby modifying the gene encoding the RNA silencing molecule such that the original specificity of the RNA silencing molecule is destroyed and a new specificity towards the target RNA is gained, thereby generating the eukaryotic non-human organism.

18. The method of claim 16 or 17, wherein said eukaryotic non-human organism is a mammal, such as a cattle or a pig, an insect, a nematode, a bird, a reptile, a fish, a crustacean, such as a shrimp, a fungi or an algae.

19. Use of a DNA editing agent in the manufacture of a medicament for treating an infectious disease, a monogenic recessive disorder, an autoimmune disease or a cancerous disease in a subject in need thereof, wherein the use comprises that said DNA editing agent which redirects a silencing specificity of a gene encoding or processed into a RNA silencing molecule to a target RNA towards a second target RNA is to be introduced to at least one cell of the subject, the target RNA and the second target RNA being distinct,

wherein the second target RNA is associated with onset or progression of said infectious disease, said monogenic recessive disorder, said autoimmune disease or said cancerous disease,

wherein the original specificity of the RNA silencing molecule is destroyed and a new specificity towards the second target RNA is gained.

20. Use of a donor oligonucleotide in the manufacture of a medicament for treating an infectious disease, a monogenic recessive disorder, an autoimmune disease or a cancerous disease in a subject in need thereof, wherein the use comprises that said donor oligonucleotide is to be introduced to at least one cell of the subject, together with a DNA editing agent which redirects a silencing specificity of a gene encoding or processed into a RNA silencing molecule to a target RNA towards a second target RNA, the target RNA and the second target RNA being distinct,

wherein the second target RNA is associated with onset or progression of said infectious disease, said monogenic recessive disorder, said autoimmune disease or said cancerous disease,

wherein the original specificity of the RNA silencing molecule is destroyed and a new specificity towards the second target RNA is gained.

21. The method of claim 16, 17 or 18, the use of claim 19 or 20, wherein said target RNA is endogenous or exogenous to the eukaryotic non-human cell.