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(54) Title: METHODS OF TREATING AMYOTROPHIC LATERAL SCLEROSES

(57) Abstract: Provided are methods and pharmaceutical compositions for treating Amyotrophic lateral scleroses (ALS) in a human subject in need thereof, by administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity or expression level of miR-9, thereby treating the ALS in the human subject in need thereof.

METHODS OF TREATING AMYOTROPHIC LATERAL SCLEROSES

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to a method of treating amyotrophic lateral scleroses by downregulation the expression level and/or activity of miR-9.

Amyotrophic lateral scleroses (ALS) is a progressive neurodegenerative disorder, characterized by the loss of upper and lower motor neurons, however, the pathogenesis and molecular mechanisms underlying ALS remain unknown and accordingly is no effective treatment. ALS patients rapidly deteriorate and have poor prognosis.

Pioneering studies from recent years discovered ALS-causing mutations in several genes including SOD1 (superoxide dismutase 1), TDP-43 (TAR DNA binding protein, also known as "TARDBP"), FUS (also known as "FUS/TLS", fused in sarcoma/translocated in liposarcoma), hnRNPAl (heterogeneous nuclear ribonucleoprotein Al) and C90RF72 (chromosome 9 open reading frame 72). Many of these genes encode for RNA-binding proteins, thus raising the hypothesis that dysregulation of RNA activity is involved in the pathogenesis of ALS.

microRNAs (miRNAs) are small non-coding RNAs that silence gene expression post transcriptionally, in a sequence-dependent manner. miRNAs emerge as major regulators in a wide spectrum of neuronal processes, and their involvement in neurodegeneration was suggested. Thus, loss of DICER was reported to cause progressive degeneration of spinal motor neurons (Haramati et al, 2010) and global downregulation of miRNAs was common to genetically unrelated forms of human ALS primarily due to failed Dicer complex activity in motor neurons. In addition, specific miRNA genes were found to be involved in feedback loops upstream or downstream of FUS [Morlando M, et al. 2012. EMBO J 31: 4502-4510; Dini Modigliani S., et al, 2014, Nature communications 5: 4335] or TDP-43 [Buratti E., et al, 2010. FEBS J 277: 2268-2281; Kawahara & Mieda-Sato, 2012. Proc Natl Acad Sci U.S.A 109: 3347-3352]. miRNAs were suggested to regulate neuromuscular junction repair (Williams AH, et al. 2009. Science 326: 1549-1554; Valdez G., et al, 2014. PLoS One 9: e93140) and to plausibly regulate neuro-inflammation in ALS (Koval ED, et al., 2013. Hum Mol Genet 22: 4127-4135) and to serve as circulating biomarkers [De Felice B, 2014.

Neurogenetics 15: 243-253; Droppelmann CA, et al. Amyotroph Lateral Scler Frontotemporal Degener 15: 321-336; Gascon E, et al. 2014. Nature medicine 20: 1444-1451].

MiR-9 (miRNA-9) is conserved from fly to man and is specifically expressed in the brain. miR-9 was previously suggested to play important roles in brain development, post-mitotic neural development and neurite morphogenesis. Previous work demonstrated that miRNAs are essential for the motor neuron function and survival in general and the centrality of neuro-specific miR-9 in particular. MiR-9 was found to be the most abundant miRNA in embryonic motor neurons [Haramati, S., et al., 2010] and it is dysregulation in several neurodegenerative disorders such as Alzheimer's disease [Cogswell, J.P., et al., 2008]. Furthermore, miR-9 has an essential role in determining motor neuron subtypes and in the organization of the spinal motor columns [Otaegi, G., et al. 2011]. In addition, miR-9 was recently found to be upregulated in the spinal cord of ALS mice (SOD1G93A; Zhou et al., Int J Clin Exp Pathol. 2013; and Marcuzzo S. et al., 2015. Mol Brain. 2015 Jan 28; 8:5. doi: 10.1186/sl3041-015-0095-0) and to promote microglial activation by targeting MCPIP1 (zinc finger CCCH-type containing 12A, also known as "ZC3H12A"; Yao H. et al., 2014. Nat. Commun. 5: 4386).

U.S. Patent No. 8,734,759 to Hornstein et al. teaches methods of diagnosing and treating motor neuron diseases.

WO 2013007874 A1 and U.S. Patent Application No. 20140259192 to Saarma Mart et al. disclose transgenic animal comprising a deletion or functional deletion of the 3'UTR of an endogenous gene.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a human subject in need thereof, the method comprising administering to the human subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression level of miR-9, thereby treating the ALS in the human subject in need thereof.

According to an aspect of some embodiments of the present invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a subject in need

thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression level of miR-9, wherein the ALS is caused by a mutation in the SOD1 gene set forth by SEQ ID NO:2, thereby treating the ALS in the subject in need thereof.

According to an aspect of some embodiments of the present invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression level of miR-9, wherein the ALS is caused by the G94A missense mutation in the SOD1 polypeptide set forth by SEQ ID NO:l, thereby treating the ALS in the subject in need thereof.

According to an aspect of some embodiments of the present invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression level of miR-9, wherein the agent is comprised in a viral vector, thereby treating the ALS in the subject in need thereof.

According to an aspect of some embodiments of the present invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression level of miR-9, wherein the active ingredient does not comprise an agent capable of downregulating a miR selected from the group consisting of: miR133a, miR133b, miR96, miR146, miR30-2, miR125b, miR125a, miR125a-5p, miR125b-5p, miR30a, miR30b, miR146 and miR146a, thereby treating the ALS in the subject in need thereof.

According to an aspect of some embodiments of the present invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression level of miR-9, wherein the pharmaceutical composition is

administered to the subject by intracerebroventricular administration (ICV), thereby treating the ALS in the subject in need thereof.

According to an aspect of some embodiments of the present invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression level of miR-9, wherein the pharmaceutical composition is administered to the subject by intrathecal administration, thereby treating the ALS in the subject in need thereof.

According to an aspect of some embodiments of the present invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression level of miR-9, wherein the pharmaceutical composition is administered to the subject by intracranial (IC) administration, thereby treating the ALS in the subject in need thereof.

According to some embodiments of the invention, the pharmaceutical composition is administered to the central nervous system (CNS) of the subject.

According to some embodiments of the invention, the pharmaceutical composition further comprises a moiety capable of directing the agent capable of downregulating the activity and/or the expression level of the miR-9 to the central nervous system of the subject.

According to some embodiments of the invention, the ALS is caused by a mutation in the SOD1 gene set forth by SEQ ID NO:2.

According to some embodiments of the invention, the mutation is the G94A missense mutation in the SOD1 polypeptide set forth by SEQ ID NO:l.

According to some embodiments of the invention, with the proviso that the active ingredient does not comprise an agent capable of downregulating a miR selected from the group consisting of: miR133a, miR133b, miR96, miR146, miR30-2, miR125b, miR125a, miR125a-5p, miR125b-5p, miR30a, miR30b, miR146 and miR146a.

According to some embodiments of the invention, the miR-9 is depicted in SEQ ID NO:3.

According to some embodiments of the invention, the agent capable of downregulating the activity and/or the expression level of the miR-9 comprises an antisense sequence of the miR-9.

According to some embodiments of the invention, the agent is an antisense oligonucleotide.

According to some embodiments of the invention, the antisense sequence is set forth by SEQ ID NO:4 or 14.

According to some embodiments of the invention, the antisense sequence is set forth by SEQ ID NO:4.

According to some embodiments of the invention, the antisense sequence is set forth by SEQ ID NO: 14.

According to some embodiments of the invention, the agent is comprised in a viral vector.

According to some embodiments of the invention, the viral vector is a recombinant adeno-associated virus pseudotype-9 (rAAV9) expression vector.

According to some embodiments of the invention, the pharmaceutical composition is administered to the subject by intracerebroventricular (ICV) administration.

According to some embodiments of the invention, the pharmaceutical composition is administered to the subject by intrathecal administration.

According to some embodiments of the invention, the pharmaceutical composition is administered to the subject by intracranial (IC) administration.

According to some embodiments of the invention, the subject is diagnosed with the ALS.

According to some embodiments of the invention, the subject is a carrier of the SODl G94A missense mutation as set forth by SEQ ID NO:l.

According to some embodiments of the invention, the method further comprising selecting a subject which is a carrier of the SODl G94A missense mutation as set forth by SEQ ID NO:l, wherein presence of at least one allele of the SODl 94A variant as set forth by SEQ ID NO:l indicates that the active ingredient is suitable for treating the subject.

According to some embodiments of the invention, administering is effected prior

to appearance of at least one ALS symptom, wherein the at least one of ALS symptom is selected from the group consisting of: difficulty walking, weakness in leg(s), weakness in hand(s), slurring of speech, trouble swallowing, muscle cramps, and twitching in arms, shoulders or tongue.

According to some embodiments of the invention, administering is effected concomitantly or after appearance of at least one ALS symptom selected from the group consisting of: difficulty walking, weakness in leg(s), weakness in hand(s), slurring of speech, trouble swallowing, muscle cramps, and twitching in arms, shoulders or tongue.

According to some embodiments of the invention, the treating comprises improvement of at least one ALS symptom selected from the group consisting of: difficulty walking, weakness in leg(s), and weakness in hand(s) as compared to prior to treating.

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

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

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

In the drawings:

FIG. 1 is a histogram depicting miRNA-9 expression in the lumbar spinal cord of SOD1G93A and of control wild type (WT) littermates mice (n=3 per group). Results of qPCR (quantitative Polymerase Chain Reaction) data were normalized to the expression levels of the same genes in control littermate mice at postnatal day 60 (P60)

and normalized to the reference noncoding RNA U6. * - p-value<0.05. Results of qPCR showed that miR-9 expression levels in WT mice is stable during the adult stage in contrast to SODl G93A mice in which miR-9 was upregulated during the disease progression.

FIGs. 2A-G demonstrate manipulation of miRNA expression within motoneurons. To modify miR-9 activity *in vivo* the present inventors utilized recombinant adeno-associated virus pseudotype 9 (rAAV9), which effectively transduces motor neurons after stereotactic intracerebroventricular (ICV) injection and drives the expression of miR-9 or of a tough decoy (TuD) vector that effectively downregulated miR-9 activity. Figure 2A - A schematic diagram depicting ICV injection of rAAV9 that drives the expression of GFP (green fluorescent protein) or of vectors for miR-9 overexpression or knockdown. Figures 2B-D - Fluorescent microscopy images depicting detection of GFP signals in the lumbar spinal cord eight weeks after ICV injection of adult mice with mock vector (Figure 2B) or with GFP-expressing virus (Figures 2C and 2D). The central canal is marked in hemisection by an arrow (Figures 2B and 2C); Asterisk (*) points towards motor corticospinal tracts. Figure 2D - High magnification of the square area (inset) of Figure 2C, showing high expression of GFP in motor axons (arrows). Figures 2E-G - Immunofluorescent staining and confocal microscopy analysis of the lumber spinal cord. GFP is shown by green staining. The motor neurons are shown by the red staining of Choline Acetyltransferase, ChAT. Separated channels showing GFP staining (Figure 2E, green) and ChAT (Figure 2F, red) and a merged image (Figure 2G) with nuclear counterstain [blue, DAPI, (4',6-diamidino-2-phenylindole)] showing the nucleus. Scale bars, 10 μιη.

FIGs. 2H-J are histograms depicting miR-9 expression level four (Figures 2H-I) and eight (Figure 2J) weeks after treatment with rAAV9 for miR-9 knockdown or for miR-9 overexpression. Figure 2H - Shown are the expression levels of miR-9 and of miR-124 (a control neuronal miRNA) by qPCR in spinal cord of mice four weeks post injection of TuD-miR-9 knockdown virus (TuD-miR-9, white bars) and a corresponding scramble control (Tud-Scramble, grey bars). * - p-value <0.05. Note the specific and significant decrease in miR-9 expression level in the spinal cord of mice treated with the miR-9 knockdown virus ("miR-9" white bar) as compared to the level of miR-9 in the spinal cord of mice treated with the Tud-Scramble virus ("miR-9" grey bar). On the

other hand, the level of the control neuronal miR-124 was not significantly changed in the spinal cord of mice treated with TuD-miR-9 virus ("miR-124" white bar) as compared to the level of miR-124 in the spinal cord of mice treated with the Tud-Scramble virus ("miR-124" grey bar). Figure 21 - Shown are the expression levels of miR-9 and of miR-124 by qPCR in spinal cord of mice four weeks post injection of a vector for overexpression of miR-9 (OE-miR-9, white bars) and a corresponding scramble control (OE-Scramble, grey bars). ** p-value > 0.01. Note the specific and highly significant increase in miR-9 expression level in the spinal cord of mice treated with the miR-9 overexpression virus ("miR-9" white bar) as compared to the level of miR-9 in the spinal cord of mice treated with the OE-Scramble virus ("miR-9" grey bar). On the other hand, the level of miR-124 was not significantly changed in the spinal cord of mice treated with OE-miR-9 virus ("miR-124" white bar) as compared to the level of miR-124 in the spinal cord of mice treated with the OE-Scramble virus ("miR-124" grey bar). Figure 2J - Expression of miR-9 in the lumber spinal cord of SOD1 G93A eight weeks after rAAV9 ICV injection with either knock down (KD) or overexpression (OE) of miR-9 and the relevant control. All samples were normalized to the relevant expression of same RNAs in control and to the expression levels of the reference noncoding RNA u6. * - p-value <0.05.

