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(54) Title: RNAi COMPOSITIONS AND METHODS FOR TREATMENT OF FRIEDREICH'S ATAXIA

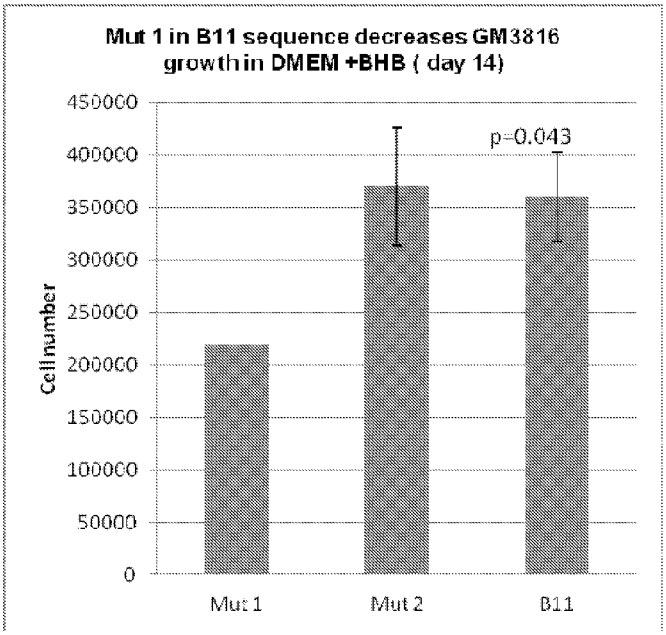

Figure 13

(57) Abstract: The invention provides ribonucleic acid (RNA) molecules, such as siRNA and shRNA molecules, that compensate for a frataxin deficiency or mutation, expression vectors encoding the same, and methods of using the same (e.g., to treat Friedreich's ataxia).

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RNAi COMPOSITIONS AND METHODS FOR TREATMENT OF FRIEDREICH'S ATAXIA

GOVERNMENT INTEREST STATEMENT

[001] This invention was made with government support under Grant Number 5R01-GM090304-03, awarded by the National Institutes of Health. The government has certain rights in the invention.

FIELD OF INVENTION

[002] Described herein are ribonucleic acid molecules, such as siRNA and shRNA molecules, that compensate for a frataxin deficiency or mutation, expression vectors encoding the same, and methods of using the same (*e.g.,* to treat Friedreich's ataxia).

BACKGROUND OF THE INVENTION

[003] Mitochondrial dysfunction has been established to contribute to the pathology of numerous diseases and is suspected in many more. A role for loss of mitochondrial function in normal aging has long been suspected. Most hypotheses focus on free radical damage to mitochondrial DNA. Mitochondrial DNA (mtDNA) lies in close proximity to the mitochondrial respiratory chain, which produces free radicals even during normal respiration. Somatic mtDNA mutations accumulate with age in post-mitotic tissues in association with a decline in mitochondrial function. MtDNA mutations are propagated during the turnover of mitochondria, which have a limited lifespan of only a few weeks, even in post-mitotic cells. Because mtDNA contributes disproportionally to respiratory complexes I, III, and IV, these complexes are disproportionally affected when mtDNA is damaged. Disproportional effects on mitochondrial respiratory complexes increase the production of free radicals by impeding the normal flux of electrons through the electron transport chain; the increase in free radicals causes further damage to mtDNA, creating a vicious cycle. That the association between the accumulation of mtDNA mutations and aging is likely not an epiphenomenon is indicated by the striking premature aging phenotype of transgenic mice with an increased mtDNA mutation rate due to expression of a proof-reading-defective mitochondrial DNA polymerase.

[004] Loss of mitochondrial function, particularly complex I function, likely contributes to Parkinson's disease (PD) as well. Complex I (NADH-ubiquinone oxidoreductase) activity is selectively decreased 15-30% in the substantia nigra (SN) in sporadic PD. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin that causes a parkinsonian syndrome in

humans and mice: MPTP is metabolized in the brain to MPP+, a complex I inhibitor that accumulates in dopaminergic neurons. Chronic inhibition of complex I with rotenone, throughout the brain, causes selective degeneration of dopaminergic neurons in the SN. Rotenone-treated rats develop all the pathological hallmarks of PD, including distribution of pathology, nigrostriatal dopaminergic neurodegeneration, formation of Lewy-Body-like cytoplasmic inclusions, and oxidative damage. The hypokinesia in rats treated with rotenone may also reflect, at least in part, a general health problem rather than loss of nigrostriatal dopaminergic neurons.

[005] A role for mitochondrial dysfunction in Friedreich's ataxia (FRDA or FA) is also recognized. FRDA is an inherited disease that causes progressive damage to the nervous system and muscle cells resulting in symptoms ranging from uncoordination, gait disturbance, and speech problems to heart disease and muscle fatigueability. FRDA has a prevalence of approximately 1 in 40,000 in Caucasians. Symptoms of FRDA include progressive ataxia of all four limbs, dysarthria, areflexia, sensory loss, and exercise intolerance. Skeletal deformities and cardiomyopathy are found in most patients, impaired glucose tolerance and diabetes mellitus are found in ~30% of patients, and reduced visual acuity, including a pigmentary retinopathy, and hearing loss are occasionally seen. Symptoms usually begin between the ages of 5 and 15 but can, on rare occasions, appear as early as 18 months or as late as 50 years of age. The first symptom to appear is usually difficulty in walking, or gait ataxia. The ataxia gradually worsens and slowly spreads to the arms and then the trunk. Foot deformities such as clubfoot, flexion (involuntary bending) of the toes, hammer toes, or foot inversion (turning inward) may be early signs. Over time, muscles begin to atrophy, especially in the feet, lower legs, and hands, and deformities develop. Other symptoms include loss of deep tendon reflexes, especially in the knees and ankles. Generally, within 10 to 20 years after the appearance of the first symptoms, afflicted individuals are confined to a wheelchair, and in later stages of the disease become completely incapacitated. Life expectancy may be affected, and many people with FRDA die in adulthood from the associated heart disease: myocardial failure is the most common cause of premature death. There are currently no approved treatments for FRDA.

[006] Friedreich's ataxia is an autosomal recessive disease caused by a triplet (GAA) repeat expansion in the first intron of the frataxin gene, which leads to decreased frataxin protein levels. Frataxin is found primarily in mitochondria where it chaperones iron for the formation of iron-sulfur clusters and may also act to store and detoxify excess iron. Iron-sulfur clusters are important prosthetic groups in the mitochondrial electron transport chain and other enzymes, including aconitase in the Krebs cycle. Decreased frataxin levels contribute to mitochondrial

dysfunction and mitochondrial iron accumulation, which are believed to lead to increased production of toxic oxidants, which can further impair mitochondrial and cellular function.

SUMMARY OF THE INVENTION

[007] In one aspect, provided herein are RNA molecules, said molecules comprising: a sense region and an antisense region which together form a duplex region, and wherein said RNA is capable of compensating for a frataxin deficiency or mutation. In some embodiments, the RNA molecules are siRNAs. In some embodiments, the RNA molecules are shRNAs. Also provided herein are pharmaceutical compositions comprising RNA molecules according to embodiments of the present invention and at least one pharmaceutically acceptable excipient.

[008] In another aspect, provided herein are expression cassettes and expression vectors, the expression vectors or cassettes comprising: a DNA that encodes an RNA comprising a sense region, an antisense region or both regions of an RNA molecule described herein, that is operably linked to an inducible or constitutive promoter and a transcription terminator. Also provided herein are host cells comprising expression vectors according to embodiments of the present invention.

[009] In a further aspect, methods for treating a subject (*e.g.*, a human) at risk for developing Friedreich's ataxia or suffering from Friedreich's ataxia or reducing the symptoms thereof are provided. In some embodiments, the methods comprise administering to the subject a therapeutically effective amount of the RNA molecules described herein or pharmaceutical compositions thereof. In some embodiments, the methods comprise administering to the subject a therapeutically effective amount of an expression vector described herein.

[0010] In yet another aspect, methods for compensating for a frataxin deficiency or mutation in a subject (*e.g.*, a human) are provided. In some embodiments, the methods comprise administering to the subject a therapeutically effective amount of the RNA molecules described herein or pharmaceutical compositions thereof. In some embodiments, the methods comprise administering to the subject a therapeutically effective amount of an expression vector described herein.

[0011] In yet a further aspect, methods for compensating for a frataxin deficiency or mutation in a cell are provided. In some embodiments, the methods comprise administering (*e.g.*, *in vitro*, *in vivo*, or *ex vivo*) to the cell an effective amount of the RNA molecules described herein or pharmaceutical compositions thereof. In some embodiments, the methods comprise administering (*e.g.*, *in vitro*, *in vivo*, or *ex vivo*) to the cell an effective amount of an expression vector described herein.

[0012] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention

belongs. Other features and advantages of the present invention will become apparent from the following detailed description examples and figures. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description. It is also contemplated that whenever appropriate, any embodiment of the present invention can be combined with one or more other embodiments of the present invention, even though the embodiments are described under different aspects of the present invention.

BRIEF DESCRIPTION OF THE FIGURES

[0013] The subject matter regarded as the invention is particularly pointed out and distinctly claimed in the concluding portion of the specification. The invention, however, both as to organization and method of operation, together with objects, features, and advantages thereof, may best be understood by reference to the following detailed description when read with the accompanying drawings in which:

[0014] Figure 1 shows creation of a library of partially self-complementary RNA molecules, part I. Described in Example 3. Sequences of 10 or more nt and not previously assigned a SEQ ID No are listed as SEQ ID Nos: 1-4.

[0015] Figure 2 shows creation of a library of partially self-complementary RNA molecules, part II. Described in Example 3. Sequences of 10 or more nt and not previously assigned a SEQ ID No are listed as SEQ ID Nos: 5-18.

[0016] Figure 3 shows creation of a library of partially self-complementary RNA molecules, part III. Described in Example 3. Sequences of 10 or more nt and not previously assigned a SEQ ID No are listed as SEQ ID Nos: 19-20.

[0017] Figure 4 shows an increase in the percentage survival (at 19 days) of human Friedreich's ataxia fibroblast GM3816 cells infected with Book 2 or Book 11, relative to the same cells infected with a control ("Ctrl3") shRNA, respectively.

[0018] Figure 5 shows an increase in growth (at 14 days) of human fibroblast GM3816 cells infected with Book 2 or Book 11, relative to the same cells infected with three different control ("Ctrl3", "Ctrl10", or "Ctrl53") shRNAs, respectively.

[0019] Figure 6 shows an increase in growth over time of human fibroblast Friedreich's ataxia GM3665 cells infected with Book 2 or Book 11, relative to the same cells infected with a control ("Ctrl3") shRNA, respectively.

[0020] Figure 7 shows an increase in growth over time of healthy human fibroblast GM8402

cells infected with Book 11, relative to the same cells infected with a control ("Ctrl3") shRNA or Book 2, respectively.

[0021] Figure 8 shows that frataxin expression is increased in human fibroblast GM3816 cells infected with Book 2 shRNA relative to relative to the same cells infected with a control shRNA Ctrl3, as measured by quantitative RT-PCR.

[0022] Figure 9 shows that frataxin expression is increased in GM3665 cells transfected with Book 2 (B2) siRNA relative to relative to the same cells infected with Control (C3).

[0023] Figure 10 shows that the siRNA version of the Book 11 shRNA increases the growth of human fibroblast GM3816 cells

[0024] Figure 11 shows that the siRNA version of the Book 11 shRNA sequence increases (similar to that induced by the corresponding shRNA sequence) the growth of human fibroblast GM3816 cells relative to a control siRNA sequence.

[0025] Figure 12 shows that the siRNA version of the Book 11 shRNA sequence increases the growth of human fibroblast GM3665 cells relative to a control siRNA sequence.

[0026] Figure 13 shows that the growth-enhancing effect of Book 11 (B11) in human fibroblast GM3816 cells treated with B11 siRNA is diminished with a mutation in the seed sequence (Mut 1) but not with a mutation in the non-seed sequence.

[0027] Figure 14 shows the effect of the Book 11 ("B11") and Book 11 Mut 1 ("Mut1") shRNA sequences on the secretion of cytokines as measured by ELISA. Panel A shows the secretion of GM-CSF and VEGF; panel B shows the secretion of MCP-1, IL-8, and IP-10. These secretion patterns matched the expression changes seen on microarray analysis of mRNAs of the same cytokines.

DETAILED DESCRIPTION OF THE INVENTION

[0028] Friedreich's ataxia (FRDA) is an autosomal recessive neurodegenerative disorder caused by mutations in the FXN gene, which encodes the protein frataxin, and for which there are no currently accepted treatments. It is the most common hereditary ataxia and causes progressive damage to the nervous system, particularly sensory neurons, resulting in symptoms ranging from ataxia, muscle fatigability, and speech problems to hypertrophic cardiomyopathy. Frataxin localizes to the mitochondrial matrix, where it chaperones iron for the assembly of iron-sulfur clusters that are then employed by a variety of respiratory and other enzymes.

[0029] In one aspect, provided herein are RNA molecules, said molecules comprising: a sense region and an antisense region which together form a duplex region, and wherein said RNA is capable of compensating for a frataxin deficiency or mutation. In some embodiments, the RNA

molecules are siRNAs. In some embodiments, the RNA molecules are shRNAs. Also provided herein are pharmaceutical compositions comprising RNA molecules according to embodiments of the present invention and at least one pharmaceutically acceptable excipient.

[0030] In another aspect, provided herein are expression cassettes and expression vectors, the expression vectors or cassettes comprising: a DNA that encodes an RNA comprising a sense region, an antisense region or both regions of an RNA molecule described herein, that is operably linked to an inducible or constitutive promoter and a transcription terminator. Also provided herein are host cells comprising expression vectors according to embodiments of the present invention.

[0031] In a further aspect, methods for treating a subject (*e.g.*, a human) at risk for developing Friedreich's ataxia or suffering from Friedreich's ataxia or reducing the symptoms thereof are provided. In some embodiments, the methods comprise administering to the subject a therapeutically effective amount of an RNA molecule described herein or pharmaceutical compositions thereof. In some embodiments, the methods comprise administering to the subject a therapeutically effective amount of an expression vector described herein.

[0032] In yet another aspect, methods for compensating for a frataxin deficiency or mutation in a subject (*e.g.*, a human) are provided. In some embodiments, the methods comprise administering to the subject a therapeutically effective amount of an RNA molecules described herein or pharmaceutical compositions thereof. In some embodiments, the methods comprise administering to the subject a therapeutically effective amount of an expression vector described herein.

[0033] In yet a further aspect, methods for compensating for a frataxin deficiency or mutation in a cell are provided. In some embodiments, the methods comprise administering (*e.g.*, *in vitro*, *in vivo*, or *ex vivo*) to the cell an effective amount of an RNA molecules described herein or pharmaceutical compositions thereof. In some embodiments, the methods comprise administering (*e.g.*, *in vitro*, *in vivo*, or *ex vivo*) to the cell an effective amount of an expression vector described herein.

[0034] In yet another aspect, methods for altering cytokine expression and secretion are provided. In another aspect, methods for altering cytokine expression are provided. In another aspect, methods for altering cytokine secretion are provided. In some embodiments, the methods comprise administering to the subject a therapeutically effective amount of an RNA molecule described herein or pharmaceutical compositions thereof. In some embodiments, the methods comprise administering to the subject a therapeutically effective amount of an expression vector described herein.

[0035] The phrase "the phenotype thereof" refers to an effect that is a partial, an equivalent, or in excess of the extent of the effect of the preceding term or terms. For example, frataxin depletion increases iron accumulation, increases oxidative stress and causes mitochondrial dysfunction. Increasing cellular frataxin gene expression results in the decrease in iron accumulation, decrease in oxidative stress, or improving mitochondrial dysfunction. Thus, the phenotype of increasing frataxin gene expression can produce a partial decrease in iron accumulation, partial decrease in oxidative stress or partial reduction of mitochondrial dysfunction. Additionally, the phenotype of increasing frataxin gene expression can produce an extent of decrease in iron accumulation, decrease in oxidative stress or reduction of mitochondrial dysfunction equivalent to that extent achieved by increasing frataxin gene expression. Alternatively, the phenotype of increasing frataxin gene expression can produce an extent of decrease in iron accumulation, decrease in oxidative stress or reduction of mitochondrial dysfunction that exceeds that extent achieved by increasing frataxin gene expression.

[0036] The term "compensating" or "compensate" in reference to frataxin deficiency or mutation refers to the property of increasing, improving, or restoring cellular biochemistry or metabolism that is impaired by a frataxin deficiency or mutation, or the phenotype thereof, wherein the compensating may involve, but does not necessarily involve, or altogether does not involve, a direct effect on frataxin levels, expression or activity. In referring to RNA molecules embodied herein as compensating for a frataxin deficiency or mutation, the RNA molecules have the property in cells, tissues, or in subjects, of increasing, improving, or restoring cellular biochemistry or metabolism that is impaired by a frataxin deficiency or mutation, wherein the RNA molecules may involve, but do not necessarily involve, or altogether does not involve, a direct effect on frataxin levels, expression or activity.

[0037] The term "subject" as used herein can be any suitable mammal, including primates, such as monkeys and humans, horses, cows, cats, dogs, rabbits, and rodents such as rats and mice. In one embodiment, the mammal to be treated in the methods provided herein is a human.

[0038] "Mismatches" refers to hybridized nucleic acid duplexes where the 2 strands are not 100% complementary. Lack of total homology is due, for example, to a deletion, insertion, inversion, or substitution.

[0039] The term "complementary" refers to the ability of polynucleotides or two regions of a single-stranded oligonucleotide to form base pairs with one another. Base pairs are typically formed by hydrogen bonds between nucleotide units in antiparallel polynucleotide strands or between nucleotide units from two regions within a single oligonucleotide strands. Complementary strands can base pair in the Watson-Crick manner (*e.g.,* A to T, A to U, C to G),

or in any other manner that allows for the formation of duplexes. As persons skilled in the art are aware, when using RNA as opposed to DNA, uracil rather than thymine is the base that is considered to be complementary to adenosine. However, when a U or T is denoted herein, the ability to substitute a T or U is implied, unless otherwise stated. As used herein, the term "substantially complementary" is used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between a nucleic acid and a nucleic acid containing the target sequence. It is understood that the sequence of a nucleic acid need not be 100% complementary to that of its target. The term encompasses a sequence complementary to another sequence with the exception of an overhang. In some cases, the sequence is complementary to the other sequence with the exception of 1-2 mismatches. In some cases, the sequences are complementary except for 1 mismatch. In some cases, the sequences are complementary except for 2 mismatches. In other cases, the sequences are complementary except for 3 mismatches.

[0040] The phrase "duplex region" or "duplex", which are used interchangeably herein, refers to the region in two complementary or substantially complementary polynucleotides or the two complementary or substantially complementary regions of a single-stranded oligonucleotide that form base pairs with one another, either by Watson-Crick base pairing or any other manner that allows for a stabilized duplex between the strands that are complementary or substantially complementary. For example, a polynucleotide strand having 21 nucleotide units can base pair with another polynucleotide of 21 nucleotide units, yet only 19 bases on each strand are complementary or substantially complementary, such that the "duplex region" has 19 base pairs. The remaining bases may, for example, exist as 5' and 3' overhangs. Further, within the duplex region, 100% complementarity is not required; substantial complementarity is allowable within a duplex region. Substantial complementarity includes 79% or greater complementarity. For example, a mismatch in a duplex region consisting of 19 base pairs results in 94.7% complementarity, rendering the duplex region substantially complementary.