FIGs. 3A-E shown the impact of miR-9 on motor neuron survival and neuro-inflammation *in vivo.* Figures 3A-B - Representative micrographs of Nissl staining of lumbar (L4-L5) sections from SOD1 G93A mice treated with the miR-9 overexpression (Figure 3A, bottom panel) and the miR-9 knock down (Figure 3B, bottom panel). The upper panels in Figures 3A and 3B depicting staining of the SOD1 G93A mice treated with the respective control vectors (Figure 3A, upper panel, control scramble of the miR-9 overexpression vector; and Figure 3B, upper panel, control scramble of miR-9 knockdown vector). The dashed line represents the border under which cells were counted. Figures 3C-D - Representative lumbar section from the treated 3.5 months old SOD1 G93A mice showing immunofluorescence for Glial Fibrillary Acidic Protein (GFAP; n = 2 for each treatment) as a standard way to assess neuro-inflammation / astocytosis. Insets - enlargements of a ventral horn area in each section. Figure 3C - Mice treated with the miR-9 overexpression vector. Figure 3D - Mice treated with the miR-9 knockdown vector. Upper panels, control [scrambles of the overexpression

(Figure 3C) and the knockdown (Figure 3D) vectors]. Figure 3E - Average number of motor neurons counted per ventral horn section in lumbar spinal cord of 3.5 months old SOD1 G93A treated mice (15 lumbar sections per animal; n = 3, each group).

FIGs. 4A-D are graphs depicting survival of SOD1^{G93A} mice following treatment with the miR-9 knockdown expression vector. Two month-old S0D1^{G93A} mice were injected by intraventricular injection with the following viral vectors: knockdown miR-9 (N=16, solid red line); scramble control knockdown vector (N=17, dotted orange line); overexpression miR-9 (N=18, solid blue line); or the scramble control of the overexpression vector (N=8, light blue, dotted line), and the injected mice were examined over time for the survival (Figure 4A), onset of symptoms (Figure 4B), weight peak (Figure 4C) and time on Rotarod (Figure 4D). Mice were sacrificed when they could not right themselves within 30 seconds of being placed on either side. Figure 4A - A histogram depicting the survival rate of the SOD1^{G93A} mice after the intracerebroventricular injection of the indicated viral vectors. End stage was defined by failure to right within 30 seconds of being placed on either side. Figure 4B - A histogram depicting onset of symptoms in the SOD1^{G93A} mice after the ICV injection of the indicated viral vectors. Disease onset was defined by initial abnormal leg extension onto lateral midline (weakness) or trembling of hind legs during tail suspension. Figure 4C - A histogram depicting weight pick of the SOD1^{G93A} mice after the ICV injection of the indicated viral vectors. The last measure time point before onset of wasting (pathological weight loss) was denoted as weight pick. Figure 4D - A histogram depicting the overall locomotive function of the S0D1^{G93A} mice after the ICV injection of the indicated viral vectors as depicted by time until failing on a Rotarod test; Repeated ANOVA test between the 3 groups. * - p-value<0.05 ** p-value > 0.01, **** p-value > 0.0001.

FIG. 5 depicts an unbiased bioinformatics analysis of miR-9 targets using next-generation sequencing. Next generation sequencing, performed on RNA that was extracted from primary motor neurons transfected with synthetic RNA mimetics miRNA-9 that recapitulate miR-9 (N=3) [miRNA-9 mimics, designed as dsRNA oligonucleotides with the miR-9-5p antisense oligo: $_{r}$U*rC*rUrUrUrGrGrUrUrArUrCrUrArGrCrUrGrUrAmUmGrA (SEQ ID NO: 8), and the miR-9 sense oligo

mAmUrAmCrAmGrCmUrAmGrAmUrAmArCmCrAmArAmGA (SEQ ID NO:9)] or with control RNAs with irrelevant sequence (N=6) [antisense mimic: rC*rG*rCrGrArCrUrArUrArCrGrCrGrCrArArUrArUmGmGrU (SEQ ID NO: 10) and the sense mimic: rAmCrCmArUmArUmUrGmCrGmCrGmUrAmUrAmGrUmCrGmCG (SEQ ID NO: 11)]. Note that "r" refers to RNA bases; "_*" refers to Phosphorothioated RNA base, and "m" refers to 2' O-methyl RNA bases. Sylamer analysis was implemented for assessing over- and under-representation of miRNA recognition sequences (seed-matches) for all known miRNAs, in 6500 expressed genes, ranked from mostly down-regulated to up-regulated in primary motor neurons overexpressing miR-9, relative to controls. Sylamer identified only two enriched motifs, both matching the miR-9 'seed' sequence (standard recognition sequences for miR-9) [in orange (mmu-miR9.7(2) and in red (mmu-miR-9.7(lA)]. Note that the only standard recognition sequences that exhibit a unique signature were those of the miR-9 miRNA. These targets expose a broad and specific signature of miR-9 impact on the transcriptome of motor neurons.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods of treating ALS and, more particularly, but not exclusively, to using an agent capable of downregulating miR-9 expression level and/or activity for treating ALS, such as ALS which results from SODl mutations, e.g., the G94A (also known as G93A) mutation in SEQ ID NO: 1.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways.

A major problem in the field of ALS is to understand the molecular mechanisms underlying motor neurodegeneration. miRNAs emerge as major regulators in a wide spectrum of neuronal processes, and their involvement in neurodegeneration is suggested. Interestingly, miR-9 is dysregulated in the brains of human patients with neurodegenerative disorders such as Alzheimer's disease and Huntington's disease.

MiR-9 was found to be upregulated in the spinal cord of the common mouse model for ALS (SODG93A) during disease progression (Figure 1 and Example 1 of the Examples section which follows and Zhou et al., Int J. Clin. Exp. Pathol. 2013).

The present inventors have surprisingly uncovered that agents which reduce or downregulate the expression level and/or activity of miR-9 can be used to treat ALS. Thus, as shown in the Examples section which follows, the present inventors used viral vectors harboring an antisense sequence directed against the miR-9 sequence (SEQ ID NO:4) and a vector overexpressing miR-9 (SEQ ID NO: 3) and administered them directly into the central nervous system (spinal cord and brain) of mice having the G93A mutation in the SODl protein (Figures 2A-J and 3A-D, Examples 2 and 3 of the Examples section which follows). Following treatment, the mice were examined and evaluated for ALS disease onset, which was defined by initial abnormal leg extension onto lateral midline (weakness) or trembling of hind legs during tail suspension (Figure 4B), onset of wasting (pathological weight loss), which was denoted as weight pick (Figure 4C), time on rotarod (figure 4D), and percent survival (Figure 4A). As is described in Example 3 of the Examples section which follows, mice that were injected with the knockdown viral vector (which downregulate miR-9) exhibit increased survival and improved neuromuscular function (Figures 4A-D). On the other hand, mice that were injected with the miR-9 overexpression vector exhibited notable decreases SOD1G93A mice survival (Figures 4A-D). These results conclusively show, for the first time, that downregulation of miR-9 in the cells of the central nervous system, such as the moto-neurons in the brain and spinal cord, is highly efficient for treating ALS, increasing the survival rate, delaying onset of symptoms in predisposed subjects (e.g., subjects carrying the SODl G93A missense mutation) and improving the functioning of the affected subjects, such as delaying the onset of wasting and ability to stably walk.

Thus, according to an aspect of some embodiments of the invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression level of miR-9, thereby treating the Amyotrophic lateral scleroses (ALS) in a subject in need thereof.

The term "treating" refers to inhibiting, preventing or arresting the development of a pathology (disease, disorder or condition) and/or causing the reduction, remission, or regression of a pathology. Those of skill in the art will understand that various methodologies and assays can be used to assess the development of a pathology, and similarly, various methodologies and assays may be used to assess the reduction, remission or regression of a pathology.

As used herein, the term "preventing" refers to keeping a disease, disorder or condition from occurring in a subject who may be at risk for the disease, but has not yet been diagnosed as having the disease.

As used herein, the term "subject" includes mammals, preferably human beings at any age which suffer from the pathology (ALS). Preferably, this term encompasses individuals who are at risk to develop the pathology.

For example, preventing the disease or delaying onset of disease symptoms can be in a predisposed subject who is carrier of a mutation causing ALS.

"Amyotrophic lateral sclerosis (ALS)" also known as *"Lou Gehrig's disease"* is a classical motor neuron disease, progressive, and ultimately fatal that disrupts signals to all voluntary muscles. The disease affects both the upper and lower motor neurons. Symptoms are usually noticed first in the arms and hands, legs, or swallowing muscles. Approximately 75 percent of people with classic ALS will develop weakness and wasting of the bulbar muscles (muscles that control speech, swallowing, and chewing). Muscle weakness and atrophy occur on both sides of the body. Affected individuals lose strength and the ability to move their arms and legs, and to hold the body upright. Other symptoms include spasticity, spasms, muscle cramps, and fasciculations. Speech can become slurred or nasal. When muscles of the diaphragm and chest wall fail to function properly, individuals lose the ability to breathe without mechanical support. Although the disease does not usually impair a person's mind or personality, several recent studies suggest that some people with ALS may develop cognitive problems involving word fluency, decision-making, and memory. Most individuals with ALS die from respiratory failure, usually within 3 to 5 years from the onset of symptoms. However, about 10 percent of affected individuals survive for 10 or more years.

Early signs of ALS include:

- Difficulty walking, tripping or difficulty doing the subject's normal daily

activities;

- Weakness in the leg, feet or ankles;
- Hand weakness or clumsiness;
- Slurring of speech or trouble swallowing;
- Muscle cramps and twitching in the arms, shoulders and tongue;

Thus, the diagnosis of ALS is usually based on:

(1) signs of degeneration of lower motor neurons, which are in the spinal cord and brainstem, by clinical examination or specialized testing;

(2) signs of degeneration of upper motor neurons in the brain, by clinical examination;

(3) progressive spread of signs within a region to other regions; and

(4) the absence of evidence of other disease processes that might explain the observed clinical and electrophysiological signs.

A thorough medical and family history and physical examination are the starting points of a neurologic work-up, which includes simple, in-office tests of muscle and nerve function.

The diagnosis of ALS can be further confirmed by an electromyogram (EMG), which can identify characteristics signals and electrical activity inside the muscles consistent with the diagnosis of ALS; magnetic resonance imaging (MRI) of the spinal cord and brain; and sometimes by testing the fluid surrounding the spinal cord by spinal tap or lumbar puncture (which involves inserting a needle into the back between two lower vertebrae).

The diagnosis of ALS may further include blood tests which exclude disorders that mimic ALS (e.g., some forms of muscular dystrophy, the neurologic condition known as spinal-bulbar muscular atrophy and adult-onset spinal muscular atrophy, the nerve-to-muscle transmission disorder known as myasthenia gravis, and various causes of compression of the spinal cord or brainstem, such as tumors and malformations), a muscle biopsy, which involves taking a small sample of muscle under local anesthesia, genetic tests (which is performed on a blood sample of the subject) to confirm the diagnosis based on familial genetic mutations associated with ALS.

Genetic testing for ALS may include screening for mutations in the SOD1 gene (superoxide dismutase 1), which account for about 20% of familial ALS and also perhaps 1-3% of sporadic ALS; mutations in the gene encoding the TDP43 protein;

mutations in the FUS gene; mutations in the gene encoding the ubiquilin 2 protein, mutations in the C90RF72 gene involving an expansion of repeated DNA sequences, which are more than twice as common as mutations in the SOD1 gene; and mutations in optineurin and valosin-containing protein (VCP) gene — combined.