[0041] "Hybridizes," refers to hybridization of a molecule to a target molecule under the conditions wherein a method of the invention is carried out. Depending on the context, the term refers to hybridization under stringent conditions or under moderate conditions. As used herein, the term "hybridizes under stringent conditions" refers to conditions for hybridization and washing under which a double-stranded nucleotide molecule at least 18 residues in length and at least 60% self-complementary typically remains hybridized. Preferably, a double-stranded nucleotide molecule at least 18 residues in length and at least 70% self-complementary is utilized.

More preferably, a double-stranded nucleotide molecule at least 19 residues in length and at least 90% self-complementary is utilized.

[0042] "Single-stranded," as used herein, refers to a nucleic acid molecule wherein all the nucleotide bases are connected to one another by covalent bonds. It will be appreciated that the term includes nucleic acid molecules and oligonucleotides comprising a duplex region.

[0043] As used herein, "double-stranded" refers to an oligonucleotide, which may be duplex in part or in full, and which may be blunt-ended or contain a 5'- and/or 3'-overhang, and also may be of a hairpin form comprising a single-stranded oligonucleotide that folds back upon itself to give a duplex region.

[0044] Unless the context dictates otherwise, each "strand" of a duplex region can either be found in a single-stranded oligonucleotide (*e.g.,* the sense and antisense strands in an shRNA) or in each of two single-stranded oligonucleotides (*e.g.,* the sense and antisense strands in an siRNA).

[0045] "Expression vector" refers to a means of expressing an RNA molecule. For example, the expression vector is a plasmid. In another example, the vector is a recombinant viral vector. In another example, the vector is a recombinant bacterial vector. In another example, the vector is a naked DNA vector. In another example, the vector is a self-replicating nucleic molecule, or virus comprising same, that is capable of expressing an RNA molecule of the present invention. Methods for constructing and utilizing recombinant vectors are well known in the art and are described, for example, in Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York), and in Brent et al. (2003, Current Protocols in Molecular Biology, John Wiley & Sons, New York).

[0046] "Biological parameter" refers to any measurable or observable phenotype of a cell, *e.g.,* a morphological characteristic, differentiation state, growth rate, cell cycle characteristic, biochemical characteristic, or another phenotype.

[0047] "Readout" refers to any means known in the art of determining, assessing, measuring, or observing a biological phenotype. It will be appreciated that the term includes biochemical assays, morphological observation, cell staining, cell sorting, and the like. The term also encompasses survival under a defined set of conditions.

[0048] The phrase, "pharmaceutically acceptable derivative", as used herein, denotes any pharmaceutically acceptable salt, ester, or salt of such ester, of such compound, or any other adduct or derivative which, upon administration to a patient, is capable of providing (directly or indirectly) a compound as otherwise described herein, or a metabolite or residue thereof. Pharmaceutically acceptable derivatives thus include among others prodrugs. A prodrug is a derivative of a compound, usually with significantly reduced pharmacological activity, which

contains an additional moiety, which is susceptible to removal *in vivo yi*elding the parent molecule as the pharmacologically active species. An example of a prodrug is an ester, which is cleaved *in vivo t*o yield a compound of interest. Another example is an N-methyl derivative of a compound, which is susceptible to oxidative metabolism resulting in N-demethylation. Pro-drugs of a variety of compounds, and materials and methods for derivatizing the parent compounds to create the prodrugs, are known and may be adapted to the present invention.

[0049] "Palindromic," as used herein, refers to a single-stranded nucleic acid molecule having a sequence that is the same sequence as the reverse complement of itself. The sequence AAGGCCTT is an example of a palindrome.

[0050] "Non self-complementary" refers, to a sequence that is not palindromic. The term encompasses a sequence that is partially self-complementary and also contains a non self-complementary region. The term also encompasses a sequence that is partially self-complementary and also contains a (non self-complementary) loop-forming region

[0051] "Constant" refers herein to a region that is unchanged or invariant within a set, including a subset, of nucleic acid molecules. It will be appreciated that the term may encompass, unless the context dictates otherwise, slight variations that occur between otherwise constant regions.

[0052] As used herein, the terms "treat" and "treatment" refer to therapeutic treatment, including prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) an undesired physiological change associated with a disease or condition. Beneficial or desired clinical results include, but are not limited to, alleviation of symptoms, diminishment of the extent of a disease or condition, stabilization of a disease or condition (*i.e.*, where the disease or condition does not worsen), delay or slowing of the progression of a disease or condition, amelioration or palliation of the disease or condition, and remission (whether partial or total) of the disease or condition, whether detectable or undetectable. "Treatment" can also mean prolonging survival as compared to expected survival if not receiving treatment. Those in need of treatment include those already with the disease or condition as well as those prone to having the disease or condition or those in which the disease or condition is to be prevented.

[0053] Oligonucleotides described herein may, but need not, be further designed to resist degradation by endogenous nucleolytic enzymes or exhibit desirable stability characteristics by using such linkages as phosphorothioate, methylphosphonate, sulfone, sulfate, ketyl, phosphorodithioate, phosphoramidate, phosphate esters, and other such linkages. Polynucleotides may also be modified to increase stability *in vivo*. Possible modifications include, but are not limited to, the addition of flanking sequences at the 5' and/or 3' ends; the use of phosphorothioate or 2' O-methyl rather than phosphodiester linkages in the backbone; and/or the inclusion of

nontraditional bases such as inosine, queosine, and wybutosine and the like, as well as acetyl-methyl-, thio- and other modified forms of adenine, cytidine, guanine, thymine, and uridine.

[0054] Oligonucleotides described herein may contain at least one modification in its base, sugar or backbone for a variety of reasons well known to one of skill in the art, for example, for its higher efficacy. The modified nucleic acid backbone comprises phosphorothioate, phosphotriester, methyl phosphonate, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. The oligonucleotides may also contain one or more substituted sugar moieties. The oligonucleotides may include one or more modified bases, for example, hypoxanthine, 6-methyladenine, 5-me pyrimidines (particularly, 5-methylcytosine), 5-hydroxymethylcytosine (HMC), glycosyl HMC and gentiobiosyl HMC, as well as synthetic nucleobases, *e.g.,* 2-aminoadenine, 2-(methylamino)adenine, 2-(imidazolylalkyl)adenine, 2-(aminoalkylamino)adenine or other heterosubstituted alkyladenines, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5-hydroxymethyluracil, 8-azaguanine, 7-deazaguanine, N^6(6-aminohexyl)adenine and 2,6-diaminopurine. Another modification of the oligonucleotides involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety, a cholesteryl moiety, cholic acid, a thioether, *e.g.,* hexyl-5-tritylthiol, a thiocholesterol, an aliphatic chain, *e.g.,* dodecandiol or undecyl residues, a phospholipid, *e.g.,* di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate, a polyamine or a polyethylene glycol chain, or adamantane acetic acid. Oligonucleotides comprising lipophilic moieties, and methods for preparing such oligonucleotides are known in the art, for example, U.S. Pat. Nos. 5,138,045, 5,218,105 and 5,459,255. The modifications described above may, for example, enhance stability against nuclease degradation and increase affinity of an oligonucleotide toward its target.

[0055] Furthermore, oligonucleotides sequences may be chemically modified or conjugated to improve their stability, *in vivo* delivery and pharmacokinetic profile. For example, without limitation, the oligonucleotide backbone may be modified to contain 2'-O-(C1-C3) alkylribonucleotides, 2'-deoxyribonucleotides, phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, alkyl phosphonates, phosphinates, phosphoroamidates, thionophosphoroamidates, thionoalkylphosphonates or phosphotriesters groups. Other modifications may involve the sugar moiety (*e.g.*, by replacing the sugar with a morpholino group), the internucleosidic bond or the purine or pyrimidine bases, *e.g.,* by introducing purines and pyrimidines variously substituted on the heterocyclic rings, for example

by alkyl, hydroxy- or halo-alkyl, halogen, hydroxyl, sulfur, amino or aza groups. Moreover, oligonucleotides can be conjugated with different groups or functionalities able to increase their activity, distribution or cellular uptake. Such groups or functionalities include, but are not limited to, lipids, aliphatic chains, poliethilenglycol chains, polyamines and phospholipids.

[0056] The sugar moieties may be unmodified or modified. Preferred modified sugar moieties oligonucleotides comprise one of the following at the 2' position: F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nNR_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2 CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-O--CH_2 CH_2 OCH_3, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) i.e., an alkoxyalkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2$ $ON(CH_3)_2$ group, also known as 2'-DMAOE, 2'-methoxy (2'-O--CH_3), 2'-aminopropoxy (2'-OCH_2 CH_2 CH_2 NH_2). A further preferred modification of this category is the bicyclic class of modifications known collectively as LNAs (Locked Nucleic Acids). One of skill in the art may use conventional methods to create such modified sugar structures. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,700,920 and 5,969,116 each of which is incorporated by reference herein in its entirety.

[0057] As used herein, the term "promoter/regulatory sequence" means a nucleic acid sequence which is required for expression of a gene product operably linked to the promoter/regulatory sequence. In some instances, this sequence may be the core promoter sequence and in other instances, this sequence may also include an enhancer sequence and other regulatory elements which are required for expression of the gene product. The promoter/regulatory sequence may, for example, be one which expresses the gene product in a spatially or temporally restricted manner.

[0058] A "constitutive" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced

in a living cell under most or all physiological conditions of that cell.

[0059] An "inducible" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a living cell substantially only when an inducer which corresponds to the promoter is present in the cell.

[0060] A "tissue-specific" promoter is a nucleotide sequence which, when operably linked with a polynucleotide which encodes or specifies a gene product, causes the gene product to be produced in a living cell substantially only if the cell is a cell of the tissue type corresponding to the promoter.

[0061] RNA interference (RNAi) is a process by which double-stranded RNA (dsRNA) is used to silence gene expression. While not wanting to be bound by theory, RNAi begins with the cleavage of longer dsRNAs into small interfering RNAs (siRNAs) by an RNaseIII-like enzyme, dicer. SiRNAs are dsRNAs that are usually about 19 to 28 nucleotides, or 20 to 25 nucleotides, or 21 to 22 nucleotides in length and often contain 2-nucleotide 3' overhangs, and 5' phosphate and 3' hydroxyl termini. One strand of the siRNA is incorporated into a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC). RISC uses this siRNA strand to identify mRNA molecules that are at least partially complementary to the incorporated siRNA strand, and then cleaves these target mRNAs or inhibits their translation. Therefore, the siRNA strand that is incorporated into RISC is known as the guide strand or the antisense strand. The other siRNA strand, known as the passenger strand or the sense strand, is eliminated from the siRNA and is at least partially homologous to the target mRNA. Those of skill in the art will recognize that, in principle, either strand of an siRNA can be incorporated into RISC and function as a guide strand. However, siRNA design (*e.g.*, decreased siRNA duplex stability at the 5' end of the antisense strand) can favor incorporation of the antisense strand into RISC.

[0062] Without wishing to be bound by theory, RISC-mediated cleavage of mRNAs having a sequence at least partially complementary to the guide strand leads to a decrease in the steady state level of that mRNA and of the corresponding protein encoded by this mRNA. Alternatively, RISC can also decrease expression of the corresponding protein via translational repression without cleavage of the target mRNA. Other RNA molecules and RNA-like molecules can also interact with RISC and silence gene expression. Examples of other RNA molecules that can interact with RISC include short hairpin RNAs (shRNAs), single-stranded siRNAs, microRNAs (miRNAs), and dicer-substrate 27-mer duplexes. The term "siRNA" as used herein refers to a double-stranded interfering RNA unless otherwise noted. Examples of RNA-like molecules that can interact with RISC include RNA molecules containing one or more chemically modified

nucleotides, one or more deoxyribonucleotides, and/or one or more non-phosphodiester linkages. For purposes of the present discussion, all RNA or RNA-like molecules that can interact with RISC and participate in RISC-mediated changes in gene expression will be referred to as "interfering RNAs." SiRNAs, shRNAs, miRNAs, and dicer-substrate 27-mer duplexes are, therefore, subsets of "interfering RNAs."

[0063] RNA of embodiments of the invention appear to act in a catalytic manner for cleavage of target mRNA, i.e., RNA is able to effect inhibition of target mRNA in substoichiometric amounts. As compared to antisense therapies, significantly less RNA is generally required to provide a therapeutic effect under such cleavage conditions.

[0064] An siRNA may be chemically synthesized, may be produced by in vitro transcription, or may be produced within a host cell. In one embodiment, siRNA is a double-stranded RNA (dsRNA) molecule with a duplex region of about 15 to about 40 nucleotides in length, preferably about 15 to about 28 nucleotides, more preferably about 19 to about 25 nucleotides in length, and more preferably about 19, 20, 21, or 22 nucleotides in length, and may contain a 3' and/or 5' overhang on each strand having a length of about 0, 1, 2, 3, 4, or 5 nucleotides. The length of the overhang is independent between the two strands, i.e., the length of the overhang on one strand is not dependent on the length of the overhang on the second strand. Preferably, the siRNA is capable of promoting RNA interference through degradation or specific post-transcriptional gene silencing (PTGS) of the target messenger RNA (mRNA).

[0065] In some embodiments, the RNA is a small hairpin (also called stem loop) RNA (shRNA). In some cases, these shRNAs are composed of an antisense strand, followed by a loop, and the analogous sense strand. Alternatively, the sense strand may precede the nucleotide loop structure and the antisense strand may follow. These shRNAs may be contained in plasmids, retroviruses, and lentiviruses and expressed from, for example, the pol III U6 promoter, or another promoter (*see, e.g.,* Stewart, *et al.* (2003) RNA April; 9(4):493-501 incorporated by reference herein in its entirety).

[0066] The target RNA cleavage reaction guided by siRNAs and other forms of RNA is highly sequence specific. In general, siRNA containing a sense nucleotide strand substantially identical in sequence to a portion of the target mRNA and an antisense nucleotide strand exactly complementary to a portion of the target mRNA are siRNA embodiments for inhibition of mRNAs cited herein. However, 100% sequence complementarity between the antisense siRNA strand and the target mRNA, or between the antisense siRNA strand and the sense siRNA strand, is not required to practice the present invention. Thus, for example, sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence are

allowed for.

[0067] In certain embodiments, the antisense strand of the siRNA has at least near-perfect contiguous complementarity of at least 19 nucleotides with the target mRNA. "Near-perfect," as used herein, means the antisense strand of the siRNA is "substantially complementary to," and the sense strand of the siRNA is "substantially identical" to at least a portion of the target mRNA. "Identity," as known by one of ordinary skill in the art, is the degree of sequence relatedness between nucleotide sequences as determined by matching the order and identity of nucleotides between the sequences. In some embodiments, the antisense strand of an siRNA having about 79% and between about 79% up to 100% complementarity, for example, 80%, 85%, 90% or 95% complementarity, to the target mRNA sequence are considered near-perfect complementarity and may be used in the present invention. "Perfect" contiguous complementarity is standard Watson-Crick base pairing of adjacent base pairs. "At least near-perfect" contiguous complementarity includes "perfect" complementarity as used herein. Computer methods for determining identity or complementarity are designed to identify the greatest degree of matching of nucleotide sequences, for example, BLASTN (Altschul, S. F., et al. (1990) J. Mol. Biol. 215:403-410), which is hereby incorporated by reference in its entirety.

[0068] One or both of the strands of double-stranded RNA may have a 3' overhang of from 1 to 6 nucleotides, which may be ribonucleotides or deoxyribonucleotides or a mixture thereof. The nucleotides of the overhang are not base-paired. For example, the RNA comprises a 3' overhang of TT or WU. In another example, the RNA comprises at least one blunt end. The termini usually have a 5' phosphate group or a 3' hydroxyl group, respectively. In other embodiments, the antisense strand has a 5' phosphate group, and the sense strand has a 5' hydroxyl group. In still other embodiments, the termini are further modified by covalent addition of other molecules or functional groups. For example, two single strands of an siRNA may be covalently linked through a non-nucleotide linker.

[0069] The sense and antisense strands of the double-stranded siRNA may be in a duplex formation of two single strands or may be a single molecule where the regions of complementarity are base-paired and are covalently linked by a hairpin loop so as to form a single strand. It is believed that the hairpin is cleaved intracellularly by a protein termed dicer to form an RNA of two individual single-stranded base-paired RNA molecules.

[0070] In a still other aspect, provided herein are methods for optimizing an RNA molecule that compensate for a frataxin deficiency or mutation, the methods comprising the steps of: (a) contacting a cell population with a set or library of expression vectors that express RNA molecule variants of a parent RNA molecule that compensate for a frataxin deficiency or mutation, wherein

the set or library of the expression vectors, or a fraction thereof, is taken up by the cell population; and (b) determining or measuring the frataxin phenotype thereof in the cell population; whereby, if a cell in the cell population exhibits an improved frataxin phenotype, then the cell carries a particular expression vector that encodes a particular RNA molecule with an improved ability relative to the parent RNA molecule to compensate for a frataxin deficiency or mutation. In some embodiments, a particular expression vector found to have an improved ability relative to the parent RNA molecule to compensate for a frataxin deficiency or mutation is isolated or amplified, then the vector or a fragment thereof is sequenced.

[0071] In some embodiments, the ds regions of the parent RNA molecule and the altered version of same share at least 70% homology. In another embodiment, the 2 regions share at least 75% homology. In another embodiment, the 2 regions share at least 80% homology. In another embodiment, the 2 regions share at least 85% homology. In another embodiment, the 2 regions share at least 90% homology. In another embodiment, the 2 regions share at least 95% homology. In another embodiment, the 2 regions share at least 97% homology. In another embodiment, the altered version of the parent RNA molecule comprises a ds region that is identical with the ds region of the particular RNA molecule. In another embodiment, the second expression vector backbone is different from the expression vector backbone utilized in the first round of screening.

[0072] In some embodiments, methods of the present invention comprises the steps of a. isolating or amplifying a particular expression vector found to express an RNA molecule (the "parent RNA") that compensates for a frataxin deficiency or mutation (the "first round of selection"); b. mutagenizing a fragment of the particular expression vector, wherein the fragment comprises a region encoding the double-stranded region of the parent RNA contained in the expression vector, thereby generating a sub-library of nucleotide molecules, the nucleotide molecules comprising variants of double-stranded region of the parent RNA; c. inserting or subcloning the sub-library into an expression vector backbone, thereby generating a sub-library of expression vectors; d. contacting a second cell population with the sub-library of expression vectors (the "second round of selection"), wherein the sub-library of expression vectors, or a fraction thereof, is taken up by the second cell population; and e. determining or measuring the frataxin phenotype in the second cell population. In this embodiment, whereby, if a cell in the cell population exhibits an improved frataxin phenotype, then the cell carries a particular expression vector that encodes a particular RNA molecule with an improved ability relative to the parent RNA to compensate for a frataxin deficiency or mutation. In another embodiment of these methods, some of the RNA molecules in the sub-library contain one or more mismatches between the 2 complementary strands. In some embodiments, the step of mutagenizing comprises the step

of copying a fragment of the particular expression vector by a low-fidelity method. In other embodiments, the mutagenized sequences are generated by a computer. In some embodiments, the computational method comprises generating each possible single mutation of the parent RNAi molecule. In some embodiments, double mutations are also generated. In some embodiments, triple mutations are also generated.

[0073] In some embodiments, wherein a mutation is introduced into a residue in the ds portion of the RNAi, a corresponding mutation is introduced in the complementary residue, to maintain base pairing. In other embodiments, a corresponding mutation is not introduced.