According to some embodiments of the invention, the ALS of the subject is a result of a mutation(s) in SODl, FUS, ubiquilin 2 protein, C90RF72, and/or optineurin and valosin-containing protein (VCP) gene.

Familial ALS, which is caused by mutations in the SODl gene, usually involves an autosomal dominant disease, but can also involve an autosomal recessive ALS.

According to an aspect of some embodiments of the invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a human subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression level of miR-9, thereby treating the ALS in the human subject in need thereof.

According to an aspect of some embodiments of the invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression level of miR-9, wherein the ALS is caused by a mutation in the SODl gene set forth by SEQ ID NO:2, thereby treating the ALS in the subject in need thereof.

According to an aspect of some embodiments of the invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression level of miR-9, wherein the ALS is caused by the G94A missense mutation in the SODl polypeptide set forth by SEQ ID NO:l, thereby treating the ALS in the subject in need thereof.

According to an aspect of some embodiments of the invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or

expression level of miR-9, wherein the agent is comprised in a viral vector, thereby treating the ALS in the subject in need thereof.

According to an aspect of some embodiments of the invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression level of miR-9, wherein the active ingredient does not comprise an agent capable of downregulating a miR selected from the group consisting of: miR133a, miR133b, miR96, miR146, miR30-2, miR125b, miR125a, miR125a-5p, miR125b-5p, miR30a, miR30b, miR146 and miR146a, thereby treating the ALS in the subject in need thereof.

According to an aspect of some embodiments of the invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression level of miR-9, wherein the pharmaceutical composition is administered to the subject by intracerebroventricular administration (ICV), thereby treating the ALS in the subject in need thereof.

According to an aspect of some embodiments of the invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression level of miR-9, wherein the pharmaceutical composition is administered to the subject by intrathecal administration, thereby treating the ALS in the subject in need thereof.

According to an aspect of some embodiments of the invention there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression level of miR-9, wherein the pharmaceutical composition is administered to the subject by intracranial (IC) administration, thereby treating the ALS in the subject in need thereof.

According to some embodiments of the invention, the ALS is caused by a mutation in the SODl gene set forth by SEQ ID NO:2.

According to some embodiments of the invention, the mutation results in the G94A missense mutation (also known as SODl "G93A" mutation) in the wild type SOD 1 polypeptide set forth by SEQ ID NO: 1.

It should be noted that the G94A missense mutation is a Glycine (while type) to Alanine (mutant variant) substitution at amino acid position 94 of the SODl polypeptide set forth by SEQ ID NO:l. The mutation is commonly referred to as G93A in SODl. The mutation is depicted in single nucleotide polymorphism (SNP) rsl21912437; at a Cytogenetic location: 21q22.1; at a Genomic location: Chr21:3 1667299 (on Assembly GRCh38) or Chr21:33039612 (on Assembly GRCh37).

According to some embodiments of the invention, the subject is a carrier the SODl G94A missense mutation (e.g., in a heterozygote or a homozygote form).

According to some embodiments of the invention, the subject has been diagnosed with ALS (e.g., as described above).

According to some embodiments of the invention, the subject is predisposed to develop ALS.

According to some embodiments of the invention, the subject is a carrier of a genetic mutation causing ALS but yet does not suffer from symptoms characterizing ALS (e.g., as described above).

According to some embodiments of the invention, the method further comprising selecting a subject which is a carrier of the SODl G94A missense mutation as set forth by SEQ ID NO:l, wherein presence of at least one allele of the SODl 94A variant as set forth by SEQ ID NO:l indicates that the active ingredient is suitable for treating the subject.

It should be noted that such a selection step (confirming presence or absence of the SODl G94A mutation) can be performed prior to treating the subject with the agent which downregulates the activity and/or expression level of miR-9.

According to some embodiments of the invention, the agent is administered to the central nervous system of the subject in need thereof.

According to some embodiments of the invention, the agent is administered into the brain and/or the spinal cord of the subject.

According to some embodiments of the invention, the agent is administered into the brain of the subject.

According to some embodiments of the invention, the agent is administered to the spinal cord of the subject.

According to some embodiments of the invention, the pharmaceutical composition is administered to the subject by intracerebroventricular (ICV) administration.

As used herein the phrase "intracerebroventricular (ICV) administration" refers to administration refers to administration into the lateral ventricles of the brain.

According to some embodiments of the invention, the pharmaceutical composition is administered to the subject by intrathecal administration.

As used herein the phrase "intrathecal administration" refers to administration into the cererbro spinal fluid or into the cisterna magna (also referred to as the cerebellomeduUary cistern) of the brain of a subject. It should be noted that intrathecal administration of the agent can result in migration of the agent into the brain ventricles. For example, intrathecal administration can be into the spinal canal (intrathecal space surrounding the spinal cord) such as near the subject's waist.

According to some embodiments of the invention, the pharmaceutical composition is administered to the subject by intracranial (IC) administration.

The phrase "intracranial (IC) administration" as used herein refers to administration into the brain parenchyma.

Methods of intracranial, intracerebroventricular and/or intrathecal administration are known in the art and are described, for example, in Pathan SA, et al. 2009. "CNS drug delivery systems: novel approaches." Recent Pat. Drug Deliv. Formul. 3: 71-89; Geiger BM, et al., 2008. Survivable Stereotaxic Surgery in Rodents. J Vis Exp. 20, pii: 880. doi: 10.3791/880; Huang X, (2010). Intracranial Orthotopic Allografting of MeduUoblastoma Cells in Immunocompromised Mice. J Vis Exp. 44, pii: 2153. doi: 10.3791/2153; Alam MI., et al. 2010. "Strategy for effective brain drug delivery". Review. European J. of Pharmaceutical Sciences, 40: 385-403; Bakhshi S., et al., 1995. "Implantable pumps for drug delivery to the brain". Journal of Neuro-Oncology 26: 133-139; each of which is fully incorporated herein by reference.

For example, intracerebral delivery of the agent of some embodiments of the invention into the parenchymal space of the brain can be achieved by directly injecting (using bolus or infusion) the agent via an intrathecal catheter, or an implantable catheter essentially as described in Haugland and Sinkjaer, 1999 ["Interfacing the body's own sensing receptors into neural prosthesis devices". Technol. Health Care, 7: 393-399; Kennedy and Bakay, 1998. "Restoration of neural output from a paralyzed patient by a direct connection". Neuroreport, 9: 1707-1711; each of which is fully incorporated herein by reference]. For example, the catheter can be implanted by surgery into the brain where it releases the agent for a predetermined time period.

Intrabrain administration of the agent can be at a single injection, at a continuous infusion, or periodic administrations, and those of skills in the art are capable of designing a suitable treatment regime depending on the condition to be treated, and the treated subject.

According to some embodiments of the invention, the IC, ICV or intrathecal administration is performed by an injection or an infusion, using e.g., a needle, a syringe, a catheter, a pump, an implantable device (e.g., as is further described hereinunder) and/or any combination(s) thereof.

According to some embodiments of the invention, the IC, ICV or intrathecal administration is performed periodically.

Additionally or alternatively, the agent can further comprise a moiety (e.g., a peptide sequence, a DNA sequence, an RNA sequence, a carbohydrate, and/or a fat molecule) which can cross the blood brain barrier (BBB) and which can direct the agent into the CNS such that administration of the agent into a non CNS tissues/organ will result in accumulate of a therapeutically effective amount of the agent in the CNS of the subject to be treated. Non-limiting examples of such moieties which cross the blood brain barrier are provided hereinunder.

Thus, according to some embodiments of the invention, the pharmaceutical composition further comprises a moiety capable of directing the agent capable of downregulating the activity or the expression of the miR-9 to the central nervous system of the subject.

According to some embodiments of the invention, the moiety is conjugated to the agent capable of downregulating the activity and/or the expression of the miR-9.

According to some embodiments of the invention, administering is effected prior to appearance of at least one ALS symptom, e.g., prior to appearance of at least two ALS symptoms, e.g., prior to appearance of at least three ALS symptoms, wherein the ALS symptom is selected from the group consisting of: difficulty walking, weakness in leg(s), weakness in hand(s), slurring of speech, trouble swallowing, muscle cramps, and twitching in arms, shoulders or tongue.

According to some embodiments of the invention, administering is effected concomitantly or after appearance of at least one ALS symptom, e.g., at least two ALS symptoms, e.g., at least three ALS symptoms, wherein the ALS symptom is selected from the group consisting of: difficulty walking, weakness in leg(s), weakness in hand(s), slurring of speech, trouble swallowing, muscle cramps, and twitching in arms, shoulders or tongue.

As used herein, the term "miR-9" refers to the microRNA (miRNA) molecule acting as post-transcriptional regulator. Exemplary miR-9 precursor polynucleotide sequences are set forth in SEQ ID NOs: 5-7. According to some embodiments of the invention, the miR-9 is depicted in SEQ ID NO:3.

MicroRNAs are typically processed from pre-miR (pre-microRNA precursors). Pre-miRs are a set of precursor miRNA molecules transcribed by RNA polymerase III that are efficiently processed into functional miRNAs, e.g., upon transfection into cultured cells. Pre-miR designs exist to all of the known miRNAs listed in the miRNA registry (see below) and can be readily designed for any research.

The term "microRNA", "miRNA", and "miR" are synonymous and refer to a collection of non-coding single-stranded RNA molecules of about 19-28 nucleotides in length, which regulate gene expression. miRNAs are found in a wide range of organisms and have been shown to play a role in development, homeostasis, and disease etiology.

Below is a brief description of the mechanism of miRNA activity.

Genes coding for miRNAs are transcribed leading to production of an miRNA precursor known as the pri-miRNA. The pri-miRNA is typically part of a polycistronic RNA comprising multiple pri-miRNAs. The pri-miRNA may form a hairpin with a stem and loop. The stem may comprise mismatched bases.

The hairpin structure of the pri-miRNA is recognized by Drosha, which is an RNase III endonuclease. Drosha typically recognizes terminal loops in the pri-miRNA

and cleaves approximately two helical turns into the stem to produce a 60-70 nucleotide precursor known as the pre-miRNA. Drosha cleaves the pri-miRNA with a staggered cut typical of RNase III endonucleases yielding a pre-miRNA stem loop with a 5' phosphate and ~2 nucleotide 3' overhang. It is estimated that approximately one helical turn of stem (-10 nucleotides) extending beyond the Drosha cleavage site is essential for efficient processing. The pre-miRNA is then actively transported from the nucleus to the cytoplasm by Ran-GTP and the export receptor Ex-portin-5.

The double-stranded stem of the pre-miRNA is then recognized by Dicer, which is also an RNase III endonuclease. Dicer may also recognize the 5' phosphate and 3' overhang at the base of the stem loop. Dicer then cleaves off the terminal loop two helical turns away from the base of the stem loop leaving an additional 5' phosphate and ~2 nucleotide 3' overhang. The resulting siRNA-like duplex, which may comprise mismatches, comprises the mature miRNA and a similar-sized fragment known as the miRNA*. The miRNA and miRNA* may be derived from opposing arms of the pri-miRNA and pre-miRNA. MiRNA* sequences may be found in libraries of cloned miRNAs but typically at lower frequency than the miRNAs.

Although initially present as a double-stranded species with miRNA*, the miRNA eventually become incorporated as a single-stranded RNA into a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC). Various proteins can form the RISC, which can lead to variability in specificity for miRNA/miRNA* duplexes, binding site of the target gene, activity of miRNA (repress or activate), and which strand of the miRNA/miRNA* duplex is loaded in to the RISC.

When the miRNA strand of the miRNA:miRNA* duplex is loaded into the RISC, the miRNA* is removed and degraded. The strand of the miRNA:miRNA* duplex that is loaded into the RISC is the strand whose 5' end is less tightly paired. In cases where both ends of the miRNA:miRNA* have roughly equivalent 5' pairing, both miRNA and miRNA* may have gene silencing activity.

The RISC identifies target nucleic acids based on high levels of complementarity between the miRNA and the mRNA, especially by nucleotides 2-7 of the miRNA.

A number of studies have looked at the base-pairing requirement between miRNA and its mRNA target for achieving efficient inhibition of translation (reviewed by Bartel 2004, Cell 116-281). In mammalian cells, the first 8 nucleotides of the

miRNA may be important (Doench & Sharp 2004 GenesDev 2004-504). However, other parts of the microRNA may also participate in mRNA binding. Moreover, sufficient base pairing at the 3' can compensate for insufficient pairing at the 5' (Brennecke et al, 2005 PLoS 3-e85). Computation studies, analyzing miRNA binding on whole genomes have suggested a specific role for bases 2-7 at the 5' of the miRNA in target binding but the role of the first nucleotide, found usually to be "A" was also recognized (Lewis et at 2005 Cell 120-15). Similarly, nucleotides 1-7 or 2-8 were used to identify and validate targets by Krek et al (2005, Nat Genet 37-495).

The target sites in the mRNA may be in the 5' UTR, the 3' UTR or in the coding region. Interestingly, multiple miRNAs may regulate the same mRNA target by recognizing the same or multiple sites. The presence of multiple miRNA binding sites in most genetically identified targets may indicate that the cooperative action of multiple RISCs provides the most efficient translational inhibition.

MiRNAs may direct the RISC to downregulate gene expression by either of two mechanisms: mRNA cleavage or translational repression. The miRNA may specify cleavage of the mRNA if the mRNA has a certain degree of complementarity to the miRNA. When a miRNA guides cleavage, the cut is typically between the nucleotides pairing to residues 10 and 11 of the miRNA. Alternatively, the miRNA may repress translation if the miRNA does not have the requisite degree of complementarity to the miRNA. Translational repression may be more prevalent in animals since animals may have a lower degree of complementarity between the miRNA and binding site.

It should be noted that there may be variability in the 5' and 3' ends of any pair of miRNA and miRNA*. This variability may be due to variability in the enzymatic processing of Drosha and Dicer with respect to the site of cleavage. Variability at the 5' and 3' ends of miRNA and miRNA* may also be due to mismatches in the stem structures of the pri-miRNA and pre-miRNA. The mismatches of the stem strands may lead to a population of different hairpin structures. Variability in the stem structures may also lead to variability in the products of cleavage by Drosha and Dicer.

The term "microRNA mimic" refers to synthetic non-coding RNAs that are capable of entering the RNAi pathway and regulating gene expression. miRNA mimics imitate the function of endogenous microRNAs (miRNAs) and can be designed as mature, double stranded molecules or mimic precursors (e.g., or pre-miRNAs). miRNA

mimics can be comprised of modified or unmodified RNA, DNA, RNA-DNA hybrids, or alternative nucleic acid chemistries (e.g., LNAs or 2'-0,4'-C-ethylene-bridged nucleic acids (ENA)). For mature, double stranded miRNA mimics, the length of the duplex region can vary between 13-33, 18-24 or 21-23 nucleotides. The miRNA may also comprise a total of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 nucleotides. The sequence of the miRNA may be the first 13-33 nucleotides of the pre-miRNA. The sequence of the miRNA may also be the last 13-33 nucleotides of the pre-miRNA. The sequence of the miRNA may comprise the sequence of SEQ ID NO: 3 or variants thereof.

Thus, the miR-9 of the present teachings may bind, attach, regulate, process, interfere, augment, stabilize and/or destabilize any target thereof. Such a target can be any molecule, including, but not limited to, DNA molecules, RNA molecules and polypeptides.

As shown in the Examples section which follows, miR-9 is part of, involved in and/or is associated with the pathogenesis of ALS, and in particular, of ALS which is caused by a mutation in SOD1 gene, such as the SOD G94A mutation in SEQ ID NO:l. MiR-9 can thus be identified via various databases including for example the micro-RNA registry (www (dot) sanger(dot) acdotuk/Software/Rfam/mirna/index(dot) shtml).

As used herein the phrase *"capable of downregulating an activity and/or expression level of miR-9"* refers to decreasing by at least 2%, e.g., by at least 5%, e.g., by at least 10%, e.g., by at least 20%, e.g., by at least 30%, e.g., by at least 40%, e.g., by at least 50%, e.g., by at least 60%, e.g., by at least 70%, e.g., by at least 80%, e.g., by at least 90%, e.g., by at least 95%, e.g., about 100% of the activity and/or the expression level of miR-9 in cells of the treated subject (e.g., cells of the CNS, e.g., motoneurons) as compared to the activity and/or the expression level of miR-9 in the same cells of a non-treated subject, or in the cells of the same subject prior to treatment.

Methods of qualifying the ability of the agent to downregulate the expression level of miR-9 are known in the art and include for example RNA detection methods such as in situ RNA hybridization, reverse transcriptase-polymerase chain reaction (RT-PCR), quantitative RT-PCR (qPCR) and the like. Non-limited examples of such method is shown in Figures 1, 2H, 21, and 2J.

Methods of qualifying the ability of the agent to downregulate the activity of miR-9 are known in the art and include for example detection of the expression level or activity of a protein which is regulated by the miR-9. For example, the expression level of the target protein can be detected by any protein detection method such as Western blot, ELISA, FACS, immunohistochemistry and/or immunofluorescence.

Following is a non-limiting list of agents capable of downregulating expression level and/or activity of miR-9.

Down-regulating the miR-9 can be affected using a polynucleotide which is hybridizable in cells under physiological conditions to the miRNA.

As used herein, the term "hybridizable" refers to capable of hybridizing, i.e., forming a double strand molecule such as RNA:RNA, RNA:DNA and/or DNA:DNA molecules.

As used herein, the term "physiological conditions" refer to the conditions present in cells, tissue or a whole organism or body. According to some embodiments of the invention, the physiological conditions include a temperature between 34-40°C, more preferably, a temperature between 35-38°C, more preferably, a temperature between 36°C and 37.5°C, most preferably, a temperature between 37°C to 37.5°C; salt concentrations (e.g., sodium chloride NaCl) between 0.8-1%, more preferably, about 0.9%; and/or pH values in the range of 6.5-8, more preferably, 6.5-7.5, most preferably, pH of 7-7.5.

Downregulation of miR-9 can be effected on the genomic and/or the transcript level using a variety of molecules which interfere with transcription (e.g., RNA silencing agents, Ribozyme, DNAzyme and antisense).

Nucleic acid agents that downregulate miR-9 activity include, but are not limited to, a target mimic, a micro-RNA resistant gene and a miRNA inhibitor.

The target mimic or micro-RNA resistant target is essentially complementary to the microRNA provided that one or more of following mismatches are allowed:

(a) a mismatch between the nucleotide at the 5' end of the microRNA and the corresponding nucleotide sequence in the target mimic or micro-RNA resistant target;

(b) a mismatch between any one of the nucleotides in position 1 to position 9 of the microRNA and the corresponding nucleotide sequence in the target mimic or micro-RNA resistant target; or

(c) three mismatches between any one of the nucleotides in position 12 to position 21 of the microRNA and the corresponding nucleotide sequence in the target mimic or micro-RNA resistant target provided that there are no more than two consecutive mismatches.

The target mimic RNA is essentially similar to the target RNA modified to render it resistant to miRNA induced cleavage, e.g. by modifying the sequence thereof such that a variation is introduced in the nucleotide of the target sequence complementary to the nucleotides 10 or 11 of the miRNA resulting in a mismatch.

Alternatively, a microRNA-resistant target may be implemented. Thus, a silent mutation may be introduced in the microRNA binding site of the target gene so that the DNA and resulting RNA sequences are changed in a way that prevents microRNA binding, but the amino acid sequence of the protein is unchanged. Thus, a new sequence can be synthesized instead of the existing binding site, in which the DNA sequence is changed, resulting in lack of miRNA binding to its target.

According to a specific embodiment, the target mimic or micro-RNA resistant target is linked to the promoter naturally associated with the pre-miRNA recognizing the target gene and introduced into the cell. In this way, the miRNA target mimic or micro-RNA resistant target RNA will be expressed under the same circumstances as the miRNA and the target mimic or micro-RNA resistant target RNA will substitute for the non-target mimic/micro-RNA resistant target RNA degraded by the miRNA induced cleavage.

Non-functional miRNA alleles or miRNA resistant target genes may also be introduced by homologous recombination to substitute the miRNA encoding alleles or miRNA sensitive target genes.

Recombinant expression is effected by cloning the nucleic acid of interest (e.g., miRNA, target gene, silencing agent, etc.) into a nucleic acid expression construct under the expression of a promoter.

In other embodiments of the invention, synthetic single stranded nucleic acids are used as miRNA inhibitors. A miRNA inhibitor is typically between about 17 to 25

nucleotides in length and comprises a 5' to 3' sequence that is at least 90 % complementary to the 5' to 3' sequence of a mature miRNA. In certain embodiments, a miRNA inhibitor molecule is 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length, or any range derivable therein. Moreover, a miRNA inhibitor has a sequence (from 5' to 3') that is or is at least 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 99.1, 99.2, 99.3, 99.4, 99.5, 99.6, 99.7, 99.8, 99.9 or 100 % complementary, or any range derivable therein, to the 5' to 3' sequence of a mature miRNA, particularly a mature, naturally occurring miRNA.

According to an embodiment, peptide nucleic acids oligonucleotide analogues (PNA ON) are used as miRNA inhibitors. Such miRNA inhibitors have been described in detail in Torres et al., *Nucleic Acids Research* (2011) 1-16, incorporated herein by reference.

MiRNA inhibitors are commercially available from Companies such as Applied Biosystems.

The miRNA inhibitors may be contacted with the cells using transient or stable transfection techniques. Thus, the miRNA inhibitors may be part of an expression vector, as described hereinbelow.

According to some embodiments, downregulating the expression of a microRNA is effected by the use of a nucleic acid sequence which specifically binds and downregulates the expression of the microRNA. A nucleic acid sequence which may be used in accordance with the present invention may be purchased from any manufacturer, as for example, from Genecopoeia (miArrest, microRNA vector based inhibitors).

According to another embodiment, there is provided an isolated polynucleotide comprising a nucleic acid sequence for downregulating an expression of miR-9 or a precursor thereof.

Exemplary polynucleotides which may be used in accordance with the present invention to downregulate the expression of miR-9 include, but are not limited to, the sequence set in SEQ ID NO: 4 or 14.

Downregulation of miR-9 can also be achieved by RNA silencing. As used herein, the phrase "RNA silencing" refers to a group of regulatory mechanisms [e.g. RNA interference (RNAi), transcriptional gene silencing (TGS), post-transcriptional gene silencing (PTGS), quelling, co-suppression, and translational repression] mediated

by RNA molecules which result in the inhibition or "silencing" of the expression of a corresponding protein-coding gene. RNA silencing has been observed in many types of organisms, including plants, animals, and fungi.

As used herein, the term "RNA silencing agent" refers to an RNA which is capable of inhibiting or "silencing" the expression of a target gene. In certain embodiments, the RNA silencing agent is capable of preventing complete processing (e.g., the full translation and/or expression) of an mRNA molecule through a post-transcriptional silencing mechanism. RNA silencing agents include noncoding RNA molecules, for example RNA duplexes comprising paired strands, as well as precursor RNAs from which such small non-coding RNAs can be generated. Exemplary RNA silencing agents include dsRNAs such as siRNAs, miRNAs and shRNAs. In one embodiment, the RNA silencing agent is capable of inducing RNA interference. In another embodiment, the RNA silencing agent is capable of mediating translational repression.

Downregulation of miR-9 can be effected by using an antisense polynucleotide capable of specifically hybridizing with a miR-9 precursor sequence (e.g., SEQ ID NOs: 5-7) or with the miR-9 mature sequence [UCUUUGGUUAUCUAGCUGUAUGA (SEQ ID NO:3)].

According to some embodiments of the invention, the agent capable of downregulating the activity or the expression level of the miR-9 comprises an antisense sequence of the miR-9 (antisense of hsa-miR-9-5p).

The antisense oligonucleotide may comprise deoxyribonucleotides (e.g., SEQ ID NO:4) or ribonucleotides (e.g., SEQ ID NO: 14).

According to some embodiments of the invention, the antisense sequence is set forth by SEQ ID NO:4 (TCATACAGCTAGATAACCAAAG) or SEQ ID NO: 14 (UCAUACAGCUAGAUAACCAAAG).

Design of antisense molecules which can be used to efficiently downregulate miR-9 must be effected while considering two aspects important to the antisense approach. The first aspect is delivery of the oligonucleotide into the cytoplasm of the appropriate cells, while the second aspect is design of an oligonucleotide which specifically binds miR-9 or miR-9 precursor sequence within cells in a way which inhibits expression or activity of miR-9.