[0074] Methods for (1) modifying an RNA molecule containing a double-stranded region, and for (2) expressing an RNA molecule containing a double-stranded region in various types of vectors, are well known in the art, and are described, for example, in Palliser D et al (An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection. Nature. 2006 Jan 5;439(7072):89-94).

[0075] In some embodiments, one or more additional rounds of enrichment are performed after the second round. The use of 2 or more rounds of enrichment generally will increase the fraction of true positive clones.

[0076] In some embodiments, the expression vector used in the first round of selection is an integrating vector. An integrating vector can facilitate identification of true positives because of the irreversible nature of its effects.

[0077] In some embodiments, the different expression vector used in the second or a subsequent round of selection produces a different form of the RNA molecule (*e.g.,* RNAi, siRNA, microRNA, or shRNA) identified in the first round of selection (having essentially the same double-stranded region), after which the different form of the RNA molecule itself (*e.g.,* an siRNA) is brought into contact with an additional cell(s), for which the frataxin phenotype thereof is determined or measured

[0078] In some embodiments, the different form of the RNA molecule used in the second or a subsequent round of selection exerts its effects in a reversible manner. For example, an expression vector with an inducible or repressible promoter is used as an alternative to a reversible form of RNAi.

[0079] In some embodiments, an improved expression vector encodes an improved RNA molecule that affects the frataxin phenotype more than the parent RNA molecule. In some embodiments, the improved expression vector exhibits greater tissue specificity than the parent RNA molecule. In some embodiments, a lower dosage is required of the improved expression vector or the corresponding RNA molecule encoded thereby, than the parent RNA molecule. In

another embodiment, the improved expression vector exhibits any other improved property known in the art, relative to the parent RNA molecule.

[0080] "Self-complementary along part of its length" refers to an RNA molecule with a region that hybridizes to another region of the molecule. In some cases, the region is perfectly complementary to the other region of the molecule. In some cases, the first region has a mismatch with respect to the other region. In some cases, the first region has more than one mismatch with respect to the other region. In some cases, the first region has a deletion with respect to the other region. In some cases, the deletion causes an internal loop that is recognized by a cellular enzyme. In some cases, the first region has an overhang or sticky end with respect to the other region. In some cases, the first region and other (complementary) region are separated by a non-complementary linker or intervening region. In some cases, the non-complementary linker region forms a loop structure.

[0081] In some embodiments, the intervening sequence of the shRNA between the sense and antisense strands forms a loop structure when the random sequence and the complementary sequence are annealed to one another. In some embodiments, the loop-forming region is not palindromic. In some embodiments, the loop-forming region is not self-complementary.

[0082] In some embodiments, the RNA molecule expressed by a vector of the present invention is a short hairpin RNA (shRNA). In some embodiments, the RNA molecule is a small inhibitory RNA (siRNA). In some embodiments, the RNA molecule is an inhibitory RNA (RNAi). In some embodiments, the RNA molecule is an agRNA (antigenic RNA). "agRNA" refers to a double stranded RNA capable of interacting with mRNA and silencing gene transcription. In some embodiments, the RNA molecule is a microRNA (miRNA). In some embodiments, the RNA molecule is an anti-sense locked-nucleic acid (LNA) oligonucleotide. A variety of types of inhibitory RNA are known in the art, for example, those enumerated or described in Banan M et al (The ins and outs of RNAi in mammalian cells. Curr Pharm Biotechnol. 2004 Oct;5(5):441-50, which is hereby incorporated by reference in its entirety.

[0083] In some embodiments, the expression vector according to embodiments of the present invention is a recombinant virus. In some embodiments, the expression vector, or a copy thereof, is capable of being packaged as a recombinant virus. In some embodiments, a recombinant RNA molecule of the present invention is capable of being packaged in a recombinant virus. In some embodiments, the packaging utilizes a packaging cell line. In some embodiments, the virus is in retrovirus form (*e.g.,* in the form of RNA that is reverse-transcribed upon transduction to generate the DNA form of the vector).

[0084] In some embodiments, the expression vectors integrate into the genome of cells in the cell

population used to test and/or identify the vectors. In some embodiments, the expression vectors integrate into the genome of the target cells (*e.g.,* for a therapeutic utility). In some embodiments, the expression vectors are carried in cells in the cell population episomally. In some embodiments, the expression vectors are carried in cells in the cell population as extra-chromosomal vectors. In some embodiments, a drug resistance gene is used to select for cells that retain an expression vector.

[0085] In some embodiments, the expression vector utilized in methods of the present invention further comprises a gene encoding a marker protein; *e.g.,* enhanced green fluorescent protein (eGFP) or enhanced farnesylated green fluorescent protein (eGFPf). In some embodiments, a marker protein is used to detect transfected or transduced cells in subsequent steps (*e.g.,* library screening or selection methods).

[0086] In some embodiments, the promoter of an RNA polymerase present in the linearized vector backbone is an RNA pol III promoter. In some embodiments, the promoter is an HI promoter. In some embodiments, the promoter is a U6 promoter. It will be appreciated that other suitable promoters are promoters for other RNA polymerases (*e.g.*, T7 RNA polymerase) known in the art.

[0087] In some embodiments, the promoter in the expression vector is 25 nt upstream of the beginning of the expressed RNA sequence in the expression plasmid. In some embodiments, one or more consecutive pyrimidines (*e.g.,* 4) immediately precedes the transcription start site in the expression plasmid. In another embodiment, the string consists of 2 pyrimidines. In some embodiments, the string consists of 4 pyrimidines. In some embodiments, the string consists of 3 pyrimidines. In some embodiments, the string consists of 5 pyrimidines. In some embodiments, the string consists of a different number of pyrimidines.

[0088] In another aspect, a method of making an RNA molecule according to embodiments of the present invention comprises contacting the expression vector with an RNA polymerase, thereby generating the RNA molecule. The step of contacting is generally performed in the presence of ribonucleotide precursors.

[0089] In some embodiments, the recombinant viruses used to package the set or library of expression vectors are recombinant retroviruses. In some embodiments, the recombinant viruses are recombinant lentiviruses. In some embodiments, the recombinant viruses are recombinant adenoviruses. In another embodiment, the recombinant viruses are derived from a vector enumerated or described in Wadhwa R et al (Vectors for RNA interference. Curr Opin Mol Ther. 2004 Aug;6(4):367-72). In some embodiments, the recombinant viruses comprise a backbone of a vector enumerated or described in Wadhwa R et al. In some embodiments, the recombinant

viruses are derived from any other type of virus known in the art that has the ability to infect or transduce a eukaryotic cell.

[0090] In another embodiment of methods of the present invention, after sequencing a PCR product of an expression vector identified as above, the ends of an aliquot of the product are digested in a PCR tube, subcloned back into the parent vector, and the shRNA construct, or a corresponding RNAi molecule with the same or a homologous double-stranded region, or a construct encoding the corresponding RNAi molecule, (and the control shRNAs) is re-added to the test cells. In this confirmatory testing, populations of cells are compared, rather than small numbers of individual survivors. This method reduces the unlikely occurrence of false positives in screening or selection methods of the present invention.

[0091] In some embodiments, the length of the duplex or self-complementary region of an RNA molecule of the present invention is 27 nt. In some embodiments, the length is 19 nt. In some embodiments, the length is 6 nt. In some embodiments, the length is 7 nt. In some embodiments, the length is 8 nt. In some embodiments, the length is 9 nt. In some embodiments, the length is 10 nt. In some embodiments, the length is 11 nt. In some embodiments, the length is 12 nt. In some embodiments, the length is 13 nt. In some embodiments, the length is 14 nt. In some embodiments, the length is 15 nt. In some embodiments, the length is 16 nt. In some embodiments, the length is 17 nt. In some embodiments, the length is 18 nt. In some embodiments, the length is 20 nt. In some embodiments, the length is 21 nt. In some embodiments, the length is 22 nt. In some embodiments, the length is 23 nt. In some embodiments, the length is 24 nt. In some embodiments, the length is 25 nt. In some embodiments, the length is 26 nt. In some embodiments, the length is 28 nt. In some embodiments, the length is 29 nt. In some embodiments, the length is 30 nt. In some embodiments, the length is more than 30 nt.

[0092] In some embodiments, an RNA molecule of the present invention has a stem or self-complementary region of 29 nt with a 3' overhang. In some embodiments, the overhang is 2 nt. In some embodiments, the RNA molecule has a stem or self-complementary region of 27 nt with a 3' overhang. In some embodiments, the overhang is 2 nt. In some embodiments, the RNA molecule has a stem or self-complementary region of 19 nt with a 3' overhang. In some embodiments, the overhang is 2 nt. In some embodiments, the RNA molecule has another of the lengths enumerated above and has a 3' overhang (*e.g.*, a 2 nt 3' overhang).

[0093] In some embodiments, an RNA molecule of the present invention has a stem or self-complementary region of 21-23 nt (in another embodiment, of 22 nt) with an intervening loop sequence of 15-25 nt (in another embodiment, of 19 nt). In some embodiments, the intervening

loop sequence is 1-30 nt. In some embodiments, the RNA molecule has a mismatch of one or more base pairs in the self-complementary region. In some embodiments, the RNA molecule has a deletion in one strand of the self-complementary region. In another embodiment, the deletion causes an internal loop that is recognized by a cellular enzyme.

[0094] In some embodiments, the length of the intervening loop region of an RNA molecule of the present invention (an shRNA) is 3-20 nt. In some embodiments, the length is 4-20 nt. In some embodiments, the length is 5-20 nt. In some embodiments, the length is 6-20 nt. In some embodiments, the length is 7-20 nt. In some embodiments, the length is 8-20 nt. In some embodiments, the length is 9-20 nt. In some embodiments, the length is 10-20 nt. In some embodiments, the length is 3-15 nt. In some embodiments, the length is 4-15 nt. In some embodiments, the length is 5-15 nt. In some embodiments, the length is 6-15 nt. In some embodiments, the length is 7-15 nt. In some embodiments, the length is 8-15 nt. In some embodiments, the length is 10-15 nt. In some embodiments, the length is 3-12 nt. In some embodiments, the length is 4-12 nt. In some embodiments, the length is 5-12 nt. In some embodiments, the length is 6-12 nt. In some embodiments, the length is 7-12 nt. In some embodiments, the length is 8-12 nt. In some embodiments, the length is 10-12 nt. In some embodiments, the length is 3-10 nt. In some embodiments, the length is 4-10 nt. In some embodiments, the length is 5-10 nt. In some embodiments, the length is 6-10 nt. In some embodiments, the length is 7-10 nt. In some embodiments, the length is 8-10 nt.

[0095] In some embodiments, the length is about 7 nt. In some embodiments, the length is about 19 nt. In some embodiments, the length is about 6 nt. In some embodiments, the length is about 8 nt. In some embodiments, the length is about 9 nt. In some embodiments, the length is about 10 nt. In some embodiments, the length is about 11 nt. In some embodiments, the length is about 12 nt. In some embodiments, the length is about 13 nt. In some embodiments, the length is about 14 nt. In some embodiments, the length is about 15 nt. In some embodiments, the length is about 16 nt. In some embodiments, the length is about 17 nt. In some embodiments, the length is about 18 nt. In some embodiments, the length is about 20 nt. In some embodiments, the length is about 21 nt. In some embodiments, the length is about 22 nt. In some embodiments, the length is about 23 nt. In some embodiments, the length is about 24 nt. In some embodiments, the length is about 25 nt. In some embodiments, the length is about 26 nt. In some embodiments, the length is about 28 nt. In some embodiments, the length is about 29 nt. In some embodiments, the length is about 30 nt. In some embodiments, the length is more than about 30 nt.

[0096] The intervening loop region of RNAi molecules of the present invention is taken, in some embodiments, from a known or naturally occurring RNAi molecule. In other embodiments, the

loop sequence is not from a known or naturally occurring RNAi molecule. It will be understood to those skilled in the art that a variety of loop sequences, including previously unrecognized ones, are suitable for methods of the present invention. Naturally occurring RNAi molecules are well known in the art, and are described for example, in Griffiths-Jones et al (Griffiths-Jones S, Grocock RJ, van Dongen S, Bateman A, Enright AJ. Nucl Acids Res, 2006, 34: D140-D144) and in Griffiths-Jones S (Nucl Acids Res, 2004, 32: D109-D111).

[0097] In some embodiments, an RNA molecule of the present invention is a substrate for an RNA-induced silencing complex (RISC). In another embodiment, a method of present invention further comprises digesting an RNA molecule of the present invention to obtain a short-interfering (siRNA) molecule. In another embodiment, the RNA molecule is a substrate for an RNase III family enzyme. In another embodiment, the enzyme is a Class I RNase III family enzyme. In another embodiment, the enzyme is a Class II RNase III family enzyme. In another embodiment, the enzyme is a Class III RNase III family enzyme. In another embodiment, the enzyme is Dicer. In another embodiment, the enzyme is Drosha. In another embodiment, the enzyme is any other enzyme that with specificity for double-stranded RNA. In another embodiment, processing by a RISC or RNase III family enzyme converts the RNA molecule to a form with a biological activity. Substrates for RISC and RNase III family enzymes are well known in the art, and are described, for example, in Jaronczyk K et al (Exploring the functions of RNA interference pathway proteins: some functions are more RISCy than others. Biochem J. 2005 May 1;387(Pt 3):561-71) and in Banan M et al (The ins and outs of RNAi in mammalian cells. Curr Pharm Biotechnol. 2004 Oct;5(5):441-50). In another embodiment, an RNA molecule of the present invention is cleaved by one of the above enzymes or complexes into a double-stranded RNA with a stem or self-complementary region of about 20 nt and a 3' overhang (*e.g.*, a 2 nt 3' overhang).

[0098] In some embodiments, the digestion occurs inside a target cell. In some embodiments, the RNA molecule is used to generate any other type of RNAi (inhibitory RNA) molecule known in the art.

[0099] The RNA can have any of a variety of structures, lengths, and/or the like. Thus, in one class of embodiments, the RNA comprises a first polyribonucleotide comprising the sense strand and a second polyribonucleotide comprising the antisense strand. The RNA can be, *e.g.*, a long double-stranded RNA that is cleaved by Dicer in the cell, such as an siRNA. For example, the first polyribonucleotide can comprise between 19 and 25 nucleotides, the second polyribonucleotide can comprise between 19 and 25 nucleotides, and the duplex region can comprise between 19 and 25 base pairs. The first and second polyribonucleotides can form a

duplex over their entire length, or they can have overhangs (*e.g.*, 5′ or 3′ overhangs; *e.g.*, 21 nt first and second polyribonucleotides can form a 19 bp double-stranded region with 2 nucleotide overhangs, 23 nt polyribonucleotides can form a 21 bp double-stranded region with 2 nucleotide overhangs, and so on). For example, in some embodiments, the first polyribonucleotide and the second polyribonucleotide each comprise a two nucleotide TT 3′ overhang (where T is 2′-deoxythymidine). Instead of comprising two polyribonucleotides, in some embodiments, the RNA comprises a self-complementary polyribonucleotide (*e.g.*, a shRNA). The RNA is optionally nuclease resistant. In another embodiment, an RNA molecule of the present invention mimics a product of an RNase III family enzyme. In another embodiment, the RNA molecule has a 20 nucleotide ds region and a 2 nucleotides 3' overhang. In another embodiment, the RNA molecule has any other structure known in the art of a product of an RNase III family enzyme.

[00100] In some embodiments, a biologically active RNA molecule of the present invention binds to a sequence shared by several genes. In some embodiments, the shared sequence is found in the 3' untranslated region (UTR) of the target mRNAs. In some embodiments, the shared sequence is found in the 5' UTR of the target mRNAs. In some embodiments, the shared sequence is found in the coding portion of the target mRNAs. In some embodiments, the shared sequence is found in an intron. In some embodiments, the shared sequence is found in a combination of the above regions.

[00101] In some embodiments, the target of an RNA molecule of the present invention is an mRNA molecule. In some embodiments, the target is another type of RNA. In other embodiments, the target is ribosomal RNA (rRNA), transfer RNA (tRNA), messenger RNA (mRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), microRNA (miRNA), or XIST RNA. In some embodiments, the target is a deoxyribonucleotide molecule. In some embodiments, the target is another type of nucleotide molecule. In some embodiments, the target is a protein molecule. In some embodiments, the target is a cofactor. In some embodiments, the target is a lipid. In some embodiments, the target is another type of cellular non-nucleotide molecule.

[00102] The complementary region between an RNA molecule of the present invention and its target sequence is, in some embodiments, 5 nt in length. In some embodiments, the length of the complementary region is 6 nt. In some embodiments, the length of the complementary region is 7 nt. In some embodiments, the length is 8 nt. In some embodiments, the length is 9 nt. In some embodiments, the length is 10 nt. In some embodiments, the length is 11 nt. In some embodiments, the length is 12 nt. In some embodiments, the length is 13 nt. In some embodiments, the length is 14 nt. In some embodiments, the length is 15 nt. In some

embodiments, the length is 16 nt. In some embodiments, the length is 17 nt. In some embodiments, the length is 18 nt. In some embodiments, the length is 19 nt. In some embodiments, the length is 20 nt. In some embodiments, the length is 21 nt. In some embodiments, the length is 22 nt. In some embodiments, the length is 23 nt. In some embodiments, the length is 24 nt. In some embodiments, the length is 25 nt. In some embodiments, the length is 26 nt. In some embodiments, the length is 27 nt. In some embodiments, the length is 28 nt. In some embodiments, the length is 29 nt. In some embodiments, the length is 30 nt. In some embodiments, the length is more than 30 nt. In some embodiments, an RNA molecule of the present invention binds different target sequences on different genes. In some embodiments, the different target sequences are not all the same length.

[00103] In some embodiments, the RNA molecule expressed by a vector of the present invention is fully complementary to its target sequence. In some embodiments, the RNA molecule is partially complementary to its target sequence. In some embodiments, the RNA molecule is complementary to its target sequence along most of the length of the RNA molecule, with a non-complementary overhang region. In some embodiments, the RNA molecule expressed by a vector of the present invention has one or more mismatched residues with respect to its target sequence. In some embodiments, the RNA molecule hybridizes to its target sequence under physiological conditions. In some embodiments, the RNA hybridizes to its target sequence under stringent conditions.

[00104] In some embodiments, expression of an RNA molecule of the present invention inside a cell causes translational repression of the target RNA molecule. In some embodiments, expression of the RNA molecule causes cleavage or degradation of the target RNA molecule. In some embodiments, whether translational repression, cleavage or degradation occurs depends on the level of complementarity between the RNA molecule of the present invention and the target RNA molecule, and the length of the complementary region.

[00105] The single-stranded and double-stranded oligonucleotides according to embodiments of the present invention may be produced by methods known to one of skill in the art, for example, by the conventional techniques of oligonucleotide synthesis and of transcription using a recombinant vector. For example each of the strands of an siRNA can be synthesized separately and then the complementary strands are hybridized so as to form RNA duplexes. Alternatively, the strands of the siRNA can be synthesized simultaneously.

[00106] Furthermore, the oligonucleotides according to embodiments of the present invention can be produced by chemical synthesis or by transcription *in vitro* or in cell culture, and then administered *in vivo*, or they are produced *in vivo* in the cells of an organism which has been

modified with a transcription vector or a DNA encoding said oligonucleotides.