The prior art teaches of a number of delivery strategies which can be used to efficiently deliver oligonucleotides into a wide variety of cell types [see, for example, Luft J Mol Med 76: 75-6 (1998); Kronenwett et al. Blood 91: 852-62 (1998); Rajur et al. Bioconjug Chem 8: 935-40 (1997); Lavigne et al. Biochem Biophys Res Commun 237: 566-71 (1997) and Aoki et al. (1997) Biochem Biophys Res Commun 231: 540-5 (1997)].

In addition, algorithms for identifying those sequences with the highest predicted binding affinity for their target mRNA based on a thermodynamic cycle that accounts for the energetics of structural alterations in both the target mRNA and the oligonucleotide are also available [see, for example, Walton et al. Biotechnol Bioeng 65: 1-9 (1999)].

Such algorithms have been successfully used to implement an antisense approach in cells. For example, the algorithm developed by Walton et al. enabled scientists to successfully design antisense oligonucleotides for rabbit beta-globin (RBG) and mouse tumor necrosis factor-alpha (TNF alpha) transcripts. The same research group has more recently reported that the antisense activity of rationally selected oligonucleotides against three model target mRNAs (human lactate dehydrogenase A and B and rat gpl30) in cell culture as evaluated by a kinetic PCR technique proved effective in almost all cases, including tests against three different targets in two cell types with phosphodiester and phosphorothioate oligonucleotide chemistries.

In addition, several approaches for designing and predicting efficiency of specific oligonucleotides using an in vitro system were also published (Matveeva et al., Nature Biotechnology 16: 1374 - 1375 (1998)].

It will be appreciated that the microRNA antisense agents (e.g. an anti-miRNA-9 oligonucleotide) of the present invention may also comprise chemical modifications, molecular modifications and/or the addition of moieties, e.g. a cholesterol moiety (e.g. antagomirs). Such molecules have been previously described in e.g. Kriitzfeldt J. et al., Nature (2005) 438:685-9 and Lennox and Behlke: Gene Therapy (2011) REVIEW: Chemical modification and design of anti-miRNA oligonucleotides, pg. 1-10.

The term "oligonucleotide" refers to a single stranded or double stranded oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring

bases, sugars and covalent internucleoside linkages (e.g., backbone) as well as oligonucleotides having non-naturally-occurring portions which function similarly to respective naturally-occurring portions.

Oligonucleotides designed according to the teachings of some embodiments of the invention can be generated according to any oligonucleotide synthesis method known in the art such as enzymatic synthesis or solid phase synthesis. Equipment and reagents for executing solid-phase synthesis are commercially available from, for example, Applied Biosystems. Any other means for such synthesis may also be employed; the actual synthesis of the oligonucleotides is well within the capabilities of one skilled in the art and can be accomplished via established methodologies as detailed in, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988) and "Oligonucleotide Synthesis" Gait, M. J., ed. (1984) utilizing solid phase chemistry, e.g. cyanoethyl phosphoramidite followed by deprotection, desalting and purification by for example, an automated trityl-on method or HPLC.

The oligonucleotide of some embodiments of the invention is of at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24 bases specifically hybridizable with the miR-9 or pre-miR-9 sequences (e.g., SEQ ID NOs: 3, 5-7).

According to some embodiments of the invention, the antisense oligonucleotide comprises the inhibitory sequence (as described above), which is hybridizable to the miR-9 or pre-miR-9 sequences (e.g., SEQ ID NOs: 3, 5-7) and additional sequences upstream and/or downstream the inhibitory sequence.

The oligonucleotides of some embodiments of the invention may comprise heterocylic nucleosides consisting of purines and the pyrimidines bases, bonded in a 3' to 5' phosphodiester linkage.

Preferably used oligonucleotides are those modified in either backbone, internucleoside linkages or bases, as is broadly described hereinunder.

Specific examples of preferred oligonucleotides useful according to some embodiments of the invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. Oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone, as disclosed in U.S. Pat. NOs: 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466, 677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkyl phosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms can also be used.

Alternatively, modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH2 component parts, as disclosed in U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623, 070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439.

Other oligonucleotides which can be used according to some embodiments of the invention, are those modified in both sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for complementation with the appropriate polynucleotide target. An example for such an oligonucleotide mimetic, includes peptide nucleic acid (PNA). A PNA oligonucleotide refers to an oligonucleotide where the sugar-backbone is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The bases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Other backbone modifications, which can be used in some embodiments of the invention are disclosed in U.S. Pat. No: 6,303,374.

Oligonucleotides of some embodiments of the invention may also include base modifications or substitutions. As used herein, "unmodified" or "natural" bases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified bases include but are not limited to other synthetic and natural bases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further bases include those disclosed in U.S. Pat. No: 3,687,808, those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., Angewandte Chemie, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y. S., Chapter 15, Antisense Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B., ed., CRC Press, 1993. Such bases are particularly useful

for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and 0-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C. [Sanghvi YS et al. (1993) Antisense Research and Applications, CRC Press, Boca Raton 276-278] and are presently preferred base substitutions, even more particularly when combined with 2'-0-methoxyethyl sugar modifications. Still further base substitutions include the non-standard bases disclosed in US Patent Nos. 8586303, 8614072, 8871469 and 9062336, all to Benner et al: for example, the non-standard dZ:dP nucleobase pair which Benner et al has shown can be incorporated into DNA by DNA polymerases to yield amplicons with multiple non-standard nucleotides.

According to some embodiments of the invention the antisense oligonucleotide is conjugated to a peptide moiety.

According to some embodiments of the invention the antisense oligonucleotide is conjugated to a lipid moiety or other chemical complex.

According to some embodiments of the invention the antisense oligonucleotide is "naked" (includes only nucleic acids).

According to some embodiments of the invention the antisense oligonucleotide is mixed with a peptide.

According to some embodiments of the invention the antisense oligonucleotide is encapsulated in lipid or other kinds of nanoparticles.

According to a specific embodiment, the agent for downregulating an activity and/or expression level of miR-9 is an antagomir.

Downregulation of miR-9 can also be effected by RNA interference. RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals mediated by short interfering RNAs (siRNAs). The corresponding process in plants is commonly referred to as post-transcriptional gene silencing or RNA silencing and is also referred to as quelling in fungi. The process of post-transcriptional gene silencing is thought to be an evolutionarily-conserved cellular defense mechanism used to prevent the expression of foreign genes and is commonly shared by diverse flora and phyla. Such protection from foreign gene expression may have evolved in response to the production of double-stranded RNAs (dsRNAs) derived from viral infection or

from the random integration of transposon elements into a host genome via a cellular response that specifically destroys homologous single-stranded RNA or viral genomic RNA.

The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. The RNAi response also features an endonuclease complex, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex.

The term "siRNA" refers to small inhibitory RNA duplexes (generally between 18-30 basepairs) that induce the RNA interference (RNAi) pathway. Typically, siRNAs are chemically synthesized as 21mers with a central 19 bp duplex region and symmetric 2-base 3'-overhangs on the termini, although it has been recently described that chemically synthesized RNA duplexes of 25-30 base length can have as much as a 100-fold increase in potency compared with 21mers at the same location. The observed increased potency obtained using longer RNAs in triggering RNAi is theorized to result from providing Dicer with a substrate (27mer) instead of a product (21mer) and that this improves the rate or efficiency of entry of the siRNA duplex into RISC.

It has been found that position of the 3'-overhang influences potency of an siRNA and asymmetric duplexes having a 3'-overhang on the antisense strand are generally more potent than those with the 3'-overhang on the sense strand (Rose et al., 2005). This can be attributed to asymmetrical strand loading into RISC, as the opposite efficacy patterns are observed when targeting the antisense transcript.

The strands of a double-stranded interfering RNA (e.g., an siRNA) may be connected to form a hairpin or stem-loop structure (e.g., an shRNA). Thus, as mentioned the RNA silencing agent of the present invention may also be a short hairpin RNA (shRNA).

The term "shRNA", as used herein, refers to an RNA agent having a stem-loop structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region. The number of nucleotides in the loop is a number between and including 3 to 23, or 5 to 15, or 7 to 13, or 4 to 9, or 9 to 11. Some of the nucleotides in the loop can be involved in base-pair interactions with other nucleotides in the loop. Examples of oligonucleotide sequences that can be used to form the loop include 5'-UUCAAGAGA-3' (SEQ ID NO: 12) (Brummelkamp, T. R. et al. (2002) Science 296: 550) and 5'-UUUGUGUAG-3' (SEQ ID NO: 13) (Castanotto, D. et al. (2002) RNA 8:1454). It will be recognized by one of skill in the art that the resulting single chain oligonucleotide forms a stem-loop or hairpin structure comprising a double-stranded region capable of interacting with the RNAi machinery.

Qualifying target sequences are selected as template for siRNA synthesis. Preferred sequences are those including low G/C content as these have proven to be more effective in mediating gene silencing as compared to those with G/C content higher than 55 %. Several target sites are preferably selected along the length of the target gene for evaluation. For better evaluation of the selected siRNAs, a negative control is preferably used in conjunction. Negative control siRNA preferably include the same nucleotide composition as the siRNAs but lack significant homology to the genome. Thus, a scrambled nucleotide sequence of the siRNA is preferably used, provided it does not display any significant homology to any other gene.

It will be appreciated that the RNA silencing agent of the present invention need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides.

In some embodiments, the RNA silencing agent provided herein can be functionally associated with a "cell-penetrating peptide". As used herein, a "cell-penetrating peptide" is a peptide that comprises a short (about 12-30 residues) amino acid sequence or functional motif that confers the energy-independent (i.e., non-endocytotic) translocation properties associated with transport of the membrane-permeable complex across the plasma and/or nuclear membranes of a cell. The cell-

penetrating peptide used in the membrane-permeable complex of the present invention preferably comprises at least one non-functional cysteine residue, which is either free or derivatized to form a disulfide link with a double-stranded ribonucleic acid that has been modified for such linkage. Representative amino acid motifs conferring such properties are listed in U.S. Pat. No. 6,348,185, the contents of which are expressly incorporated herein by reference. The cell-penetrating peptides of the present invention preferably include, but are not limited to, penetratin, transportan, plsl, TAT(48-60), pVEC, MTS, and MAP.

Exemplary miR-9 silencing agents include, but are not limited to, Anti-miR™ miRNA Inhibitors available from Ambion Inc. for inhibition of miR-9.

Another agent capable of downregulating miR-9 is a DNAzyme molecule capable of specifically cleaving miR-9 or DNA sequence encoding miR-9. DNAzymes are single-stranded polynucleotides which are capable of cleaving both single and double stranded target sequences (Breaker, R.R. and Joyce, G. Chemistry and Biology 1995;2:655; Santoro, S.W. & Joyce, G.F. Proc. Natl, Acad. Sci. USA 1997;943:4262) A general model (the "10-23" model) for the DNAzyme has been proposed. "10-23" DNAzymes have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. This type of DNAzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions (Santoro, S.W. & Joyce, G.F. Proc. Natl, Acad. Sci. USA 199; for rev of DNAzymes see Khachigian, LM [Curr Opin Mol Ther 4:119-21 (2002)].

Examples of construction and amplification of synthetic, engineered DNAzymes recognizing single and double-stranded target cleavage sites have been disclosed in U.S. Pat. No. 6,326,174 to Joyce et al. DNAzymes of similar design directed against the human Urokinase receptor were recently observed to inhibit Urokinase receptor expression, and successfully inhibit colon cancer cell metastasis in-vivo (Itoh et al, 20002, Abstract 409, Ann Meeting Am Soc Gen Ther wwwdotasgtdotorg). In another application, DNAzymes complementary to bcr-abl oncogenes were successful in inhibiting the oncogenes expression in leukemia cells, and lessening relapse rates in autologous bone marrow transplant in cases of CML and ALL.