[00107] Chemical synthesis of oligonucleotides can be carried out according to the conventional phosphoramidite methods. For example, each of the strands of an siRNA can be synthesized according to β-cyanoethyl phosphoramidite chemistry on a solid support using 2'-O-tert-butyl-dimethylsilyl (TBDMS) as a group for protecting the 2'-position of the ribonucleotide. Other protective groups can be used; silyl ether, which protects the 5'-hydroxyl end of the ribonucleotide, can be used in combination with a labile orthoester which protects the 2'-hydroxyl of the ribonucleotide.

[00108] Alternatively, transcription by means of a recombinant vector can be carried out using double-stranded DNA encoding an oligonucleotide described herein, for example, encoding at least one or the two strands of an siRNA or a shRNA. Such DNAs cloned into appropriate expression vectors allow separate or simultaneous transcription of the two complementary strands of said siRNA. In some embodiments, provided herein are expression cassettes, the expression cassettes comprising: comprises at least one of the foregoing DNAs, under the control of appropriate transcriptional regulatory elements, in particular an inducible or noninducible promoter and a transcription terminator.

[00109] In some embodiments, also provided herein are expression vectors into which one of the foregoing expression cassettes is inserted. These vectors are constructed and introduced into host cells by conventional recombinant DNA and gene therapy methods, which are known in themselves. Many vectors into which a nucleic acid molecule of interest can be inserted in order to introduce it into and to maintain it in a eukaryotic or prokaryotic host cell are known in themselves; the choice of an appropriate vector depends on the use envisioned for this vector (for example, replication of the sequence of interest, expression of this sequence, maintaining the sequence in extrachromosomal form or else integration into the host's chromosomal material), and also on the nature of the host cell. Use may be made, *inter alia*, of viral vectors such as adenoviruses, retroviruses, lentiviruses and AAVs into which the sequence of interest has been inserted beforehand, or else nonviral vectors such as plasmids. Preferably, said vector is a DNA vector (recombinant plasmid or virus) comprising a duplex oligodeoxynucleotide described herein; such a vector encoding nucleic acids (*e.g.,* an siRNA or shRNA) described herein are useful for the *in vitro* or *in vivo* production of said nucleic acids.

[00110] Pharmaceutical compositions comprising any of the aforementioned RNA molecules and expression vectors and classes and subclasses of special interest are embraced herein.

[00111] Embodied herein are compounds as described above and pharmaceutical compositions thereof. It will be appreciated that the compounds and compositions, according to the methods of

the present invention, may be administered using any amount and any route of administration effective for the treatment of Friedreich's ataxia and/or where compensation of a frataxin deficiency or mutation has a therapeutically useful role. Thus, the expression "effective amount" as used herein, refers to compensation for a frataxin deficiency or mutation, when it refers to a compensation to exhibit a therapeutic effect, then "therapeutically effective amount" is used. The exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the particular therapeutic agent, its mode and/or route of administration, and the like. The RNA molecules and expression vectors are preferably formulated in dosage unit form for ease of administration and uniformity of dosage. The expression "dosage unit form" as used herein refers to a physically discrete unit of therapeutic agent appropriate for the patient to be treated. It will be understood, however, that the total daily usage of the RNA molecules and expression vectors and compositions of the present invention will be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective dose level for any particular patient or organism will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts.

[00112] Furthermore, after formulation with an appropriate pharmaceutically acceptable carrier in a desired dosage, the pharmaceutical compositions of this invention can be administered to humans and other animals orally, rectally, parenterally, intracisternally, intravaginally, intraperitoneally, subcutaneously, intradermally, intra-ocularly, topically (as by powders, ointments, or drops), buccally, as an oral or nasal spray, or the like, depending on the severity of the disease or disorder being treated. In certain embodiments, the RNA molecules and expression vectors of the invention may be administered at dosage levels of about 0.001 mg/kg to about 50 mg/kg, preferably from about 0. 1 mg/kg to about 10 mg/kg for parenteral administration, or preferably from about 1 mg/kg to about 50 mg/kg, more preferably from about 10 mg/kg to about 50 mg/kg for oral administration, of subject body weight per day, one or more times a day, to obtain the desired therapeutic effect. It will also be appreciated that dosages smaller than 0.001 mg/kg or greater than 50 mg/kg (for example 50-100 mg/kg) can be administered to a subject. In certain embodiments, RNA molecules and expression vectors are administered orally or parenterally.

[00113] In some embodiments of methods and compositions of the present invention, the pharmaceutical compositions are administered orally, and are thus formulated in a form suitable for oral administration, i.e. as a solid or a liquid preparation. Suitable solid oral formulations include tablets, capsules, pills, granules, pellets and the like. Suitable liquid oral formulations include solutions, suspensions, dispersions, emulsions, oils and the like. In some embodiments of the present invention, the active ingredient is formulated in a capsule. In accordance with this embodiment, the compositions of the present invention comprise, in addition to the active compound and the inert carrier or diluent, a hard gelatin capsule.

[00114] In some embodiments, the pharmaceutical compositions are administered by intravenous, intra-arterial, subcutaneous or intra-muscular injection of a liquid preparation. Suitable liquid formulations include solutions, suspensions, dispersions, emulsions, oils and the like. In some embodiments, the pharmaceutical compositions are administered intravenously and are thus formulated in a form suitable for intravenous administration. In some embodiments, the pharmaceutical compositions are administered intra-arterially and are thus formulated in a form suitable for intra-arterial administration. In some embodiments, the pharmaceutical compositions are administered intra-muscularly and are thus formulated in a form suitable for intra-muscular administration.

[00115] In some embodiments, the pharmaceutical compositions are administered topically to body surfaces and are thus formulated in a form suitable for topical administration. Topical formulations include, in some embodiments, gels, ointments, creams, lotions, drops and the like.

[00116] In some embodiments, the pharmaceutical composition is administered as a suppository, for example a rectal suppository or a urethral suppository. In some embodiments, the pharmaceutical composition is administered by subcutaneous implantation of a pellet. In some embodiments, the pellet provides for controlled release of active agent over a period of time.

[00117] In some embodiments, the active compound is delivered in a vesicle, *e.g.,* a liposome.

[00118] In other embodiments, carriers or diluents used in methods of the present invention include, but are not limited to, a gum, a starch (*e.g.,* corn starch, pregeletanized starch), a sugar (*e.g.,* lactose, mannitol, sucrose, dextrose), a cellulosic material (*e.g.,* microcrystalline cellulose), an acrylate (*e.g.,* polymethylacrylate), calcium carbonate, magnesium oxide, talc, or mixtures thereof.

[00119] In other embodiments, pharmaceutically acceptable carriers for liquid formulations are aqueous or non-aqueous solutions, suspensions, emulsions or oils. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions,

including saline and buffered media. Examples of oils are those of animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, olive oil, sunflower oil, fish-liver oil, another marine oil, or a lipid from milk or eggs.

[00120] In some embodiments, parenteral vehicles (for subcutaneous, intravenous, intra-arterial, or intramuscular injection) include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's and fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Examples are sterile liquids such as water and oils, with or without the addition of a surfactant and other pharmaceutically acceptable adjuvants. In general, water, saline, aqueous dextrose and related sugar solutions, and glycols such as propylene glycols or polyethylene glycol are preferred liquid carriers, particularly for injectable solutions. Examples of oils are those of animal, vegetable, or synthetic origin, for example, peanut oil, soybean oil, olive oil, sunflower oil, fish-liver oil, another marine oil, or a lipid from milk or eggs.

[00121] In some embodiments, the compositions further comprise binders (*e.g.,* acacia, cornstarch, gelatin, carbomer, ethyl cellulose, guar gum, hydroxypropyl cellulose, hydroxypropyl methyl cellulose, povidone), disintegrating agents (*e.g.,* cornstarch, potato starch, alginic acid, silicon dioxide, croscarmelose sodium, crospovidone, guar gum, sodium starch glycolate), buffers (*e.g.,* Tris-HCl., acetate, phosphate) of various pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (*e.g.,* Tween 20, Tween 80, Pluronic F68, bile acid salts), protease inhibitors, surfactants (*e.g.,* sodium lauryl sulfate), permeation enhancers, solubilizing agents (*e.g.,* glycerol, polyethylene glycerol), anti-oxidants (*e.g.,* ascorbic acid, sodium metabisulfite, butylated hydroxyanisole), stabilizers (*e.g.,* hydroxypropyl cellulose, hyroxypropylmethyl cellulose), viscosity increasing agents(*e.g.,* carbomer, colloidal silicon dioxide, ethyl cellulose, guar gum), sweeteners (*e.g.,* aspartame, citric acid), preservatives (*e.g.,* Thimerosal, benzyl alcohol, parabens), lubricants (*e.g.,* stearic acid, magnesium stearate, polyethylene glycol, sodium lauryl sulfate), flow-aids (*e.g.,* colloidal silicon dioxide), plasticizers (*e.g.,* diethyl phthalate, triethyl citrate), emulsifiers (*e.g.,* carbomer, hydroxypropyl cellulose, sodium lauryl sulfate), polymer coatings (*e.g.,* poloxamers or poloxamines), coating and film forming agents (*e.g.,* ethyl cellulose, acrylates, polymethacrylates) and/or adjuvants.

[00122] In some embodiments, the pharmaceutical compositions provided herein are controlled-release compositions, i.e. compositions in which the active compound is released over a period of time after administration. Controlled- or sustained-release compositions include formulation in lipophilic depots (*e.g.,* fatty acids, waxes, oils). Alternatively, the agent may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or

other modes of administration. In one embodiment, a pump may be used (see Langer, supra; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989). In some embodiments, polymeric materials are used; *e.g.,* in microspheres in or an implant. In yet some embodiments, a controlled release system is placed in proximity to the target cell, thus requiring only a fraction of the systemic dose (see, *e.g.,* Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984); and Langer R, Science 249: 1527-1533 (1990). In some embodiments, the composition is an immediate-release composition, i.e. a composition in which of the active compound is released immediately after administration.

[00123] The compositions also include, in some embodiments, incorporation of the active material into or onto particulate preparations of polymeric compounds such as polylactic acid, polglycolic acid, hydrogels, etc, or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts.) Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance.

[00124] Also contemplated are particulate compositions coated with polymers (*e.g.,* poloxamers or poloxamines) and the RNA coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors.

[00125] Also comprehended are RNA molecules and expression vectors modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone or polyproline. The modified RNA molecules and expression vectors are known to exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified RNA molecules and expression vectors. Such modifications may also increase the compound's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the compound, and greatly reduce the immunogenicity and reactivity of the compound. As a result, the desired in vivo biological activity may be achieved by the administration of such polymer-compound abducts less frequently or in lower doses than with the unmodified compound.

[00126] In some embodiments, the RNA molecules provided herein are delivered in appropriate compositions as further provided herein via intra-thecal injection. In some embodiments, the RNA molecules provided herein are delivered in appropriate compositions as further provided herein via intra-thecal injection for the treatment of Friedreich's ataxia.

[00127] In some embodiments, the methods of the present invention comprise administering an active compound as the sole active ingredient. However, also encompassed within the scope of

the present invention are methods for treating diseases and disorders that comprise administering the active compound in combination with one or more therapeutic agents appropriate for the disease or disorder that is being treated, as is well known in the art.

[00128] All sequence citations, accession numbers, references, patents, patent applications, scientific publications or other documents cited are hereby incorporated by reference.

[00129] The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various uses and conditions.

EXAMPLES

EXAMPLE 1: DUAL RNAi EXPRESSION BY A RETROVIRAL VECTOR

[00130] A retroviral vector was designed to express simultaneously two shRNAs. The vector contained enhanced, farnesylated green-fluorescent protein (eGFPf), which allows straightforward flow-sorting of infected or transfected cells, and a G418 resistance gene, which facilitates selection of infected or transfected cells. The eGFP gene of the retroviral vector pQCXIX® (Clontech) was replaced with eGFPf, and two copies of a Pol III-dependent H1-promoter cassette (from pSuper-Retro) were cloned into the inactivated long-terminal repeat of pQCXIX, to create the vector pQe2. In each H1-promoter cassette of pQe2, 2 unique restriction enzyme sites were incorporated to allow independent cloning of shRNA constructs into each cassette. pQe2 was used to knock down expression of proteins important in spindle-checkpoint function; both shRNA knockdown (by Western and other analyses) and eGFP expression was validated. Thus, expression of target genes can be knocked down by RNA molecules. In addition, expression of both a particular gene of interest (*e.g.,* frataxin) can be suppressed in normal cells to induce a phenotype, including a disease phenotype, and one or more random targets can be suppressed as well to alter or reverse the phenotype.

EXAMPLE 2: VECTOR MODIFICATION

[00131] pSuper-Retro (Oligoengine®, Seattle, WA), which can be packaged as a retrovirus and includes the gene encoding enhanced green fluorescent protein (eGFP) and a G418-resistance gene, was used in the cloning methods below. The Bgl II-Mlu I fragment of the spacer sequence was replaced with a Bgl II-Bbs I-Mlu I fragment. By then cutting with Bbs I, filling in with Klenow, and cutting with Not I, a linearized vector was created. Other than Pme I, which was

eliminated in creating the spacer sequence, pSuper-Retro lacked all the relevant restriction enzyme sites needed for the procedure shown in Figures 1-3, and thus was suitable for the procedure described in Example 3.

[00132] In the development of alternate vectors, the Bgl II cloning site and the spacer sequence between Bgl II and Hind III of pSuper-Retro were replaced with Xcm I and Sfi I to eliminate the unique Pme I site. (The region from just before the Bgl II site back to the unique BlpI site was PCR amplified, using a primer with a tail containing the sequences for Xcm I, Sfi I, and Hind III, and the vector's Blp I -Hind III fragment was replaced with the PCR product digested with Blp I and Hind III). A spacer sequence was added between XcmI and SfiI by amplifying the old spacer sequence, up to but not including the PmeI site, using primers with tails containing the sequences for XcmI and SfiI. The rationale for adding back a spacer sequence was, in this experiment, to simplify the elimination of single-cut vector and thereby maximize the efficiency of the library ligation.

[00133] Many other vectors could be used and many other restriction enzyme combinations are suitable for the methods in this and the other Examples herein. For example, enzyme pairs that can be used to create non-complementary loop sequences (with the resulting loop sequences in parentheses) include, EcoN I/Aar I (CCTCCCGC), Sma I/Aar I (CCCC), Stu I/Apa I (AGGC), Bsu36 I/Aar I (CCTCAC), Bbv CI/Aar I (CCTCAC), Ear I/Aar I (TCTTCCGC), etc.

EXAMPLE 3: CREATION OF A LIBRARY OF PARTIALLY SELF-COMPLEMENTARY RNA MOLECULES

[00134] Figures 1-3 illustrate an approach used for generating a library of expression vectors for shRNA:

Creating the reverse complement of the random stem sequence, covalently linking the two, and starting the second extension

[00135] As in the previous method, a single-stranded DNA molecule "single-stranded nucleic acid intermediate I" with a region of random sequence sandwiched between 2 constant regions ("first constant region" and "second constant region," 5' and 3', respectively, to region of the random sequence) was synthesized (referred to as "ss I" in Figure 1). The second constant region contains 1 strand of a Pme I recognition site just downstream of the NsNsN26 sequence. In ss I, "NsNsN26" refers to 28 random nt, the first 2 of which are followed by phosphorothioate bonds (to create asymmetric BtgZ I cutting after the second extension, as described hereinbelow and depicted in Figure 7). ss I also contained 1 strand of Not I and BtgZ I recognition sequences, as depicted for ds Ib in Figure 1.

[00136] Simple extension from a recessed primer, containing 2 mismatches, created ds Ib, containing the reverse complements of: (a) a fragment of the first constant region, (b) the NsNsN26 sequence, and (c) the second constant region.

[00137] Use of a mismatched primer created one strand of an Aar I recognition sequence in the reverse complement of the second constant region, just upstream of the n26nn sequence; which was not present in the first copy of the second constant region. In addition, the mismatched primer eliminated the Pme I recognition sequence in the reverse complement of the second constant region. This created the asymmetry used to generate a non-complementary loop between NsNsN26 and n26nn after insertion into the vector (see hereinbelow and Figure 3).

[00138] Ligation of a hairpin-loop linker ("linker B") to the recessed-primer end of ds Ib covalently linked the 2 strands of ds Ib, containing the NsNsN26 and n26nn sequences, and completed the reverse complement of the first constant region, thereby generating nucleic acid intermediate II ("n.a. II"). (The compatible sticky ends of ds Ib and linker B are from Sal I and Xho I sites, respectively; digestion with Sal I and Xho I cut homodimers of ds Ib or linker B, respectively, but did not cut the desired, heterodimeric product, thereby facilitating gel separation by size.) n.a. II contains, in 5'-3' order, (a) a first copy of the first constant region; (b) a first copy of the region of random sequence ("NsNsN26"); (c) a first copy of the second constant region; (d) the hairpin-loop linker; (e) the reverse complement of the second constant region; (f) the reverse complement of the region of random sequence ("n26nn"); and (g) the reverse complement of the first constant region.

[00139] To facilitate the second extension (starting at the bottom of Figure 1 and continuing at the top of Figure 2), a nick site was created with the nicking enzyme N.BbvC, which cuts only 1 strand of DNA (indicated by arrowhead in Figure 1), then the resulting 5' fragment was extended with the strand-displacing DNA polymerase Bst (depicted at bottom of Figure 1 and top of Figure 2) to create the reverse complement of n.a. II, thereby generating double-stranded intermediate III (ds III). ds III contains, in 5'-3' order with respect to the top strand, the following regions, all <u>double-stranded</u>: (a) a second, inverted copy of the first constant region; (b) a second, inverted copy of the random region (n28); (c) a second, inverted copy of the second constant region; (d) a copy of the hairpin-loop linker; (e) a first copy of the second constant region; (f) a first copy of the random region (N28); and (g) a first copy of the first constant region. In ds III of this embodiment, the regions are synthesized in the <u>reverse order</u> from the previous Example, and thus are depicted in the reverse order from the previous Example.

[00140] The phosphorothioate bonds originally appearing in ssI (top of Figure 1) created a restriction site asymmetry in ds III, wherein BtgZ I only cuts 1 end of ds III. Digestion of ds III

with BtgZ I cut the DNA immediately before the first random nucleotide of the newly synthesized N28, as well as 4 nucleotides further in on the opposite strand, leaving a recessed 3' end (Figure 2; ds IIIB). Filling in with Klenow regenerated the four nucleotides in the strand opposite the newly synthesized N28, creating a blunt end. The asymmetric BtgZI digestion allows inclusion of a TTTTT termination sequence after the last random nucleotide while changing the complementary AAAAA to 5 pyrimidines just upstream of the H1 transcription start site at the first random nucleotide.

Finishing the second extension to create the vector insert, and insertion into the vector

[00141] The asymmetric BtgZ I digestion also eliminated 1 of the 2 Not I sites. Digestion with Not I created the library insert (ds IIIC), which was ligated into the vector backbone (first ligation step, Figure 2), thereby generating circular intermediate IV. The top of Figure 3 depicts the vector insert sequence between the N28 and n28 sequences. As a result of the mismatch in primer A (Figure 1), a unique Aar I site was present at 1 end of the insert and a unique Pme I site at the other end. Digestion with Pme I created a blunt end followed by AAACC in the sense strand. Digestion with Aar I cut the DNA 3 nucleotides before the first random nucleotide of the original n28, as well as 4 nucleotides further in on the opposite strand, leaving a recessed 3' end. Filling in with Klenow fragment created a blunt end with GCT in the transcribed strand immediately following N28 ("linear intermediate V"). Uni-molecular, blunt-end ligation of linear intermediate V (second ligation step, Figure 3), generated circular product VI, containing a non-complementary CTAAAC loop sequence between N28 and n28.