Another agent capable of downregulating miR-9 is a ribozyme molecule capable of specifically cleaving a precursor RNA of miR-9. Ribozymes are being increasingly

used for the sequence-specific inhibition of gene expression by the cleavage of mRNAs encoding proteins of interest [Welch et al., Curr Opin Biotechnol. 9:486-96 (1998)]. The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications. In the therapeutics area, ribozymes have been exploited to target viral RNAs in infectious diseases, dominant oncogenes in cancers and specific somatic mutations in genetic disorders [Welch et al., Clin Diagn Virol. 10:163-71 (1998)]. Most notably, several ribozyme gene therapy protocols for HIV patients are already in Phase 1 trials. More recently, ribozymes have been used for transgenic animal research, gene target validation and pathway elucidation. Several ribozymes are in various stages of clinical trials. ANGIOZYME was the first chemically synthesized ribozyme to be studied in human clinical trials. ANGIOZYME specifically inhibits formation of the VEGF-r (Vascular Endothelial Growth Factor receptor), a key component in the angiogenesis pathway. Ribozyme Pharmaceuticals, Inc., as well as other firms have demonstrated the importance of anti-angiogenesis therapeutics in animal models. HEPTAZYME, a ribozyme designed to selectively destroy Hepatitis C Virus (HCV) RNA, was found effective in decreasing Hepatitis C viral RNA in cell culture assays (Ribozyme Pharmaceuticals, Incorporated - WEB home page).

An additional method of regulating the expression of miR-9 in cells is via triplex forming oligonucleotides (TFOs). Recent studies have shown that TFOs can be designed which can recognize and bind to polypurine/polypirimidine regions in double-stranded helical DNA in a sequence-specific manner. These recognition rules are outlined by Maher III, L. J., et al., Science, 1989;245:725-730; Moser, H. E., et al., Science, 1987;238:645-630; Beal, P. A., et al, Science,1992;251:1360-1363; Cooney, M., et al., Science,1988;241:456-459; and Hogan, M. E., et al., EP Publication 375408. Modification of the oligonucleotides, such as the introduction of intercalators and backbone substitutions, and optimization of binding conditions (pH and cation concentration) have aided in overcoming inherent obstacles to TFO activity such as charge repulsion and instability, and it was recently shown that synthetic oligonucleotides can be targeted to specific sequences (for a recent review see Seidman and Glazer, J Clin Invest 2003;112:487-94).

In general, the triplex-forming oligonucleotide has the sequence correspondence:

oligo	3'-A	G	G	T
duplex	5'-A	G	C	T
duplex	3'-T	C	G	A

However, it has been shown that the A-AT and G-GC triplets have the greatest triple helical stability (Reither and Jeltsch, BMC Biochem, 2002, Septl2, Epub). The same authors have demonstrated that TFOs designed according to the A-AT and G-GC rule do not form non-specific triplexes, indicating that the triplex formation is indeed sequence specific.

Thus for any given sequence in the miR-9 regulatory region a triplex forming sequence may be devised. Triplex-forming oligonucleotides preferably are at least 15, more preferably 25, still more preferably 30 or more nucleotides in length, up to 50 or 100 bp.

Transfection of cells (for example, via cationic liposomes) with TFOs, and formation of the triple helical structure with the target DNA induces steric and functional changes, blocking transcription initiation and elongation, allowing the introduction of desired sequence changes in the endogenous DNA and resulting in the specific downregulation of gene expression. Examples of such suppression of gene expression in cells treated with TFOs include knockout of episomal supFGl and endogenous HPRT genes in mammalian cells (Vasquez et al., Nucl Acids Res. 1999;27:1176-81, and Puri, et al, J Biol Chem, 2001;276:28991-98), and the sequence- and target specific downregulation of expression of the Ets2 transcription factor, important in prostate cancer etiology (Carbone, et al, Nucl Acid Res. 2003;31:833-43), and the pro-inflammatory ICAM-1 gene (Besch et al, J Biol Chem, 2002;277:32473-79). In addition, Vuyisich and Beal have recently shown that sequence specific TFOs can bind to dsRNA, inhibiting activity of dsRNA-dependent enzymes such as RNA-dependent kinases (Vuyisich and Beal, Nuc. Acids Res 2000;28:2369-74).

Additionally, TFOs designed according to the abovementioned principles can induce directed mutagenesis capable of effecting DNA repair, thus providing both downregulation and upregulation of expression of endogenous genes (Seidman and Glazer, J Clin Invest 2003;112:487-94). Detailed description of the design, synthesis and administration of effective TFOs can be found in U.S. Patent Application Nos. 2003

017068 and 2003 0096980 to Froehler et al, and 2002 0128218 and 2002 0123476 to Emanuele et al, and U.S. Pat. No. 5,721,138 to Lawn.

Expressing the miR-9 downregulating agents of the present invention in cells such as motoneuron cells may be effected using expression constructs encoding the miR-9 downregulating agents and capable of expressing same in motoneuron.

According to some embodiments of the invention, the agent is comprised in a nucleic acid construct (or an expression vector).

The nucleic acid construct (also referred to herein as an "expression vector") of the present invention typically includes additional sequences which render this vector suitable for replication and integration in prokaryotes, eukaryotes, or preferably both (e.g., shuttle vectors). In addition, typical cloning vectors may also contain a transcription and translation initiation sequence, transcription and translation terminator and a polyadenylation signal.

Eukaryotic promoters typically contain two types of recognition sequences, the TATA box and upstream promoter elements. The TATA box, located 25-30 base pairs upstream of the transcription initiation site, is thought to be involved in directing RNA polymerase to begin RNA synthesis. The other upstream promoter elements determine the rate at which transcription is initiated.

Enhancer elements can stimulate transcription up to 1,000 fold from linked homologous or heterologous promoters. Enhancers are active when placed downstream or upstream from the transcription initiation site. Many enhancer elements derived from viruses have a broad host range and are active in a variety of tissues. For example, the SV40 early gene enhancer is suitable for many cell types. Other enhancer/promoter combinations that are suitable for the present invention include those derived from polyoma virus, human or murine cytomegalovirus (CMV), the long term repeat from various retroviruses such as murine leukemia virus, murine or Rous sarcoma virus and HIV. See, Enhancers and Eukaryotic Expression, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. 1983, and Bell MP et al., J Immunol. (2007) 179(3): 1893-900, both of which are incorporated herein by reference.

In the construction of the expression vector, the promoter is preferably positioned approximately the same distance from the heterologous transcription start site as it is

from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

Polyadenylation sequences can also be added to the expression vector in order to increase the efficiency of the miR-9 downregulating agent. Two distinct sequence elements are required for accurate and efficient polyadenylation: GU or U rich sequences located downstream from the polyadenylation site and a highly conserved sequence of six nucleotides, AAUAAA, located 11-30 nucleotides upstream. Termination and polyadenylation signals that are suitable for the present invention include those derived from SV40.

In addition to the elements already described, the expression vector of the present invention may typically contain other specialized elements intended to increase the level of expression of cloned nucleic acids or to facilitate the identification of cells that carry the recombinant DNA. For example, a number of animal viruses contain DNA sequences that promote the extra chromosomal replication of the viral genome in permissive cell types. Plasmids bearing these viral replicons are replicated episomally as long as the appropriate factors are provided by genes either carried on the plasmid or with the genome of the host cell.

The vector may or may not include a eukaryotic replicon. If a eukaryotic replicon is present, then the vector is amplifiable in eukaryotic cells using the appropriate selectable marker. If the vector does not comprise a eukaryotic replicon, no episomal amplification is possible. Instead, the recombinant DNA integrates into the genome of the engineered cell, where the promoter directs expression of the desired nucleic acid.

The expression vector of the present invention can further include additional polynucleotide sequences that allow, for example, the translation of several proteins from a single mRNA such as an internal ribosome entry site (IRES) and sequences for genomic integration of the promoter-chimeric polypeptide.

Examples of mammalian expression vectors include, but are not limited to, pcDNA3, pcDNA3.1 (+/-), pGL3, pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pSinRep5, DH26S, DHBB, pNMTl, pNMT41, pNMT81, which are available from Invitrogen, pCI which is available from Promega, pMbac, pPbac, pBK-RSV and pBK-CMV which are available from Strategene, pTRES which is available from Clontech, and their derivatives.

Expression vectors containing regulatory elements from eukaryotic viruses such as retroviruses can be also used. SV40 vectors include pSVT7 and pMT2. Vectors derived from bovine papilloma virus include pBV-lMTHA, and vectors derived from Epstein Bar virus include pHEBO, and p205. Other exemplary vectors include pMSG, pAV009/A$^+$, pMTO10/A$^+$, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV-40 early promoter, SV-40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. Thus, the type of vector used by the present invention will depend on the cell type transformed. The ability to select suitable vectors according to the cell type transformed is well within the capabilities of the ordinary skilled artisan and as such no general description of selection consideration is provided herein. For example, bone marrow cells can be targeted using the human T cell leukemia virus type I (HTLV-I) and kidney cells may be targeted using the heterologous promoter present in the baculovirus Autographa californica nucleopolyhedro virus (AcMNPV) as described in Liang CY et al., (Arch Virol. 2004;149:51-60).

Various methods can be used to introduce the expression vector of the present invention into the motoneuron. Such methods are generally described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (1989, 1992), in Ausubel et al., Current Protocols in Molecular Biology, John Wiley and Sons, Baltimore, Md. (1989), Chang et al., Somatic Gene Therapy, CRC Press, Ann Arbor, Mich. (1995), Vega et al., Gene Targeting, CRC Press, Ann Arbor Mich. (1995), Vectors: A Survey of Molecular Cloning Vectors and Their Uses, Butterworths, Boston Mass. (1988) and Gilboa et at. [Biotechniques 1986;4:504-512] and include, for example, stable or transient transfection, lipofection, electroporation and infection with recombinant viral vectors. In addition, see U.S. Pat. Nos. 5,464,764 and 5,487,992 for positive-negative selection methods.

Introduction of nucleic acids by viral infection offers several advantages over other methods such as lipofection and electroporation, since higher transfection efficiency can be obtained due to the infectious nature of viruses.

According to some embodiments of the invention, the agent is comprised in a viral vector.

Viral vectors for gene therapy are well known in the art. See for example, Katsuya Inagaki et al. Molecular Therapy (2006) 14, 45-53, which is fully incorporated herein by reference. Adeno-associated viruses (AAV) are commercially available, e.g., from, SIRION Biotech GmbH, Am Klopferspitz 19, 82152 Martinsried, GERMANY.

According to some embodiments of the invention, the viral vector is the recombinant adeno-associated virus pseudotype-9 (rAAV9) expression vector for gene transfer.

According to some embodiments of the invention, the active ingredient does not comprise an agent capable of downregulating a miR selected from the group consisting of: miR133a, miR133b, miR96, miR146, miR30-2, miR125b, miR125a, miR125a-5p, miR125b-5p, miR30a, miR30b, miR146 and miR146a.

According to an aspect of some embodiments of the invention, there is provided a method of treating Amyotrophic lateral scleroses (ALS) in a human subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity and/or expression of miR-9, wherein the administering is to the central nervous system (CNS) of the subject, and/or wherein the pharmaceutical composition further comprises a moiety capable of directing the agent capable of downregulating the activity or the expression of the miR-9 to the central nervous system of the subject, and/or wherein the agent is comprised in a viral vector, and/or wherein the pharmaceutical composition is administered to the subject by intracerebroventricular administration (ICV), and/or wherein the pharmaceutical composition is administered to the subject by intrathecal administration, and/or wherein the pharmaceutical composition is administered to the subject by intracranial (IC) administration, and/or wherein the ALS is caused by a mutation in the SODl gene set forth by SEQ ID NO:2, and/or wherein the ALS is caused by the G94A missense mutation in the SODl polypeptide set forth by SEQ ID NO:l, and/or wherein the active ingredient does not comprise an agent capable

of downregulating a miR selected from the group consisting of: miR133a, miR133b, miR96, miR146, miR30-2, miR125b, miR125a, miR125a-5p, miR125b-5p, miR30a, miR30b, miR146 and miR146a, thereby treating the ALS in the subject in need thereof.

The agent capable of downregulating an activity or expression of miR-9 of some embodiments of the invention can be administered to an organism per se, or in a pharmaceutical composition where it is mixed with suitable carriers or excipients.

According to some embodiments of the invention, the agent capable of downregulating an activity and/or expression of miR-9 is comprised in a pharmaceutical composition along with a pharmaceutically acceptable carrier.

According to an aspect of some embodiments of the invention, there is provided a pharmaceutical composition for treating Amyotrophic lateral scleroses (ALS) in a human subject, comprising as an active ingredient an agent capable of downregulating an activity and/or expression of miR-9, wherein the agent is comprised in a viral vector, and a pharmaceutically acceptable carrier.

According to an aspect of some embodiments of the invention, there is provided a pharmaceutical composition for treating Amyotrophic lateral scleroses (ALS) comprising as an active ingredient an agent capable of downregulating an activity and/or expression of miR-9, with the proviso that the active ingredient does not comprise an agent capable of downregulating a miR selected from the group consisting of: miR133a, miR133b, miR96, miR146, miR30-2, miR125b, miR125a, miR125a-5p, miR125b-5p, miR30a, miR30b, miR146 and miR146a, and a pharmaceutically acceptable carrier.

According to an aspect of some embodiments of the invention, there is provided a pharmaceutical composition for treating Amyotrophic lateral scleroses (ALS) comprising as an active ingredient an agent capable of downregulating an activity and/or expression of miR-9, wherein the pharmaceutical composition is formulated for administration into the central nervous system (CNS) of a subject, and a pharmaceutically acceptable carrier.

According to an aspect of some embodiments of the invention, there is provided a pharmaceutical composition for treating Amyotrophic lateral scleroses (ALS) comprising as an active ingredient an agent capable of downregulating an activity and/or expression of miR-9, wherein the pharmaceutical composition further comprises a moiety capable of directing the agent capable of downregulating the activity or the

expression of the miR-9 to the central nervous system of the subject, and a pharmaceutically acceptable carrier.

According to an aspect of some embodiments of the invention, there is provided a pharmaceutical composition for treating Amyotrophic lateral scleroses (ALS) comprising as an active ingredient an agent capable of downregulating an activity and/or expression of miR-9, wherein the pharmaceutical composition is formulated for intracerebroventricular administration (ICV) administration, and a pharmaceutically acceptable carrier.

According to an aspect of some embodiments of the invention, there is provided a pharmaceutical composition for treating Amyotrophic lateral scleroses (ALS) comprising as an active ingredient an agent capable of downregulating an activity and/or expression of miR-9, wherein the pharmaceutical composition is formulated for intrathecal administration, and a pharmaceutically acceptable carrier.

According to an aspect of some embodiments of the invention, there is provided a pharmaceutical composition for treating Amyotrophic lateral scleroses (ALS) comprising as an active ingredient an agent capable of downregulating an activity and/or expression of miR-9, wherein the pharmaceutical composition is formulated for intracranial (IC) administration, and a pharmaceutically acceptable carrier.

According to some embodiments of the invention, the miR-9 is depicted in SEQ ID NO:3.

According to some embodiments of the invention, the agent capable of downregulating the activity and/or the expression of the miR-9 comprises an antisense sequence of the miR-9.

According to some embodiments of the invention, the antisense sequence is set forth by SEQ ID NO:4.

According to some embodiments of the invention, the viral vector is a recombinant adeno-associated virus pseudotype-9 (rAAV9) expression vector for gene transfer.

According to some embodiments of the invention, the subject is diagnosed with the ALS.

According to some embodiments of the invention, the subject is a carrier of the SOD1 G94A missense mutation as set forth by SEQ ID NO:l.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to the agent capable of downregulating an activity and/or expression of miR-9 accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intracardiac, e.g., into the right or left ventricular cavity, into the common coronary artery, intravenous, intraperitoneal, intranasal, intraocular injections and/or intra central nervous system (CNS) administration.

Conventional approaches for drug delivery to the central nervous system (CNS) include: neurosurgical strategies (e.g., intracerebral injection or intracerebroventricular infusion); molecular manipulation of the agent (e.g., production of a chimeric fusion molecule that comprises a transport peptide that has an affinity for an endothelial cell surface molecule in combination with an agent that is itself incapable of crossing the BBB) in an attempt to exploit one of the endogenous transport pathways of the BBB; pharmacological strategies designed to increase the lipid solubility of an agent (e.g.,

conjugation of water-soluble agents to lipid or cholesterol carriers); and the transitory disruption of the integrity of the BBB by hyperosmotic disruption (resulting from the infusion of a mannitol solution into the carotid artery or the use of a biologically active agent such as an angiotensin peptide).

According to some embodiments of the invention, the pharmaceutical composition is formulated for administration into the central nervous system (CNS) of a subject.

According to some embodiments of the invention, the pharmaceutically acceptable carrier is formulated for intrabrain and/or intra-spinal cord administration.

According to some embodiments of the invention, the pharmaceutical composition is formulated for intracerebroventricular (ICV) administration.

According to some embodiments of the invention, the pharmaceutical composition is formulated for intrathecal administration.

According to some embodiments of the invention, the pharmaceutical composition is formulated for intracranial (IC) administration.

According to some embodiments of the invention, the pharmaceutical composition further comprises a moiety capable of directing the agent capable of downregulating the activity and/or the expression of the miR-9 to the central nervous system of the subject.

One may administer the pharmaceutical composition in a local rather than systemic manner, for example, via injection of the pharmaceutical composition directly into a tissue region of a patient.

Pharmaceutical compositions of some embodiments of the invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with some embodiments of the invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or

liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to some embodiments of the invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The pharmaceutical composition of some embodiments of the invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of some embodiments of the invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients (i.e., an agent which is capable of downregulating the activity and/or expression level of miR-9) effective to prevent, alleviate or ameliorate symptoms of a disorder (e.g., ALS) or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from in vitro and cell culture assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures in vitro, in cell cultures or experimental animals. The data obtained from these in vitro and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.l).

Dosage amount and interval may be adjusted individually to provide brain and/or spinal levels of the active ingredient are sufficient to induce or suppress the biological effect (minimal effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

Compositions of some embodiments of the invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

According an aspect of some embodiments of the invention, there is provided a kit for treating Amyotrophic lateral scleroses (ALS) comprising the pharmaceutical composition of some embodiments of the invention and instructions for treating ALS in a subject (e.g., a human subject).

As used herein the term "about" refers to ± 10% .

The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of means "including and limited to".

The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the

additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example, description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

When reference is made to particular sequence listings, such reference is to be understood to also encompass sequences that substantially correspond to its complementary sequence as including minor sequence variations, resulting from, e.g., sequencing errors, cloning errors, or other alterations resulting in base substitution, base deletion or base addition, provided that the frequency of such variations is less than 1 in

50 nucleotides, alternatively, less than 1 in 100 nucleotides, alternatively, less than 1 in 200 nucleotides, alternatively, less than 1 in 500 nucleotides, alternatively, less than 1 in 1000 nucleotides, alternatively, less than 1 in 5,000 nucleotides, alternatively, less than 1 in 10,000 nucleotides.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al.

(eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

GENERAL MATERIALS AND EXPERIMENTAL METHODS

Mammalian in vivo model: Expression in nervous tissue of adult mice -

The *in vivo* experimental model that was established is based on expression of the recombinant adeno-associated virus pseudotype-9 (rAAV9) expression vector for gene transfer, a versatile tool that efficiently transduces cortical and spinal motor neurons. As proof of concept in preliminary experiments, eGFP-encoding rAAV9 was introduced into the lateral ventricle of P60 wild type mice. Eight weeks post-injection, eGFP was still expressed throughout the lumbar spinal cord and in the dorsal corticospinal tract, which contains mostly axons originated from the motor cortex.

The procedure included introduction of a 33 gauge Hamilton syringe into the lateral ventricle to stereotactic coordinates -0.58 mm posterior, 1.15 mm lateral, -1.97 mm ventral bregma (following Paxinos & Watson Mouse Brain Atlas 1998).

4xl0 [10] genome copies in a volume of 5 µï (at 0.25 µï/min) were introduced. Ten minutes later, the needle was withdrawn and scalp was stitched with acrylic thread.

Knockdown of specific miRNAs was accomplished by delivering Tough Decoy using the rAAV9 vector (rAAV9-TuD-9) (obtained through a collaboration with G. Gao, U. Mass. Medical School). rAAV9-TuD-scramble was used as control. This vector gives long lasting expression of the encoded sequence: expression of a G-Luciferase reporter co-expressed from the virus was present twelve weeks after transduction, establishing prolonged expression of the rAAV9-TuD-9 vector.

Furthermore, knockdown of miRNA expression was verified by RT-PCR, data normalized to the expression of U6 and Sno234 and then to the expression levels in the control experiment.

EXAMPLE 1

MIR-9 IS UPREGULATED IN THE SPINAL CORD OF ALS (SODG93A) MOUSE MODEL

Experimental Results

Mir-9 is upregulated in the spinal cord of ALS (SOD G93A mutants) mouse model - In the present study the present inventors focused on the participation of miR-9 expression in the pathogenesis of ALS. First, the level of miR-9 was measured in most common ALS mouse model, SOD1 G93A high copy number transgene males [Gurney, M.E., et al., 1994. Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation. Science, 264(5166): 1772-5.]. The present inventors performed multiple time-point analysis of miR-9 levels by RT-qPCR in RNA extracted from lumber spinal cord at postnatal day 60 (P60), 90 (P90) and 120 (P120) of mutants and corresponding littermate controls that did not harbor the transgene (N=3 per each group). As shown in Figure 1, miR-9 expression levels were comparable with control at P60 and P90. The expression levels of miR-9 in control littermates remained relatively stable from P60 to P120. In contrast, SOD1 G93A mutants exhibited upregulation of miR-9 which was observed at P120. This finding is in accordance with recent other reports that characterized miR-9 levels in spinal cord [Zhou, F., et al., miRNA-9 expression is upregulated in the spinal cord of G93A-SOD1 transgenic mice. Int J Clin Exp Pathol, 2013. 6(9): 1826-38] or brain [Marcuzzo, S., et al., Up-regulation of neural

and cell cycle-related microRNAs in brain of amyotrophic lateral sclerosis mice at late disease stage. Mol Brain, 2015. 8(1): p. 5] of ALS G93A mouse model.

EXAMPLE 2

DIRECT MANIPULATION OF MIR-9 EXPRESSION IN CENTRAL NERVOUS SYSTEM AND SPINAL CORD OF ALS MOUSE MODEL SOD G93A

Experimental Results

***Efficient transduction of motorneurons using intracerebroventricular** (ICV) **injection of rAAV9** -* The present inventors sought to directly manipulate miR-9 expression in the central nervous system and spinal cords of adult SOD1 G93A mice. To this end, the present inventors utilized a platform for somatic trangenesis of adult mouse brains with recombinant adeno-associated viruses, psuedotype 9 (rAAV9) [Xie, J., et al., *Long-term, efficient inhibition of microRNA function in mice using rAAV vectors.* Nat Methods. 9(4): p. 403-9]. The present inventors used this viral system to inject the lateral ventricle of adult mice at P60 (postnatal day 60). Intracerebroventricular (ICV) injection of rAAV9 that drives the expression of GFP demonstrated the efficiency in transducing rAAV9 into motor neurons (Figures 2A-G) which is corresponded with previous studies [Zhang, H., et al., Several rAAV vectors efficiently cross the blood-brain barrier and transduce neurons and astrocytes in the neonatal mouse central nervous system. Mol Ther. 19(8): p. 1440-8; Thomsen, G.M., et al., Delayed disease onset and extended survival in the SOD1G93A rat model of amyotrophic lateral sclerosis after suppression of mutant SOD1 in the motor cortex. J Neurosci, 2014. 34(47): p. 15587-600]. In parallel, the present inventors performed ICV injection of rAAV9 into P2 (postnatal day 2) neonate mice that resulted in comparable dissemination of the virus (data not shown).

Manipulation of miR-9 RNA levels using the rAAV9 viruses - miR-9 overexpression in SOD1 G93A spinal cords, resulted in upregulation of miR-9, relative to control vector that harbored a scrambled miRNA control sequence (Figures 2I and 2J). Likewise, knockdown miR-9 was performed by using a tough decoy (TuD) vector that is specifically inhibiting miR-9 activity and resulted in downregulation of miR-9 levels in SOD1 G93A spinal cords, relative to control TuD viruses (Figures 2H and 2J). It should be noted that since the analysis included whole spinal cord tissue, and AAV9

has motor-neuron preference, these measurements probably reflect an underestimate of the changes in miR-9 levels in motor neurons.

EXAMPLE 3

THE EFFECT OF OVEREXPRESSION OF DOWNREGULATION OF MIR-9 ON ALS PATHOGENESIS

Experimental Results

Overexpression of miR-9 results in a decrease in the number of motoneurons in the ventral horn of SOD G93A mice - By using the above system the present inventors have further characterized the impact of miR-9 on ALS pathogenesis. To this end the present inventors transduced the CNS of P2 (postnatal 2) mice, which were segregated by P30 (postnatal day 30) by genotype and gender. The final experimental cohort included therefore more than 20 SODl G93A male littermates that were divided into four sub-groups by the viral vector. N > 4 in each of the following groups: Males with miR-9 overexpression; males with control overexpression virus; males with miR-9 knockdown TuD virus; and males with control KD virus. Figures 3A-D show the effect of the miR-9 overexpression (Figures 3A and 3C) and miR-9 knockdown (Figures 3B and 3D) vectors on the level of miR-9 level in the motoneurons in the lumbar region. In addition, Figure 3E demonstrates that overexpression of miR-9 results in a significant decrease in the number of motoneurons in the ventral horn of the SOD G93A mice.