[00142] The transcribed strands of the inserts contained 5 pyrimidines upstream of the transcription start site (to increase the efficiency of starting transcription at +1, which pol III prefers to be a purine), followed by a 29-nt stem containing a 28-nt random sequence, followed by a non-complementary loop sequence, followed by the reverse complement of the 29-nt random sequence, followed by 5 thymidines (to terminate pol III transcription, which occurs after the second thymidine). Thus, the vectors encoded shRNAs with 29-nt stems and 2-nt overhangs.

[00143] To test the efficacy of the method, *E. coli* were transfected with circular IM IV, and 300,000 colonies were plated out. Plasmid DNA from 15 of these colonies was isolated, and inserts were sequenced. The sequences of all 15 inserts contained random sequences and their reverse complements separated by the Aar I-Pme I fragment depicted in the top of Figure 3, exactly as predicted. In addition, the pool of intermediates was sequenced. The sequence data confirmed the presence of the expected constant sequences, and lack of bias in the random region, verifying the efficacy of the method. The base usage of the random sequences was 50.9% A/T

and 49.1% G/C, demonstrating that the random region exhibits random character.

[00144] DNA was prepared from the remaining (~300,000) colonies, digested sequentially with Aar I and Pme I. and re-ligated. The ligation mix was used to transfect *E. coli*, and 1,000,000 colonies were plated out. Plasmid DNA was isolated from 5 of these colonies; all 5 had inserts of the proper size.

[00145] Following completion of the method, the random (n29) regions of 14 clones were sequenced. The sequences exhibited no detectable skewing, demonstrating that the method was efficacious, and the final product corresponded exactly to the desired product.

[00146] In addition, inserts from individual "clones" from the completed library were sequenced in their entirety. The sequence from these representative "clones" contained the N28 random sequence, followed by a "G" residue (supplied by the 3' constant region of the original ss DNA molecule), followed by the loop sequence, followed by a "C" residue, followed by the reverse complement of N28, (depicted as "n28."). The sequence corresponds to the bottom (upside-down) strand at the bottom of Figure 3. Thus, the final product corresponded exactly to the desired product, re-confirming that the method was efficacious.

[00147] Other methods of generating libraries of RNA molecules, such as shRNA, for selecting RNA molecules that enhance the survival of Friedriech Ataxia cells, are provided in U.S. Published Application No. 2009/285788, which is hereby incorporated by reference in its entirety.

EXAMPLE 4: DEFINITION OF AN *IN VITRO* MODEL SYSTEM FOR SELECTION OF FRIEDREICH'S ATAXIA CELLS WITH ENHANCED SURVIVAL CAPABILITIES

[00148] Primary FRDA fibroblasts are far more sensitive to oxidative stress than normal control fibroblasts. (Jauslin et al. (Hum Molec Gen 11: 3055, 2002) used L-buthionine (S,R)-sulfoximine (BSO) to block the rate-limiting enzyme in glutathione synthesis, and found a concentration (0.05 mM) at which virtually all primary FRDA fibroblasts lose viability, but more than 90% of normal control fibroblasts retain viability.)

[00149] In other experiments, a time range of 16-48 h was used, and concentrations of 0.001, 0.05, and 0.1 mM BSO were tested. Glutathione depletion of these cells with BSO at 1 mM in media supplemented with 0.3 mg/ml fully saturated human transferrin, which exacerbates the tendencies of these cells to accumulate mitochondrial iron, rendered all the cells in a 6-well plate nonviable after 48 hours; while the majority of age- and passage-matched controls remained viable. By washing the cells, adding back their normal medium, and looking for colony formation, the minimum conditions under which 100% of the cells die were confirmed.

EXAMPLE 5: USE OF THE shRNA LIBRARY TO IDENTIFY RNA MOLECULES WITH ABILITY TO INHIBIT DEATH OF FRDA FIBROBLASTS

[00150] Two shRNA sequences ("Book 2" and "Book 11") were identified that at doubled the growth of primary Friedreich's-ataxia fibroblasts, a cell model system of the genetic disorder Friedreich's ataxia (FA). Briefly, the library from Example 3 of 300,000 random shRNA sequences was used in a retroviral vector system that also contains green fluorescent protein (GFP) as a reporter gene. The library was packaged as retroviruses, and FA cells were infected with the library. The GFP-positive clones were sorted and then the cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing beta-hydroxybutyrate (BHB), a condition for which FA cells are more sensitive than their normal counterparts. After several cycles of selection and recovery, two putative hit sequences ("books") were isolated by PCR from the genomic DNA (as the library was packaged in a retroviral vector, which as result integrates into genomic DNA) of surviving cells. Two of the clones (or "books") that were able to increase growth were isolated and sequenced. The DNA coding strand for the the shRNA sequences (including the poly-T transcription termination sequences) of those two books (Book 2 and Book 11) are provided below, with the loop-encoding sequences underlined. The shRNAs themselves are RNA versions of these sequences, with T changed to U.

Book 2: 5' – GCCAATCGGGGAGGTCGGCGCATATCATGCTAAACCATGATATGCGCCGACC TCCCCGATTGGCTTTTT - 3' (SEQ ID No: 21).

Book 11: 5' – CAACAGAATGGAAGAGTGCTGGGTGGTGGCTAAACCCACCACCCAGCACTC TTCCATTCTGTTGTTTTT - 3' (SEQ ID No: 22)

[00151] Book 11 and Book 2 were tested individually versus a randomly chosen clone. Book 11 and Book 2 were found to have a statistically significant increase in the growth in a ketone-based medium of human fibroblasts in all three commercially available cell lines widely used as model systems for FA, including primary Friedreich's ataxia (FA) GM3816 fibroblasts (See Figures 4 and 5) with a moderate phenotype (i.e. growth defect) and primary FA GM3665 fibroblasts (See Figure 6) with a more severe phenotype – due to the larger triplet repeat expansion in the FA gene. Using an antibody to Argonaut, shRNA guide strands were eluted, deep sequenced, which showed that both Book 2 and Book 11 are expressed in the cells and processed into the RNA-induced silencing complex (RISC).

[00152] Both books were tested in an unrelated, hematopoietic cell line, U937, without an effect

on these cells. Both books were also tested on normal control fibroblasts from three individuals, in addition to FA fibroblasts from a total three different affected individuals. Book 2 was specific, as it did not affect the normal fibroblasts (See Figure 7). Book 11 affected the normal fibroblasts slightly, but not as much as the FA fibroblasts See Figures 6 and 7). Book 2 increases frataxin expression (See Figure 8), which could account for its specificity. Book 2 sequences that were transfected/delivered into GM3665 primary FA fibroblasts with larger trinucleotide repeat expansions (having lower frataxin levels), show a nearly 2.5-fold increase in frataxin level. This increase is clinically significant as it represents the difference between severe, early-onset disease, and mild, late-onset disease (Figure 9).

[00153] Book 11 does not seem to increase frataxin expression, but has a pronounced effect on growth. In the most severely affected FA fibroblasts, Book 11 increased growth nearly 10-fold when used as an siRNA (See Figure 11), while in moderately affected FA fibroblasts a 2-fold increase in growth was seen (See Figure 10). Thus, Book 11 was shown to increase the growth rate of primary FA fibroblasts, but does not increase the growth rate of control primary fibroblasts, thereby demonstrating disease specificity.

[00154] The sequences of the siRNA version of the Book 11 siRNA ("B11 siRNA") used in this Example, as well as the sequences of two mutated versions of the B11 siRNA ("MUT 1" and "MUT 2") are shown below as well with the mutations shown enlarged and in bold

B11 siRNA

5′- AACAGAAUGGAAGAGUGCUGGGUdGdG -3′ (SEQ ID No: 23)
3′- UGUUGUCUUACCUUCUCACGACCCA C C -5′ (SEQ ID No: 24)

B11 MUT 1 siRNA

5′- AACA**C**AAUGGAAGAGUGCUGGGUdGdG -3′ (SEQ ID No: 25)
3′- UGUUGU**G**UUACCUUCUCACGACCCA C C -5′ (SEQ ID No: 26)

B11 MUT 2 siRNA

5′- AACAGAAUGGAAGAGU**C**CUGGGUdGdG -3′ (SEQ ID No: 27)
3′- UGUUGUCUUACCUUCUCA**G**GACCCA C C -5′ (SEQ ID No: 28)

[00155] As described above, the siRNA version of the Book 11 sequence was used and shown to recapitulate the phenotype induced by the corresponding shRNA sequence in the human FA fibroblasts (See Figures 10-13). Small interfering siRNAs are double-stranded RNAs not covalently linked through a loop sequence as they are in short-hairpin-loop shRNAs. Thus, they are not integrated in the genome and are preferred when a reversible or transient effect is desired.

[00156] Important sequences in siRNAs and shRNAs are the "seed" sequences, which are usually nucleotides 2 through 7 (or so) at the 5' end of the guide strand. Thus, B11 MUT 1

should change the seed sequence of Book 11 (B11) for the upper strand, but not for the lower strand, while B11 MUT 2 should change the seed sequence for the lower strand, but not for the upper strand. Figure 13 shows that with MUT 1, the growth-enhancement phenotype of B11 is affected, but not with MUT 2, which implicates the upper strand (SEQ ID No: 23) as the B11 guide strand for growth enhancement.

[00157] Further, Book 11 and Book 11mt were transfected into primary Friedreich's ataxia fibroblasts and expressed as shRNAs. mRNA was prepped from the cells and analyzed using microarray analyses. There were statistically significant differences in expression of many genes, specifically a large number of genes involved in cytokine secretion and secretory pathways. Accordingly, cytokines were measured in the media of the cells with Book 11 and Book 11mt on board (using an ELISA assay) and the differences were matched up with the microarrays. Book 11 appears to alter the secretion of an array of cytokines that underlie the survival/growth advantage (see Figures 14A and 14B).

[00158] The medium of cells with Book 11 on board are tested to determine whether it is sufficient to enhance the survival/growth of cells without Book 11 on board.

EXAMPLE 6: FURTHER IMPROVEMENT OF RNA MOLECULES WITH ABILITY TO INHIBIT DEATH OF FRDA FIBROBLASTS

[00159] To identify improvements of sequences identified after RNAi library screening described in one of the above Examples, random mutagenesis is used. In other experiments, an error-prone copying method, such as, error-prone PCR is utilized. Random mutagenesis by error-prone PCR takes advantage of the low fidelity of Taq polymerase in the presence of $Mn2^{+}$, high $Mg2^{+}$, and unequal dNTP concentrations, and is well known in the art. Because a randomly mutagenized RNAi sequence requires, under some conditions, a matched reverse complement for shRNA, iterative selection uses a recapitulation of the library synthesis protocol described in Example 3. The cell or cells in which the desired effect occurred are isolated, and using error-prone PCR, the sequence corresponding to that of the oligonucleotide of Figure 1 is amplified, *e.g.,* by using perfectly matched primers that extend from the edge of the N28 sequence, plus one nucleotide on the downstream side (so that the 29th nucleotide in the final shRNA stem is mutagenized as well), all the way to the ends of the oligonucleotide sequence, this creates a library of "half-books."

[00160] After random mutagenesis, library construction is performed as described in Example 3. The first, mismatched extension primer is, in some embodiments, an equimolar mix of four primers, each ending in a different nucleotide (complementary to the random nucleotide just

downstream of the N28), (without the need for mixing four sub-libraries as done in the initial library generation), each with a different "29th" nucleotide. Although the strand of DNA complementary to the equivalent of the oligonucleotide at the top of Figure 4 is present in the single-extension reaction, only single-extension products of the recessed, first extension primer anneal to the hairpin-loop linker.

[00161] The sub-library for a given sequence is introduced into target cells as described in one of the above Examples, except that the original sequence is included among the controls. In some experiments, increased effectiveness of an shRNA construct in this context is defined as (1) a larger number of surviving cells under the original conditions used for selection, (2) longer survival under the original conditions used for selection, or (3) survival under more stringent conditions. For initially identified RNA molecules that show subtle improvement over the control shRNAs (such as survival for slightly longer under the original conditions used for selection), the second criterion will likely be the most important for selecting more effective sequences. For RNA molecules that rescue cells for extended periods in the initial confirmatory assay, the third criterion will be the most important for selecting more effective sequences; for such sequences, more stringent conditions are tested to establish new minimum conditions for 100% loss of viability.

[00162] The entire gene encoding an RNAi molecule of the present invention (i.e. both halves of the double-stranded region, and the intervening region; or "whole books") is copied by a low-fidelity method, then the sub-library of whole-books is inserted or subcloned into an expression vector, etc, and the resulting sub-library is introduced into target cells as described for the above method.

[00163] In cases wherein a secondary sequence significantly improves upon a primary sequence, another round of iterative selection is performed on the secondary sequence. Although the selection assay described herein for FRDA cells is based on oxidant stress, this assay can also be used to obtain RNA molecules that improve aspects of FRDA cells unrelated to anti-oxidant defenses per se. The reason for this is that the selection assay is performed under oxidant stress conditions that allow survival of normal, control fibroblasts; therefore, an intervention that makes FRDA cells more like normal cells will improve survival in the assay. Some shRNA are found to affect the tri-nucleotide repeat expansion that inhibits frataxin expression, or the triplex DNA that is formed by the tri-nucleotide repeat expansion, which is measured by an increase in frataxin expression, either using Northern or Western blots.

EXAMPLE 7: FURTHER IMPROVEMENT OF RNA MOLECULES

[00164] A set of sequences is generated, having appropriate flanking sequences for subcloning and an internal portion comprising a gene encoding an RNA molecule with the following components: (a) a portion of residues 1-22 are kept constant, based on the RNA sequence identified in the above Example, while the remainder are randomized; (b) the next 3-20 residues are constant and non-palindromic; (c) the next 22 residues are complementary to the first 22 residues. In other experiments, the seed sequence (approximately residues 1-8 of the ds region) is kept constant, while the remainder of the ds region is varied. In other experiments, the seed sequence is varied, while the remainder of the ds region is kept constant. In other experiments, residues 2-8 of the seed sequence are kept constant, while residues

[00165] An oligonucleotide synthesizer is programmed with the computer-generated sequences. Each of the 65,500 shRNA-encoding sequences is annealed with its complement and then ligated as a pool into an appropriate expression vector, thus creating a library of 65,500 random shRNA-encoding sequences that represent a random sampling of the 18 trillion possible 22-mer shRNA-encoding sequences.

[00166] After randomization of the shRNA by either mutagenesis or computer randomization, the sub-library for a given sequence is introduced into target cells as described in one of the above Examples, except that the original sequence is included among the controls. In some experiments, increased effectiveness of an shRNA construct in this context is defined as (1) a larger number of surviving cells under the original conditions used for selection, (2) longer survival under the original conditions used for selection, or (3) survival under more stringent conditions. For initially identified RNA molecules that show subtle improvement over the control shRNAs (such as survival for slightly longer under the original conditions used for selection), the second criterion will likely be the most important for selecting more effective sequences. For RNA molecules that rescue cells for extended periods in the initial confirmatory assay, the third criterion will be the most important for selecting more effective sequences; for such sequences, more stringent conditions are tested to establish new minimum conditions for 100% loss of viability.

[00167] In other experiments, the sub-library is tested using one of the protocols described for Example 5, in order to identify improved sequences.

EXAMPLE 8: SECOND-GENERATION RANDOM shRNA-EXPRESSION LIBRARY

[00168] First, a second-generation random shRNA-expression library was constructed, with 3 million clones with stems of random sequence and further containing mismatches engineered

between the two halves of the stem. With the increased size of the second-generation library, and with the mismatches to increase potency, more potent initial hits are expected to be more readily identified. This hypothesis has been borne out by recent results in a IL3-withdrawal model system, where the initial hit sequences from the second-generation library were much more potent than the initial hit sequences from the first-generation 300,000-clone library.

[00169] Second, the identified small RNA (shRNA/siRNA) "book 2," not only protects primary Friedreich's ataxia (FA) fibroblasts, but also increases expression of the disease gene, frataxin. FA is unusual among genetic disorders in that most FA disease alleles have a GAA repeat expansion in the first intron, decreasing frataxin expression. The exonic sequences of frataxin are intact; thus, re-expression of the gene in affected cells could be curative. Severely affected individuals have mRNA and protein levels that are ~10% of normal, whereas mildly affected individuals have mRNA and protein levels that are ~20% of normal. Book 2 consistently increases frataxin expression more than two-fold.

EXAMPLE 9: HIT OPTIMIZATION OF BOOK 2

[00170] Book 2 is hit-optimized using the method of random mutagenesis and re-screening. Briefly, to randomly mutagenize book 2 and create a sub-library, 1-3 mutations are introduced per clone. The screening protocol used to identify book 2 is repeated. This protocol comprises packaging the sub-library as retroviruses, introducing the library into primary FA fibroblasts, using flow cytometry to select for cells that have taken up clones, and culturing the cells in medium with beta-hydroxybutyrate (BHB) in place of glucose (to stress mitochondria, which are impaired in FA). To enrich for true positives, the cells are cycled between BHB-based medium (stress) and glucose-based medium (recovery). Because primary FA fibroblasts are very slow growing (especially in BHB-based media), these cycles require several months. Also, because the cells are primary, they generally last about three enrichment cycles before undergoing senescence, necessitating an intermediate recovery (by PCR) of the remaining library clones, repackaging as retroviruses, and re-introduction into the cells for further cycling to enrich for true positives.

[00171] At the end of the cycling to enrich for true positives, individual candidate shRNAs are retrieved (by PCR), re-cloned, and sequenced. Those that are most abundant are tested individually, first by phenotypic effects on growth/survival of primary FA fibroblasts (using book 2 as a positive control and a random book as a negative control). Favorable clones are then tested in primary FA fibroblasts for their effects on frataxin mRNA expression (using quantitative RT-PCR). The most favorable clones are further tested for frataxin protein expression (using ELISA). Sub-clones of book 2 that increase frataxin expression to a higher level than book 2 are identified.

EXAMPLE 10: PHENOTYPIC SCREENING OF THE SECOND-GENERATION LIBRARY IN PRIMARY FA CELLS

[00172] As discussed above, the rationale for screening the second-generation library is based in part on the finding that in an IL3-withdrawal model initial hit sequences were more potent than those obtained with the first-generation library. The rationale for phenotypic screening is the success using this approach with the first-generation library to identify book 2. The screening protocol used to identify book 2 is used, with the exception that it is scaled up ten fold. The second-generation library, packaged as retroviruses, is introduced into primary FA fibroblasts, and cells that have taken up clones are selected for by flow cytometry. The cells are cultured in medium with BHB in place of glucose. To enrich for true positives, the cells are cycled between BHB-based medium and glucose-based medium, as described in Example 8 for sub-library testing.

[00173] At the end of the cycling to enrich for true positives, individual candidate shRNAs are retrieved (by PCR), re-cloned, and sequenced. Those that are most abundant are tested individually, first by phenotypic effects on growth/survival of primary FA fibroblasts (using book 2 as a positive control and a random book as a negative control). Favorable clones are then tested in primary FA fibroblasts for their effects on frataxin mRNA expression (using quantitative RT-PCR). The most favorable clones are hit-optimized by random mutagenesis and re-screening (Example 12).

EXAMPLE 11: REPORTER-BASED SCREENING OF THE SECOND-GENERATION LIBRARY

[00174] The rationale for reporter-based screening of the second-generation library is 1) the availability of a Green Fluorescence Protein (GFP) reporter construct for frataxin expression already integrated into an appropriate cell line, and 2) the goal of identifying shRNAs that increase frataxin expression. The second-generation library, packaged as retroviruses, is introduced into primary FA fibroblasts, and cells that have taken up clones are selected for by flow cytometry (using the red fluorophore, mCherry, which marks the library plasmid), and the cells are cultured in regular, glucose-based medium. After the cells recover, flow cytometry is used to sort for the highest expressers of the reporter construct (GFP), those cells are then returned to regular culture medium, are re-expanded, and the sorting is repeated for the highest GFP expressers. By cycling in this way, the true positives are enriched.

[00175] At the end of the cycling, individual candidate shRNAs are retrieved (by PCR), re-cloned, and sequenced. Those that are most abundant are tested individually, first by using the GFP reporter construct, then by quantitative RT-PCR in primary FA fibroblasts. The most favorable clones are hit-optimized by random mutagenesis and re-screening.

EXAMPLE 12: HIT-OPTIMIZATION OF shRNAs FROM SECOND-GENERATION-LIBRARY SCREENS

[00176] The hit books obtained in Example 10 and Example 11 are optimized using the method of random mutagenesis and re-screening. Briefly, to randomly mutagenize, 1-3 mutations per clone are introduced to create a sub-library. The screening protocol used to identify the clone is then repeated, either phenotypic screening or reporter-based screening. At the end of the cycling to enrich for true positives, individual candidate shRNAs are retrieved (by PCR), re-cloned, and sequenced. Those that are most abundant are tested individually, either by phenotypic effects on growth/survival of primary FA fibroblasts (books identified in Example 10), or by expression of the GFP reporter construct (books identified in Example 11). Favorable clones are then tested in primary FA fibroblasts for their effects on frataxin mRNA expression (using quantitative RT-PCR). The most favorable clones are further tested for frataxin protein expression (using ELISA). Sub-clones of books are identified in Example 11 and Example 12 that increase frataxin expression to a higher level than the initial hit books from which they are derived.

EXAMPLE 13: SCREENING OF THE SECOND-GENERATION LIBRARY IN MOUSE CELLS

[00177] An aspect of developing small-RNA therapeutics from our random shRNA-expression library, for any disease application, is to demonstrate translation of *in vitro* efficacy to *in vivo* efficacy. As transgenic mice with GAA repeat expansions in the frataxin gene have been engineered, it makes sense to these mice as a model to demonstrate translation of *in vitro* efficacy to *in vivo* efficacy. Both transgenic mice with GAA repeat expansions in the endogenous murine frataxin, as well as transgenic mice with GAA-repeat-expansion alleles of human frataxin knocked in (and both murine frataxin alleles knocked out) are available. (With the former, Western blot are used to confirm that increases in mRNA levels translate to increases in protein levels *in vivo*; with the latter, standard ELISA assays are used.) Several in vivo siRNA delivery technologies are commercially available, including Injectin (BioCellChallenge), AteloGene (Koken), and in vivo-jetPEI (Polyplus transfection). These products deliver siRNAs to various organs within mice, with varying efficiency.

[00178] However, prior to carrying out *in vivo* experiments, shRNAs that are most effective in murine cells are identified. Clones identified in Examples 9-12 are tested in mouse cell lines that have GAA repeat expansions in the murine frataxin gene, using quantitative RT-PCR to assess frataxin expression and clones that translate from human cells to murine cells are identified. Mouse cell lines that have GAA repeat expansions in the murine frataxin gene are also tested for conditions favorable for phenotypic screening, much as was done for primary human FA fibroblasts, and much as was done successfully for murine fibroblasts with disease-associated missense mutations in the murine frataxin gene.

[00179] After establishing appropriate conditions (likely with BHB-based medium), the second-generation library is packaged as retroviruses, is introduced into the mouse cells that have GAA repeat expansions in the murine frataxin gene, is selected by flow cytometry for cells that have taken up clones, and the cells are cultured in the conditions that have been pre-established. To enrich for true positives, the cells is cycled between stress medium and non-stress medium, as described above in the Examples 9-12.

[00180] At the end of the cycling to enrich for true positives, individual candidate shRNAs are retrieved (by PCR), re-cloned, and sequenced. Those that are most abundant are tested individually, first by phenotypic effects on growth/survival of the murine, GAA-repeat-expansion cells. Favorable clones are then tested in the same cells for their effects on murine frataxin mRNA expression (using quantitative RT-PCR). The most favorable clones are hit-optimized by random mutagenesis and re-screened as described above for clones identified in human cells (Example 9 and Example 12).

[00181] It will be appreciated by those skilled in the art that changes could be made to the embodiments described above without departing from the broad inventive concept thereof. It is understood, therefore, that this invention is not limited to the particular embodiments disclosed, but it is intended to cover modifications that are within the spirit and scope of the invention, as defined by the appended claims.

WHAT IS CLAIMED IS:

1. An RNA comprising a sense region and an antisense region which together form a duplex region, and wherein said RNA is capable of compensating for a frataxin deficiency or mutation.

2. The RNA of claim 1, wherein said duplex region is from 15 to 30 basepairs in length.

3. The RNA of claim 2, wherein said duplex region is from 17 to 23 basepairs in length.

4. The RNA of claim 1, wherein said RNA is an siRNA.

5. The RNA of claim 4, wherein said siRNA has a 5' end with a phosphate group and a 3' end with a hydroxyl group.

6. The RNA of claim 4, wherein said siRNA has at least one 2 nucleotide (nt) overhang at a 3' end.

7. The RNA of claim 6, wherein said siRNA has a 2 nt overhang at both 3' ends.

8. The RNA of claim 7, wherein said 2 nt overhangs are TT or UU.

9. The RNA of claim 1, wherein said molecule contains at least one modified oligonucleotide other than a ribonucleotide or a deoxyribonucleotide.

10. The RNA of claim 1, wherein said duplex region contains at least one mismatched base pair.

11. The RNA of claim 1, wherein said duplex region comprises a sequence that is at least 80% identical to a sequence of at least 19 contiguous nucleotides from the first, from the 5' end, 29 nucleotides of either SEQ ID No: 21 or SEQ ID No: 22.

12. The RNA of claim 11, wherein said duplex region comprises a sequence that is at least 90% identical to a sequence of at least 19 contiguous nucleotides from the first, from the 5' end, 29 nucleotides of either SEQ ID No: 21 or SEQ ID No: 22.

13. The RNA of claim 12, wherein said duplex region comprises a sequence that is identical to a sequence of at least 19 contiguous nucleotides from the first, from the 5' end, 29 nucleotides of either SEQ ID No: 21 or SEQ ID No: 22.

14. The RNA of any one of claims 11 to 13, wherein said duplex region comprises the sequence of nucleotides 2 thru 7 of SEQ ID No: 23.

15. The RNA of claim 1, wherein said RNA is an shRNA.

16. The RNA of claim 15, wherein said shRNA has a loop of 3-20 nt connecting said sense and antisense regions.

17. The RNA of claim 15, wherein said shRNA has a 2 nt overhang at its 3' end.

18. The RNA of claim 17, wherein said 2 nt overhang is TT or UU.

19. The RNA of claim 15, wherein said molecule contains at least one modified oligonucleotide other than a ribonucleotide or a deoxyribonucleotide.

20. The RNA of claim 15, wherein said duplex region contains at least one mismatched base pair.

21. The RNA of claim 15, wherein said duplex region comprises a sequence that is at least 80% identical to a sequence of at least 19 contiguous nucleotides from the first, from the 5' end, 29 nucleotides of either SEQ ID No: 21 or SEQ ID No: 22.

22. The RNA of claim 21, wherein said duplex region comprises a sequence that is at least 90% identical to a sequence of at least 19 contiguous nucleotides from the first, from the 5' end, 29 nucleotides of either SEQ ID No: 21 or SEQ ID No: 22.

23. The RNA of claim 22, wherein said duplex region comprises a sequence that is identical to a sequence of at least 19 contiguous nucleotides from the first, from the 5' end, 29 nucleotides of either SEQ ID No: 21 or SEQ ID No: 22.

24. The RNA of any one of claims 21 to 23, wherein said duplex region comprises the sequence of nucleotides 2 thru 7 of SEQ ID No: 23.

25. A pharmaceutical composition comprising an RNA according to any one of claims 1-13 or 15-23 and at least one pharmaceutically acceptable excipient.

26. The pharmaceutical composition of claim 25, wherein said duplex region comprises the sequence of nucleotides 2 thru 7 of SEQ ID No: 23.

27. An expression cassette for an RNA comprising a DNA that encodes an RNA comprising the sense region, the antisense region or both according to any one of claims 1-13 or 15-23, that is operably linked to an inducible or constitutive promoter and to a transcription terminator.

28. The expression cassette of claim 27, wherein said duplex region comprises the sequence of nucleotides 2 thru 7 of SEQ ID No: 23.

29. The expression cassette of claim 27, wherein said DNA encodes an shRNA.

30. An expression vector comprising the expression cassette according to claim 27.

31. The expression vector of claim 30, wherein said vector is a recombinant plasmid.

32. The expression vector of claim 30, wherein said vector is packaged in a virus.

33. The expression vector of claim 30, wherein said vector is an integrating vector.

34. The expression vector of claim 30, wherein said vector further comprises a gene encoding a marker.

35. A host cell transformed or transfected with the expression vector according to any one of claims 30-34.

36. A method for treating a subject at risk for developing Friedreich's ataxia or suffering from Friedreich's ataxia or reducing the symptoms thereof comprising administering to the

subject a therapeutically effective amount of the pharmaceutical composition of claim 25.

37. The method of claim 36, wherein said duplex region comprises the sequence of nucleotides 2 thru 7 of SEQ ID No: 23.

38. The method according to claim 36, wherein the subject is a human.

39. The method according to claim 36, further comprising the step of administering an additional agent for treating said subject.

40. A method for treating a subject at risk for developing Friedreich's ataxia or suffering from Friedreich's ataxia or reducing the symptoms thereof comprising administering to the subject a therapeutically effective amount of the expression vector according to any one of claims 30-34.

41. The method according to claim 40, wherein the subject is a human.

42. The method according to claim 40, further comprising the step of administering an additional agent for treating said subject.

43. A method for compensating for a frataxin deficiency or mutation in a subject comprising administering to the subject a therapeutically effective amount of the pharmaceutical composition of claim 25.

44. The method according to claim 43, wherein the subject is a human.

45. A method for compensating for a frataxin deficiency or mutation in a subject comprising administering to the subject a therapeutically effective amount of the expression vector according to any one of claims 30-34.

46. The method according to claim 45, wherein the subject is a human.

47. A method for compensating for a frataxin deficiency or mutation in a cell comprising administering to the cell an effective amount of the RNA according to any one of claims

1-13 or 15-23 or a pharmaceutical composition thereof.

48. The method of claim 47, wherein said duplex region comprises the sequence of nucleotides 2 thru 7 of SEQ ID No: 23.

49. The method according to claim 47, wherein administering to the cell comprises administering to the cell *in vitro*.

50. The method according to claim 47, wherein administering to the cell comprises administering to the cell *in vivo*.

51. The method according to claim 47, wherein administering to the cell comprises administering to the cell *ex vivo*.

52. A method for compensating for a frataxin deficiency or mutation in a cell comprising administering to the cell an effective amount of the expression vector according to any one of claims 30-34.

53. The method according to claim 52, wherein administering to the cell comprises administering to the cell *in vitro*.

54. The method according to claim 52, wherein administering to the cell comprises administering to the cell *in vivo*.

55. The method according to claim 52, wherein administering to the cell comprises administering to the cell *ex vivo*.

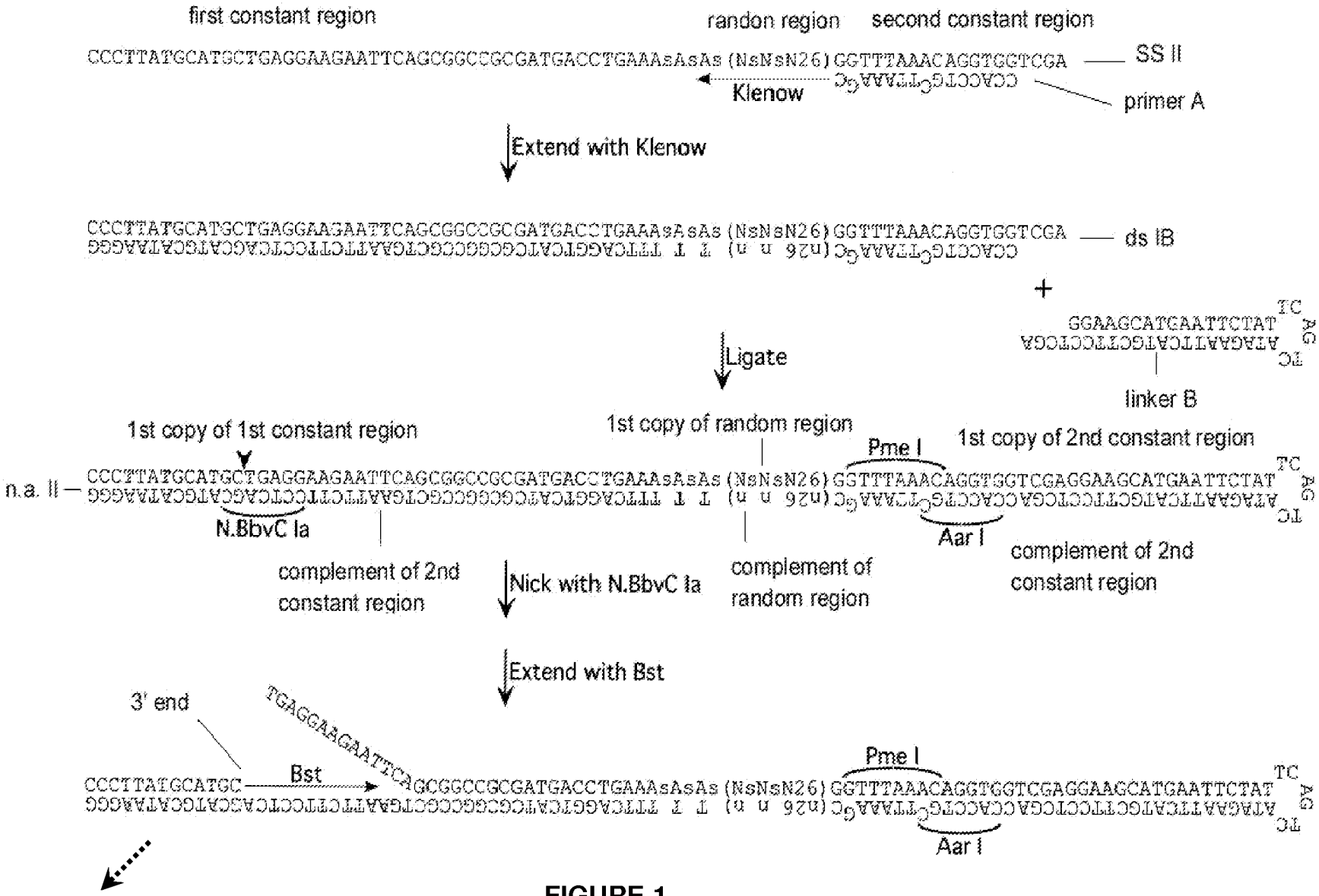


FIGURE 1

Continued on Figure 2

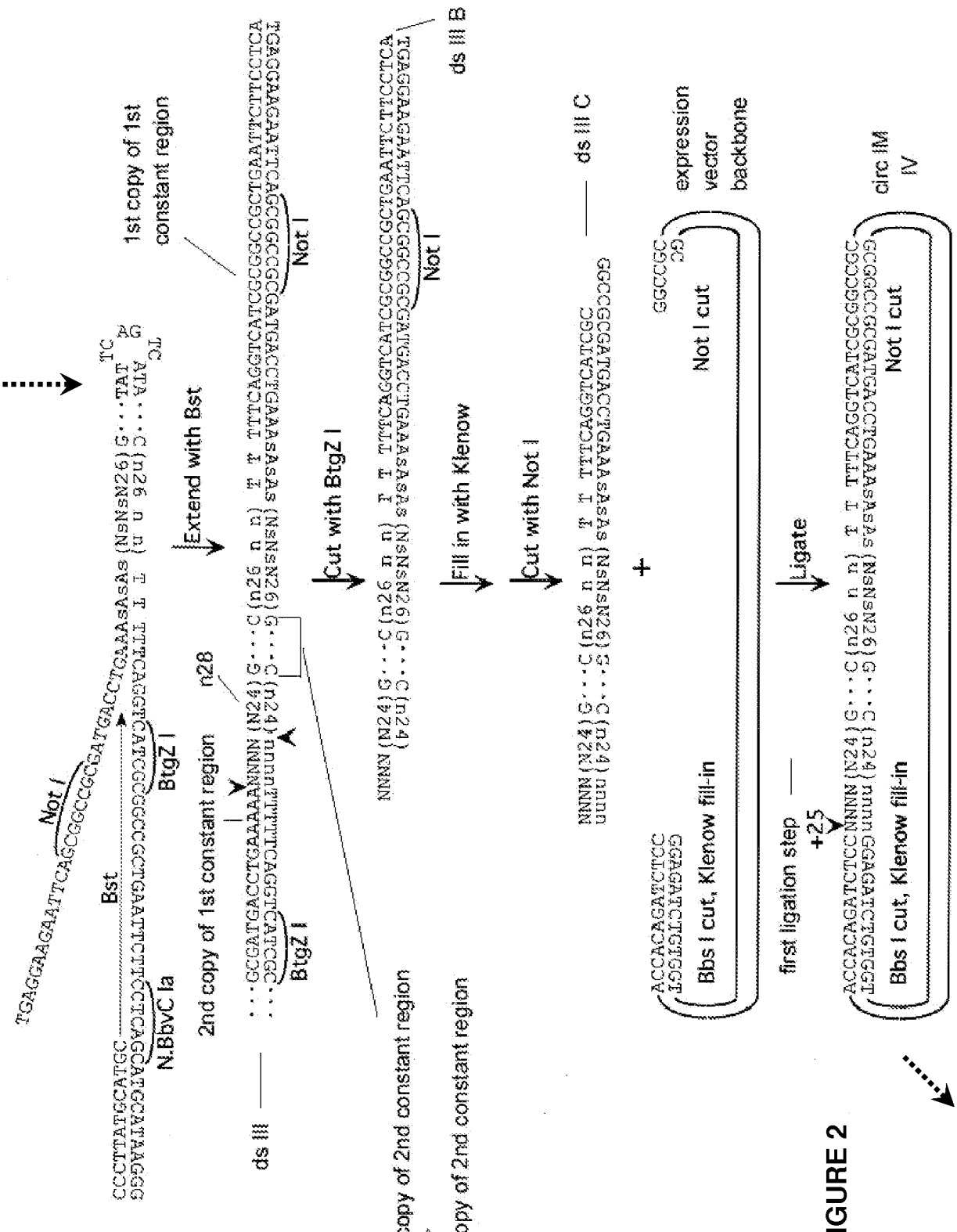

Continued from Figure 1
Not I
Bst
N.BbvC Ia
BtgZ I
1st copy of 1st constant region
2nd copy of 1st constant region
n28
Extend with Bst
Not I
Cut with BtgZ I
BtgZ I
ds III
- 2nd copy of 2nd constant region
- linker
- 1st copy of 2nd constant region
Fill in with Klenow
Not I
ds III B
Cut with Not I
ds III C
+
expression vector backbone
Not I cut
Bbs I cut, Klenow fill-in
first ligation step
Ligate
+25
circ IM IV
Bbs I cut, Klenow fill-in
Not I cut
Continued on Figure 3

FIGURE 2

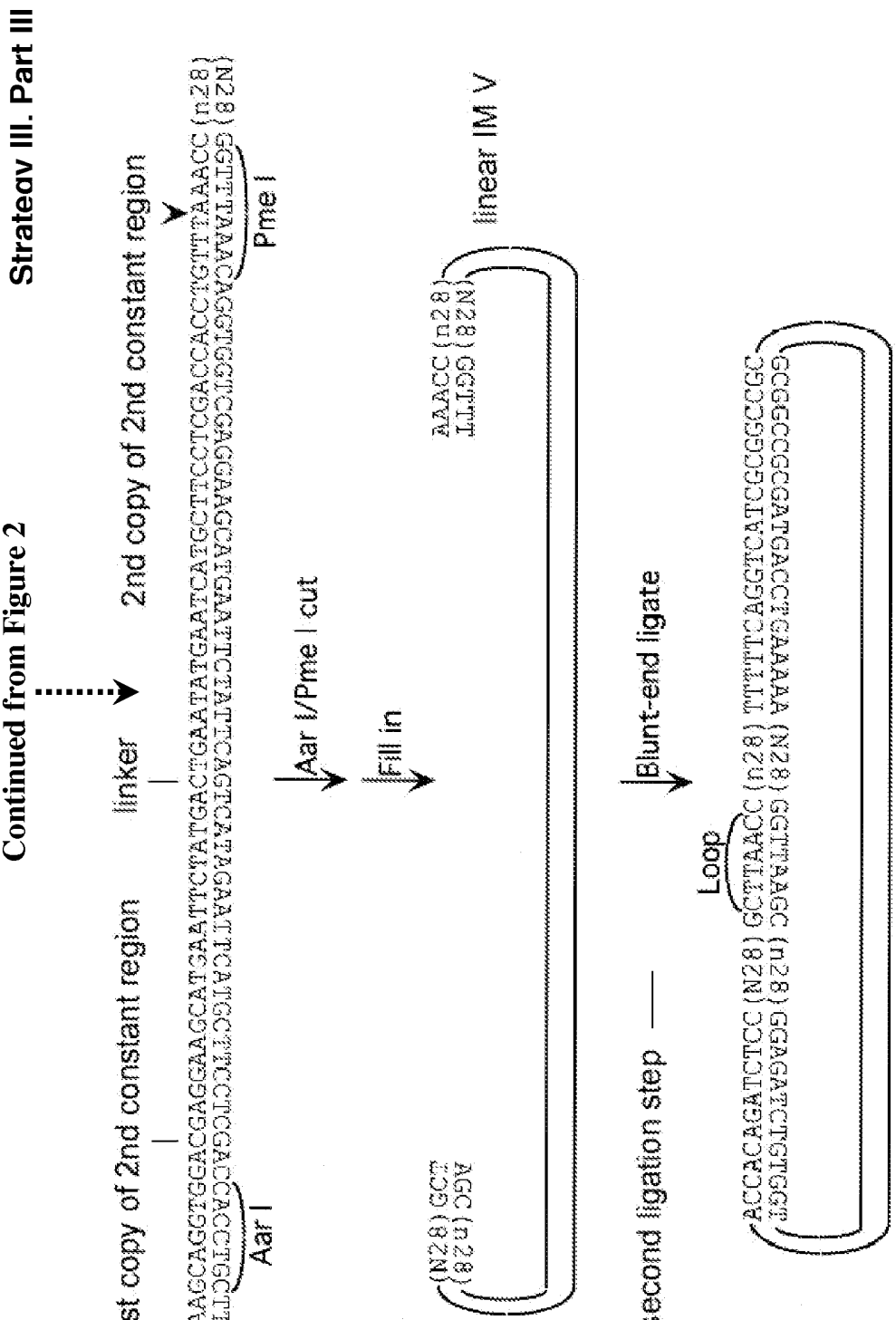

Strategy III. Part III
Continued from Figure 2
linker
2nd copy of 2nd constant region
1st copy of 2nd constant region
insert
Aar I
Pme I
Aar I/Pme I cut
Fill in
linear IM V
second ligation step
Blunt-end ligate
Loop

FIGURE 3

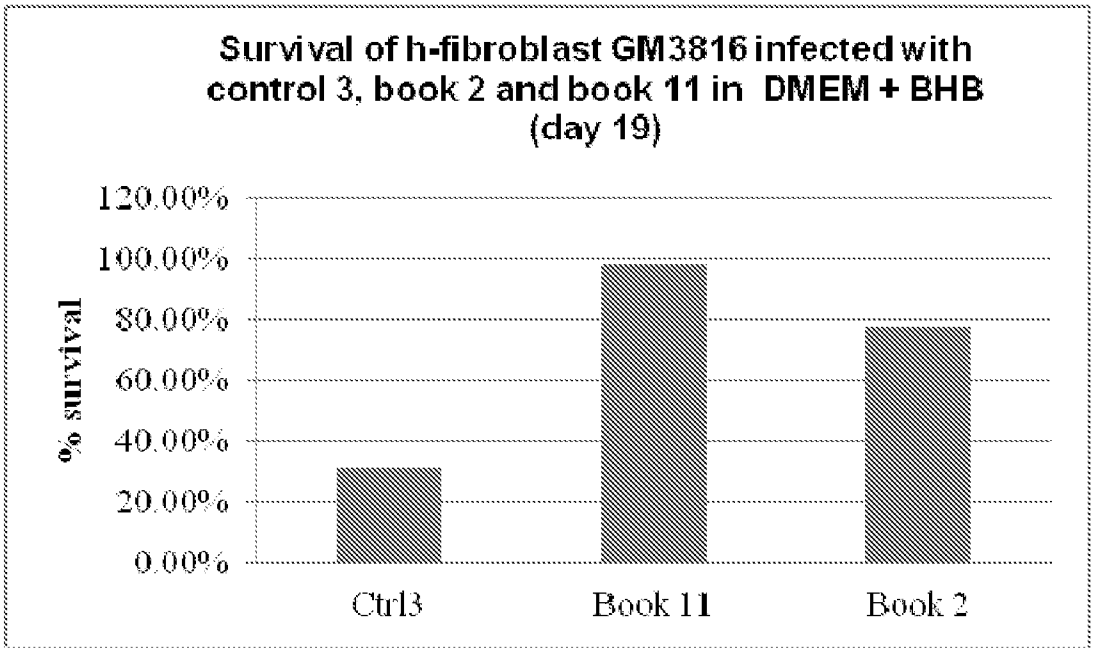

Survival of h-fibroblast GM3816 infected with control 3, book 2 and book 11 in DMEM + BHB (day 19)
% survival
120.00%
100.00%
80.00%
60.00%
40.00%
20.00%
0.00%
Ctrl3
Book 11
Book 2

Figure 4

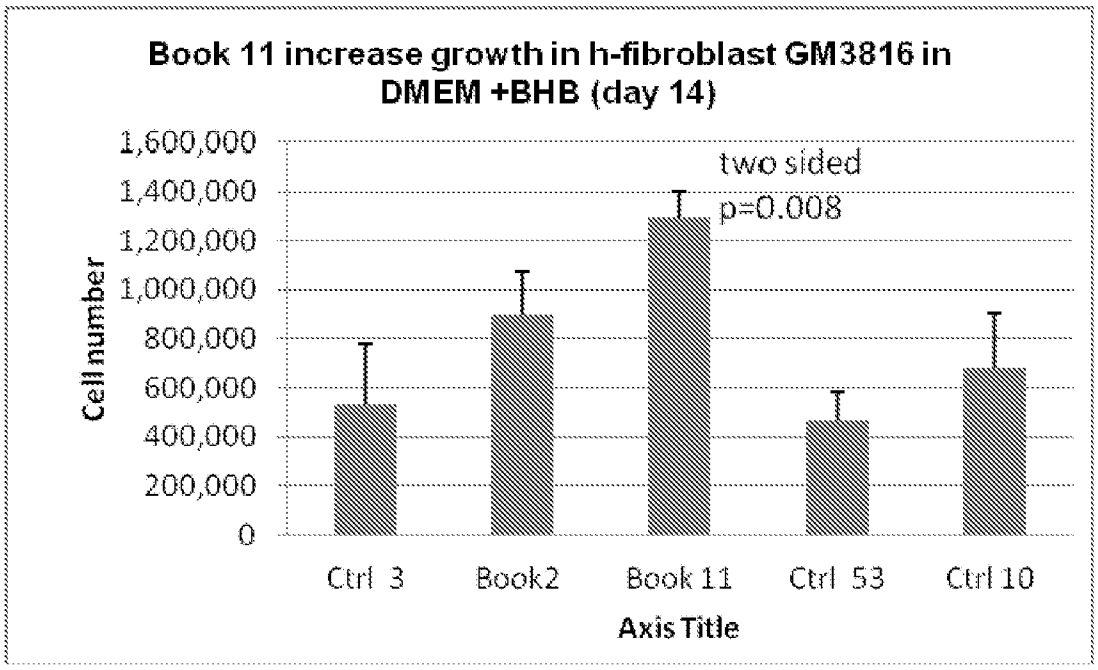

Book 11 increase growth in h-fibroblast GM3816 in DMEM +BHB (day 14)
1,600,000
1,400,000
1,200,000
1,000,000
800,000
600,000
400,000
200,000
0
Cell number
two sided
p=0.008
Ctrl 3
Book2
Book 11
Ctrl 53
Ctrl 10
Axis Title

Figure 5

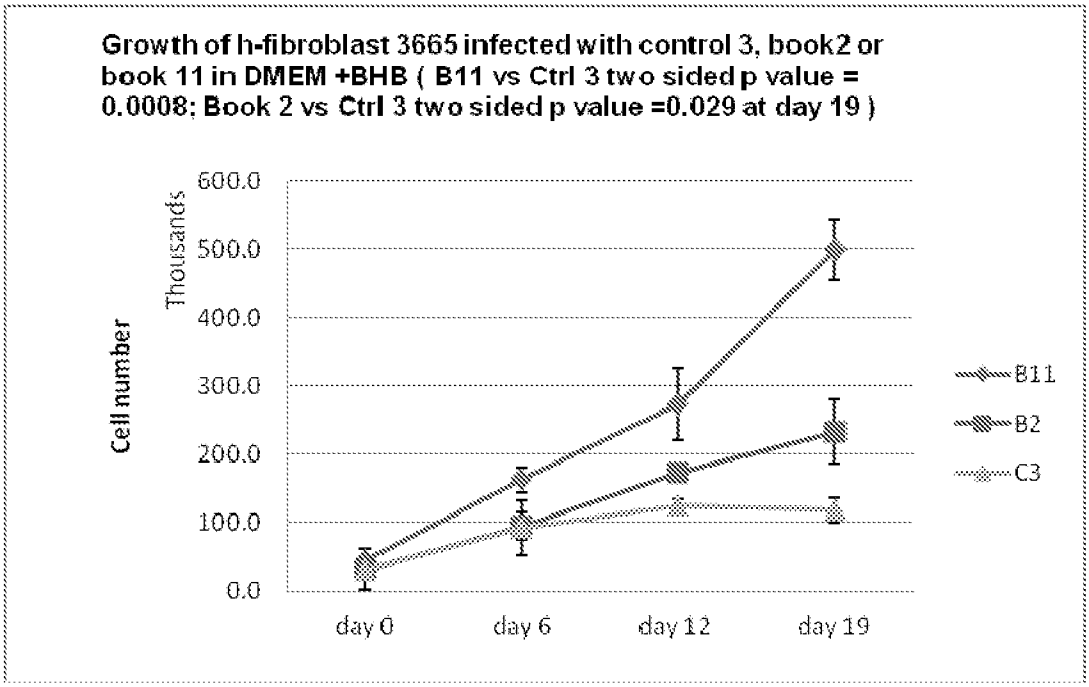

Growth of h-fibroblast 3665 infected with control 3, book2 or book 11 in DMEM +BHB (B11 vs Ctrl 3 two sided p value = 0.0008; Book 2 vs Ctrl 3 two sided p value =0.029 at day 19)
Cell number
Thousands
600.0
500.0
400.0
300.0
200.0
100.0
0.0
day 0
day 6
day 12
day 19
B11
B2
C3

Figure 6

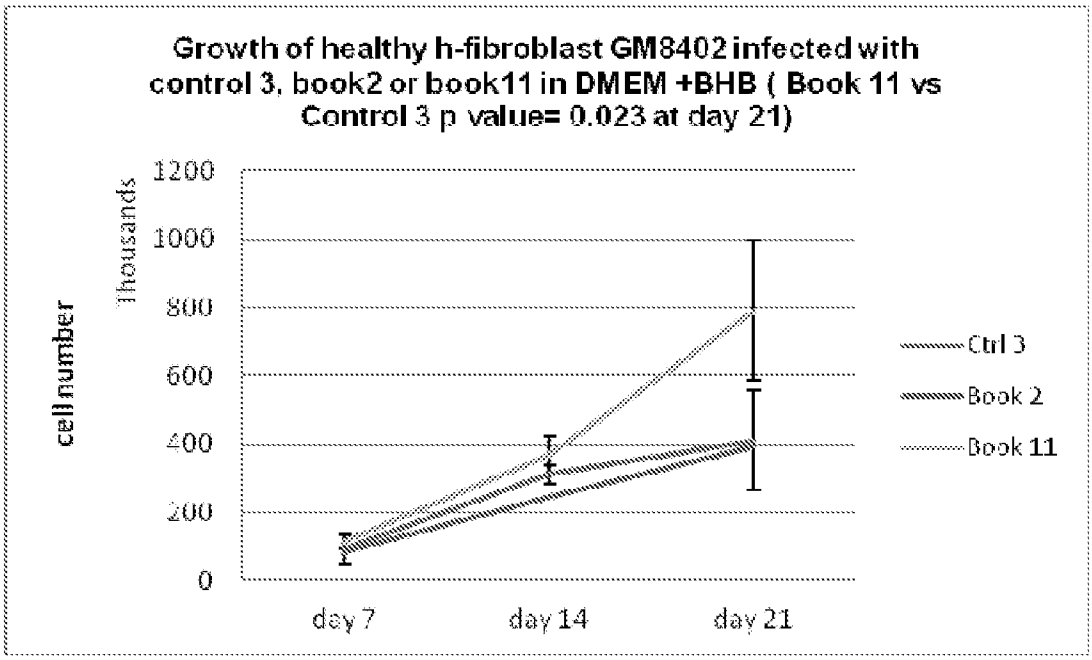

Growth of healthy h-fibroblast GM8402 infected with control 3, book2 or book11 in DMEM +BHB (Book 11 vs Control 3 p value= 0.023 at day 21)
cell number
thousands
1200
1000
800
600
400
200
0
day 7
day 14
day 21
Ctrl 3
Book 2
Book 11

Figure 7

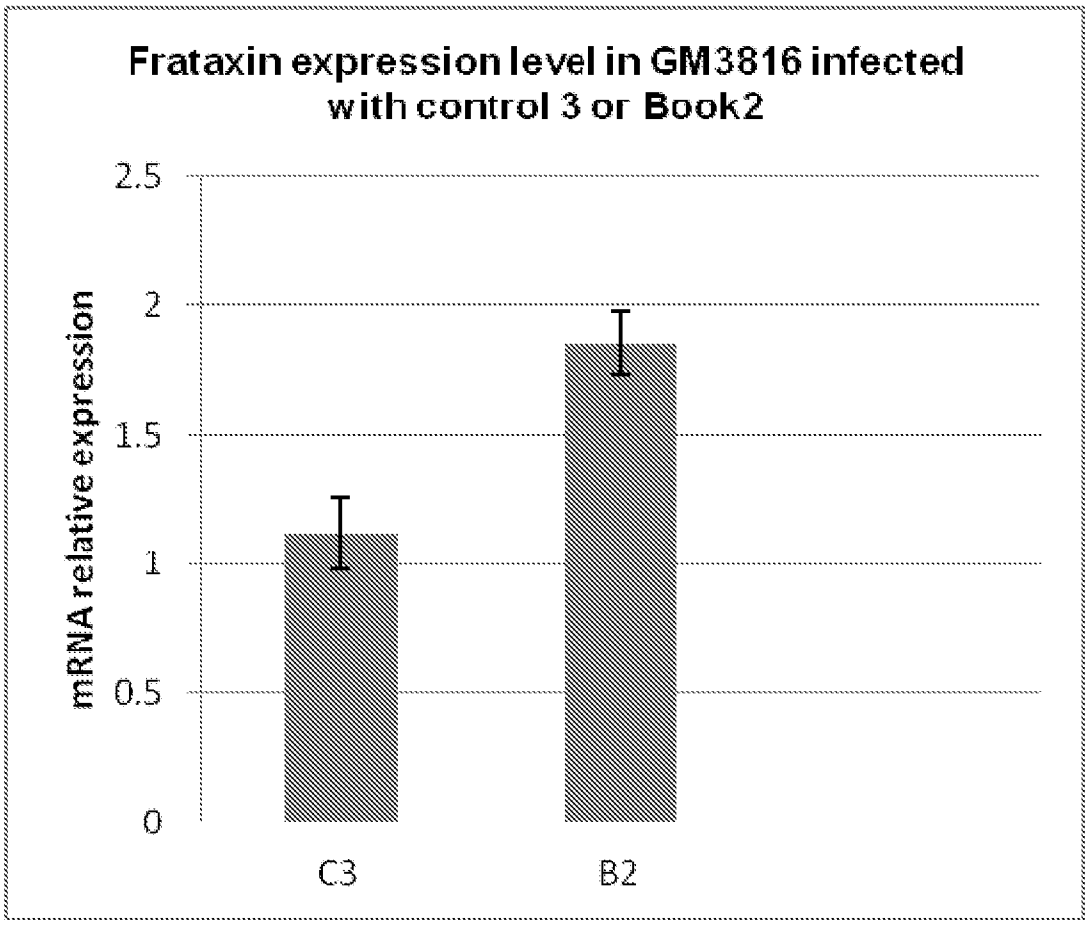

Frataxin expression level in GM3816 infected with control 3 or Book2
mRNA relative expression
2.5
2
1.5
1
0.5
0
C3
B2

Figure 8

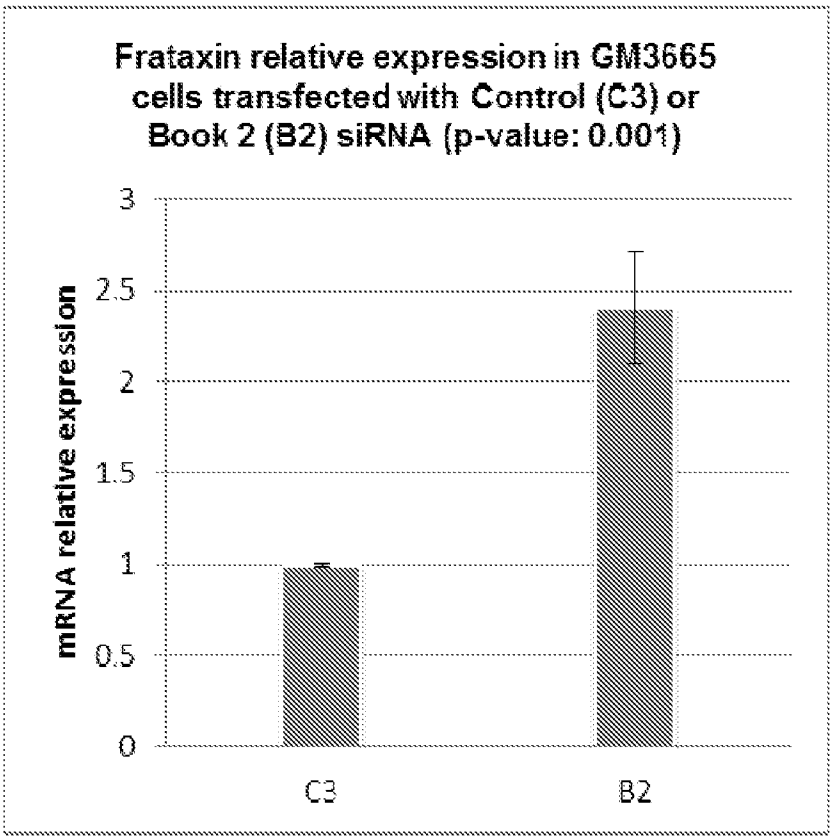

Frataxin relative expression in GM3665 cells transfected with Control (C3) or Book 2 (B2) siRNA (p-value: 0.001)
mRNA relative expression
3
2.5
2
1.5
1
0.5
0
C3
B2

Figure 9

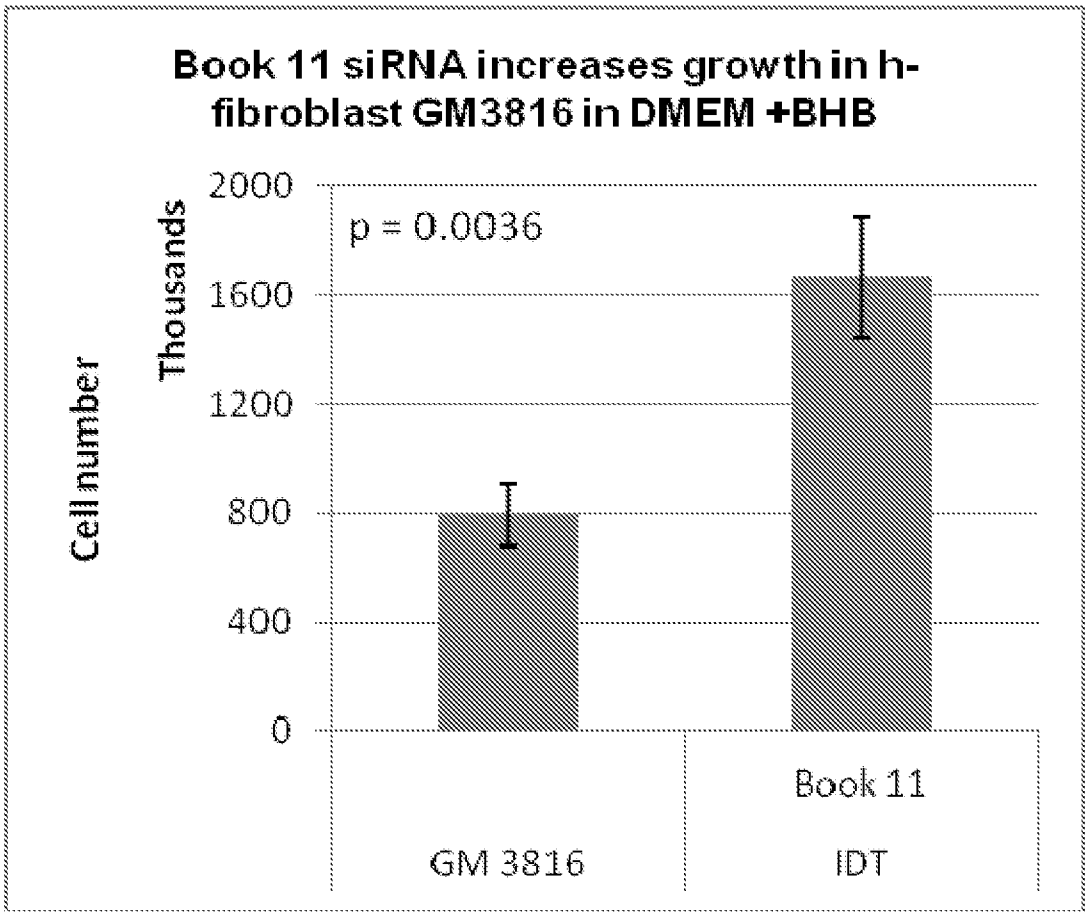

Book 11 siRNA increases growth in h-fibroblast GM3816 in DMEM +BHB
Cell number
Thousands
2000
1600
1200
800
400
0
p = 0.0036
GM 3816
Book 11
IDT

Figure 10

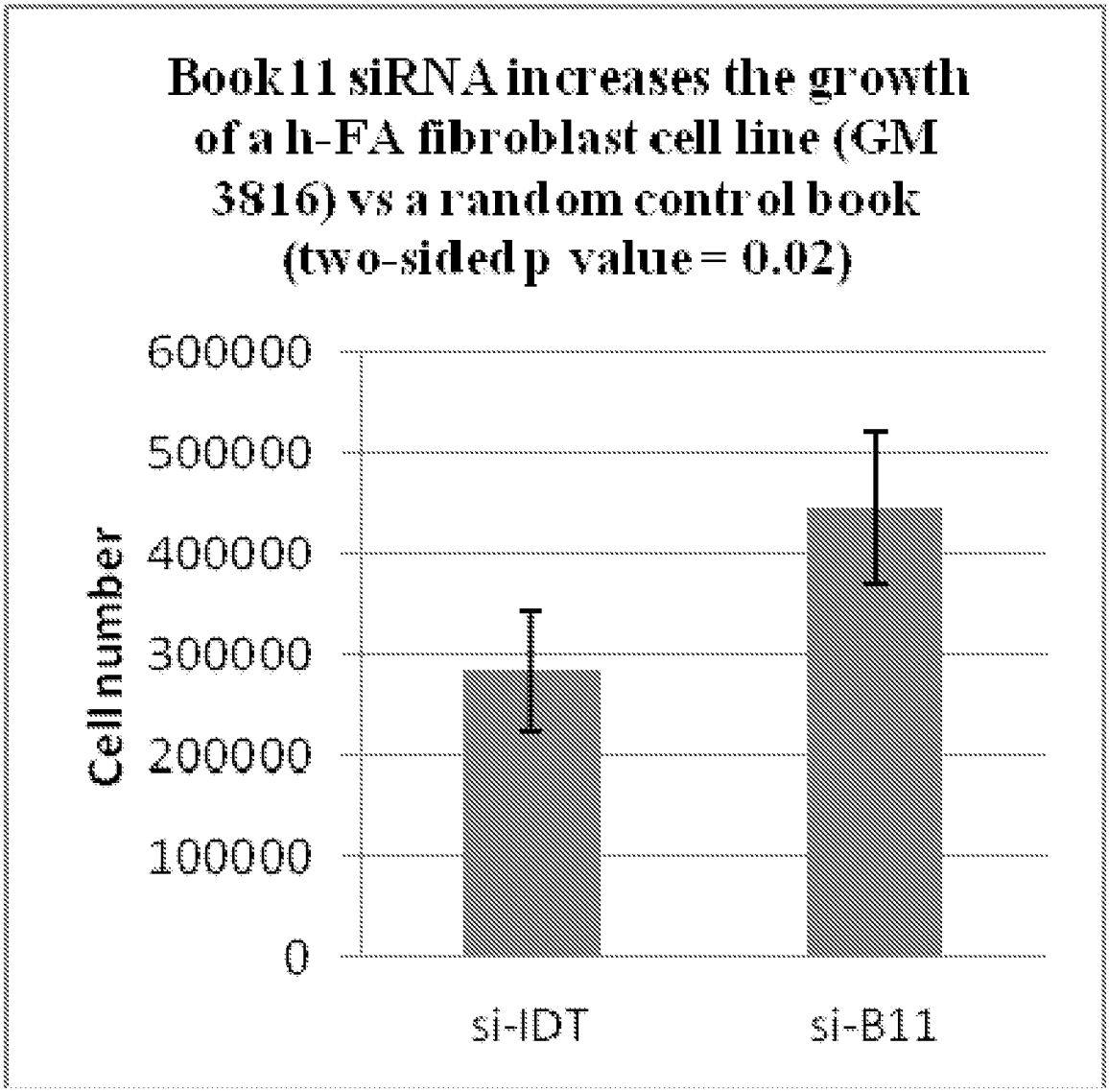

Book11 siRNA increases the growth of a h-FA fibroblast cell line (GM 3816) vs a random control book (two-sided p value = 0.02)
Cell number
600000
500000
400000
300000
200000
100000
0
si-IDT
si-B11

Figure 11

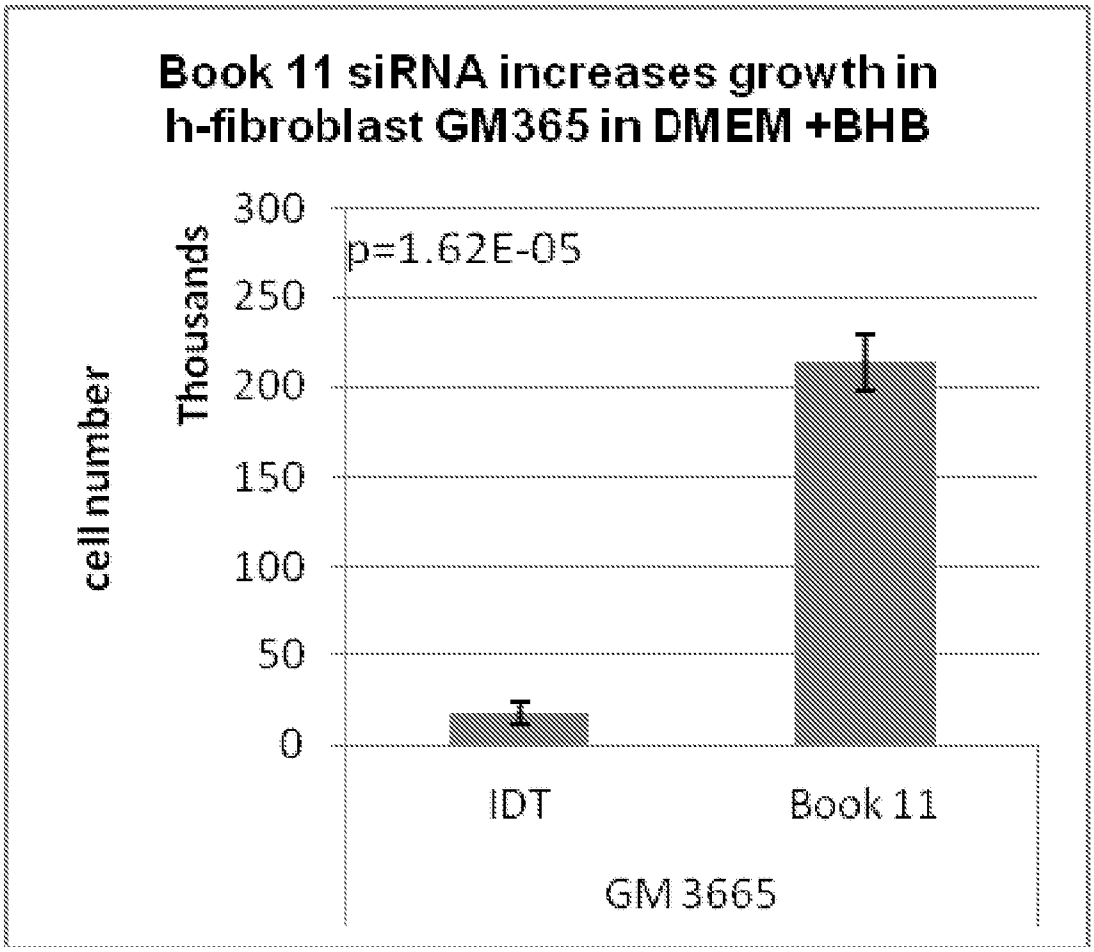

Book 11 siRNA increases growth in h-fibroblast GM365 in DMEM +BHB
cell number
Thousands
300
250
200
150
100
50
0
p=1.62E-05
IDT
Book 11
GM 3665

Figure 12

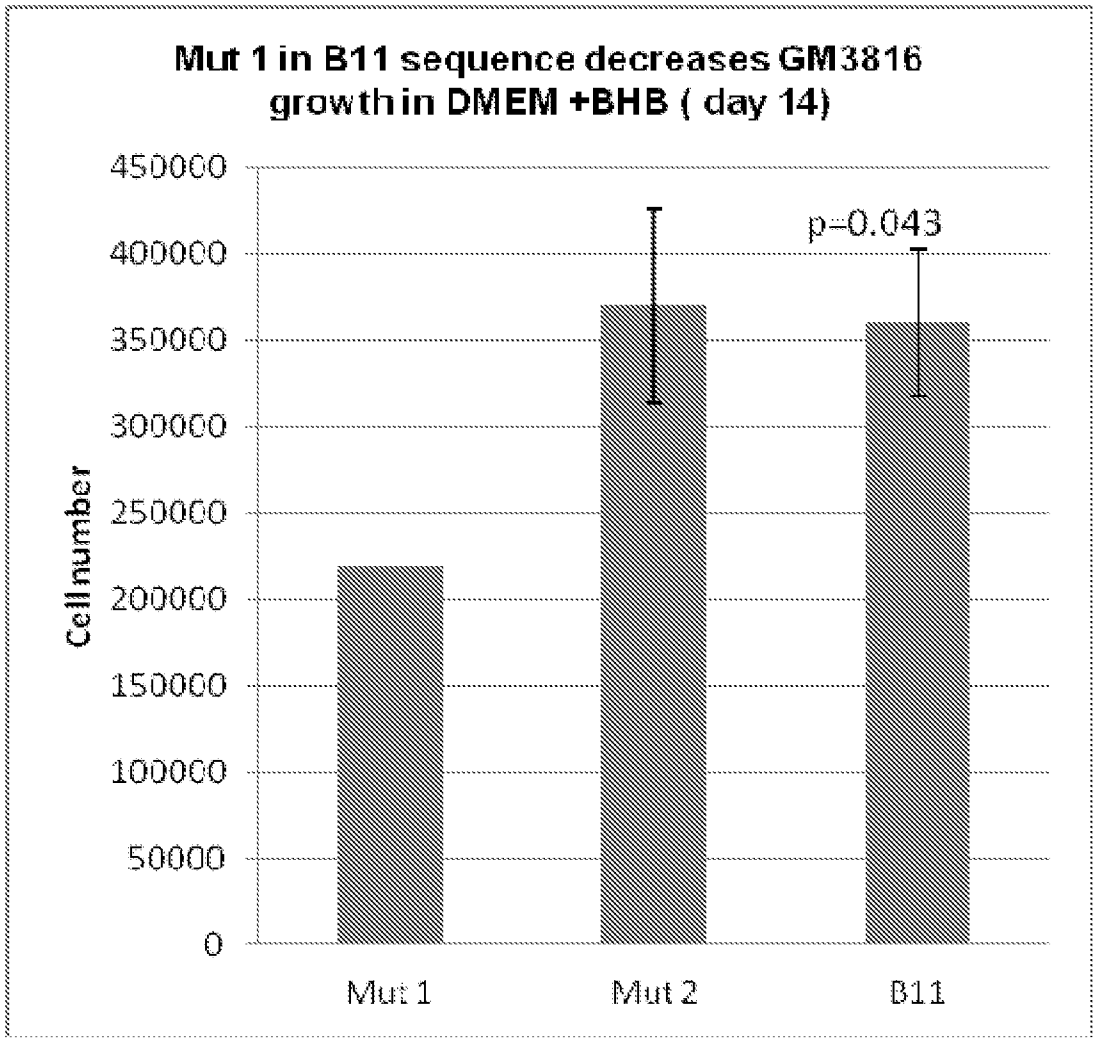
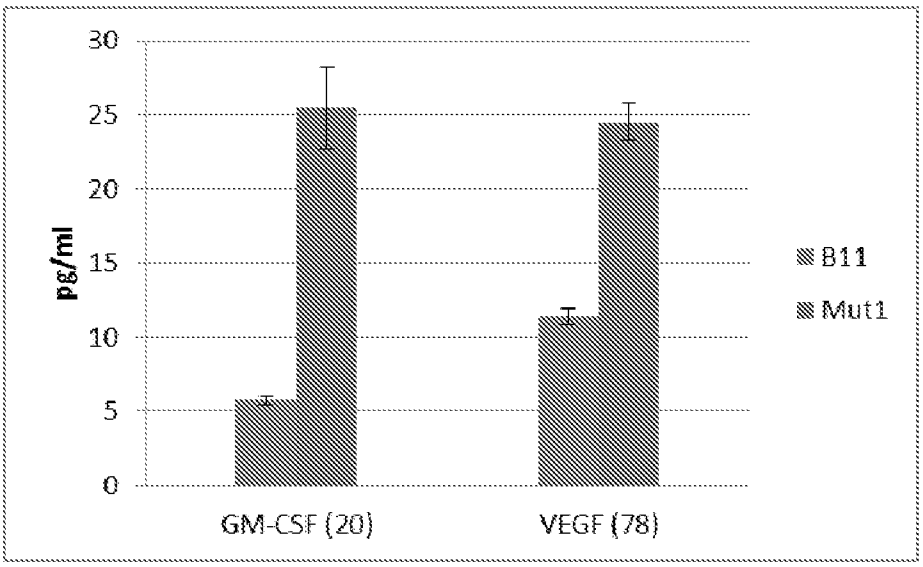

Mut 1 in B11 sequence decreases GM3816 growth in DMEM +BHB (day 14)
Cell number
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
p=0.043
Mut 1
Mut 2
B11

Figure 13

A


30
25
20
15
10
5
0
pg/ml
B11
Mut1
GM-CSF (20)
VEGF (78)

B

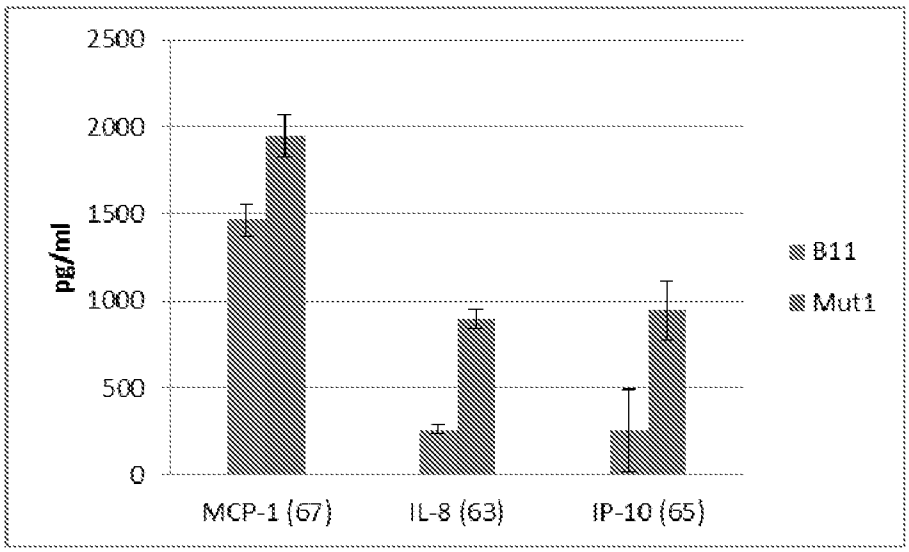

2500
2000
1500
1000
500
0
pg/ml
B11
Mut1
MCP-1 (67)
IL-8 (63)
IP-10 (65)

Figure 14