Down-regulation of miR-9 expression level results in increased survival of SODl G93A mice - As is shown in Figures 4A-D, reduction of miR-9 expression within motor neurons is beneficial for the SODl G93A mice in terms of higher survival rate (Figure 4A, red solid line), delayed onset of symptoms (Figure 4B, red solid line), higher weight peak (Figure 4C, red solid line) and extended time on rotarod (Figure 4D, triangle symbols) as compared to control mice treated with the scrambled control vector.

Up-regulation of miR-9 expression level results in decreased survival and worsening of neurodegenerative symptoms in SODl G93A mice - The survival of SODl G93A mice was reduced by miR-9 overexpression (Figure 4A, blue solid line) as compared to mice treated with the control vector (Figure 4A, light blue dotted line).

Onset of disease, that was defined as the time mice failed to abduct hind limbs when grabbed from their tails, was consistent (Figure 4B).

Onset of disease and onset of wasting (weight loss) was also identified earlier in miR-9 overexpressing SOD1 G93A mice (Figure 4C, blue solid line) and was delayed in miR-9 knockdown mice (Figure 4C, red solid line). Together these data suggest that in adult SOD1 mice, reduction in miR-9 levels is beneficial and higher levels of miR-9 impose additional insult and facilitate disease progression.

To further understand which miRNAs and protein are involved in the pathway downstream of miR-9, mRNA targets of miR-9 were studied by overexpression of miR-9 and RNA sequencing approach. RNA was extracted from primary motor neurons transfected with synthetic RNA mimetics miRNA-9 that recapitulate miR-9 or with control RNAs with irrelevant sequence. As shown in Figure 5, Sylamer analysis, which was implemented to assessing over- and under-representation of miRNA recognition sequences (seed-matches) for all known miRNAs, in 6500 expressed genes, ranked from mostly down-regulated to up-regulated in primary motor neurons overexpressing miR-9, identified only two enriched motifs, both matching the miR-9 'seed' sequence.

Analysis and Discussion

Without being bound by any theory, the present inventor has hypothesized that normalizing the expression of miR-9 may be beneficial if the elevated expression of miR-9 has a role in the progress of ALS. In order to explore this hypothesis the present inventors manipulated miR-9 levels in the adult mouse via somatic viral transgenesis.

The data presented herein demonstrate that reduction of miR-9 expression in spinal cord and brain of SOD1G93A mice increases survival and improves neuromuscular function. On the other hand, miR-9 overexpression resulted in notable decreases SOD1G93A mice survival.

The novel platform for manipulation of miRNA expression in a tissue-specific manner *in vivo* allows the present inventors to evaluate the roles of miRNAs in motor neurons as well as to provide a definitive answer as to their role in the pathogenesis of neurodegenerative diseases such as ALS. Finally, this study serves as a platform for a novel ALS therapy approach by miRNA.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are used, they should not be construed as necessarily limiting.

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WHAT IS CLAIMED IS:

1. A method of treating Amyotrophic lateral scleroses (ALS) in a human subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity or expression level of miR-9, thereby treating the ALS in the human subject in need thereof.

2. A method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity or expression level of miR-9, wherein said ALS is caused by a mutation in the SOD1 gene set forth by SEQ ID NO:2, thereby treating the ALS in the subject in need thereof.

3. A method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity or expression level of miR-9, wherein said ALS is caused by the G94A missense mutation in the SOD1 polypeptide set forth by SEQ ID NO:l, thereby treating the ALS in the subject in need thereof.

4. A method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity or expression level of miR-9, wherein said agent is comprised in a viral vector, thereby treating the ALS in the subject in need thereof.

5. A method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity or expression level of miR-9, wherein said active ingredient does not comprise

an agent capable of downregulating a miR selected from the group consisting of: miR133a, miR133b, miR96, miR146, miR30-2, miR125b, miR125a, miR125a-5p, miR125b-5p, miR30a, miR30b, miR146 and miR146a, thereby treating the ALS in the subject in need thereof.

6. A method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity or expression level of miR-9, wherein said pharmaceutical composition is administered to the subject by intracerebroventricular administration (ICV), thereby treating the ALS in the subject in need thereof.

7. A method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity or expression level of miR-9, wherein said pharmaceutical composition is administered to the subject by intrathecal administration, thereby treating the ALS in the subject in need thereof.

8. A method of treating Amyotrophic lateral scleroses (ALS) in a subject in need thereof, the method comprising administering to the subject a pharmaceutical composition comprising as an active ingredient an agent capable of downregulating an activity or expression level of miR-9, wherein said pharmaceutical composition is administered to the subject by intracranial (IC) administration, thereby treating the ALS in the subject in need thereof.

9. The method of any one of claims 1, 2, 3, 4, and 5, wherein said pharmaceutical composition is administered to the central nervous system (CNS) of the subject.

10. The method of any one of claims 1-9, wherein said pharmaceutical composition further comprises a moiety capable of directing said agent capable of

downregulating said activity or said expression level of said miR-9 to the central nervous system of the subject.

11. The method of any one of claims 1, 4, 5, and 6-10, wherein said ALS is caused by a mutation in the SOD1 gene set forth by SEQ ID NO:2.

12. The method of claim 11, wherein said mutation is the G94A missense mutation in the SOD1 polypeptide set forth by SEQ ID NO:l.

13. The method of any one of claims 1-4, and 6-12, with the proviso that said active ingredient does not comprise an agent capable of downregulating a miR selected from the group consisting of: miR133a, miR133b, miR96, miR146, miR30-2, miR125b, miR125a, miR125a-5p, miR125b-5p, miR30a, miR30b, miR146 and miR146a.

14. The method of any one of claims 1-13, wherein said miR-9 is depicted in SEQ ID NO:3.

15. The method of any one of claims 1-14, wherein said agent capable of downregulating said activity or said expression level of said miR-9 comprises an antisense sequence of said miR-9.

16. The method of claim 15, wherein said antisense sequence is set forth by SEQ ID NO:4.

17. The method of any of claims 1-3, and 5-16, wherein said agent is comprised in a viral vector.

18. The method of claim 4 or 17, wherein said viral vector is a recombinant adeno-associated virus pseudotype-9 (rAAV9) expression vector.

19. The method of any of claims 1-5, and 9-18, wherein said pharmaceutical composition is administered to the subject by intracerebroventricular administration (ICV).

20. The method of any of claims 1-5 and 9-18, wherein said pharmaceutical composition is administered to the subject by intrathecal administration.

21. The method of any of claims 1-5 and 9-18, wherein said pharmaceutical composition is administered to the subject by intracranial (IC) administration.

22. The method of any one of claims 1-21, wherein the subject is diagnosed with the ALS.

23. The method of any one of claims 1, 2, and 4-22, wherein the subject is a carrier of the SODl G94A missense mutation as set forth by SEQ ID NO:l.

24. The method of any one of claims 1-22, further comprising selecting a subject which is a carrier of the SODl G94A missense mutation as set forth by SEQ ID NO:l, wherein presence of at least one allele of said SODl 94A variant as set forth by SEQ ID NO:l indicates that the active ingredient is suitable for treating the subject.

25. The method of any one of claims 1-24, wherein said administering is effected prior to appearance of at least one ALS symptom, wherein said at least one of ALS symptom is selected from the group consisting of: difficulty walking, weakness in leg(s), weakness in hand(s), slurring of speech, trouble swallowing, muscle cramps, and twitching in arms, shoulders or tongue.

26. The method of any one of claims 1-24, wherein said administering is effected concomitantly or after appearance of at least one ALS symptom selected from the group consisting of: difficulty walking, weakness in leg(s), weakness in hand(s), slurring of speech, trouble swallowing, muscle cramps, and twitching in arms, shoulders or tongue.

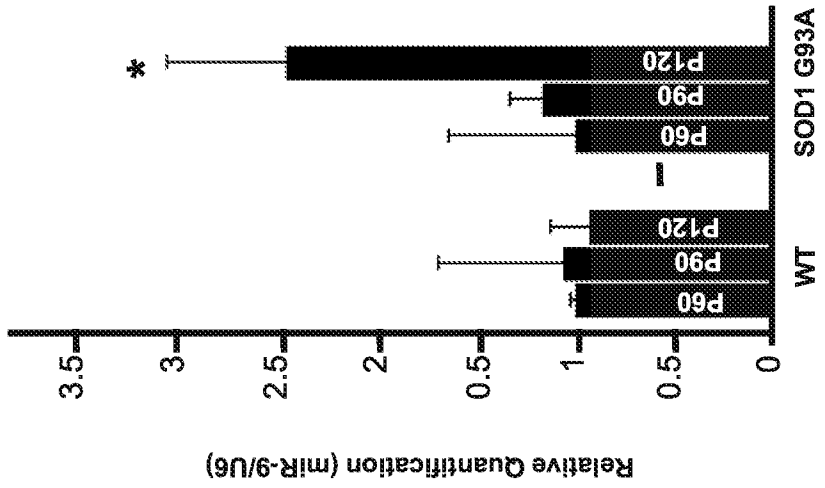

Relative Quantification (miR-9/U6)
3.5
3
2.5
2
0.5
1
0.5
0
WT
SOD1 G93A
P60
P90
P120
P60
P90
P120
*
*

FIG. 1

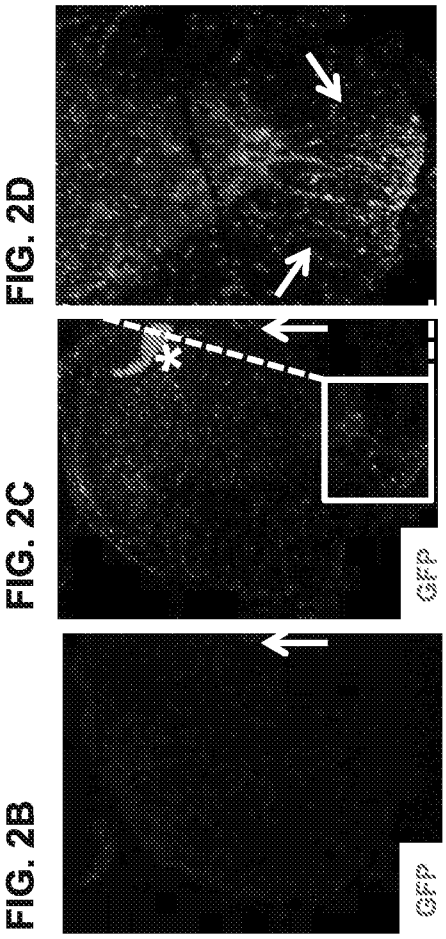


FIG. 2B

FIG. 2C

FIG. 2D

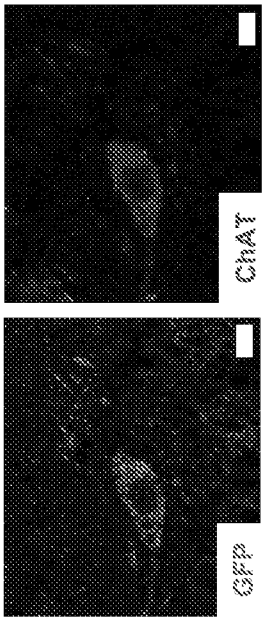


FIG. 2F

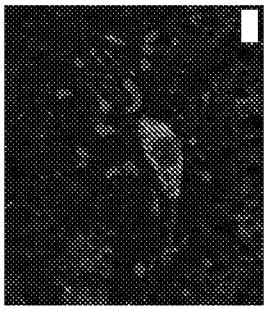


FIG. 2G

FIG. 2E

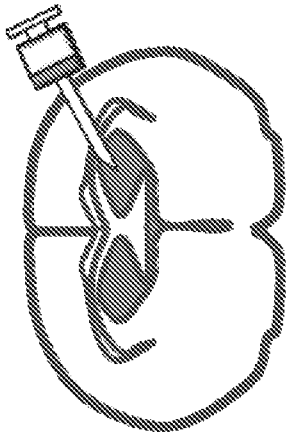
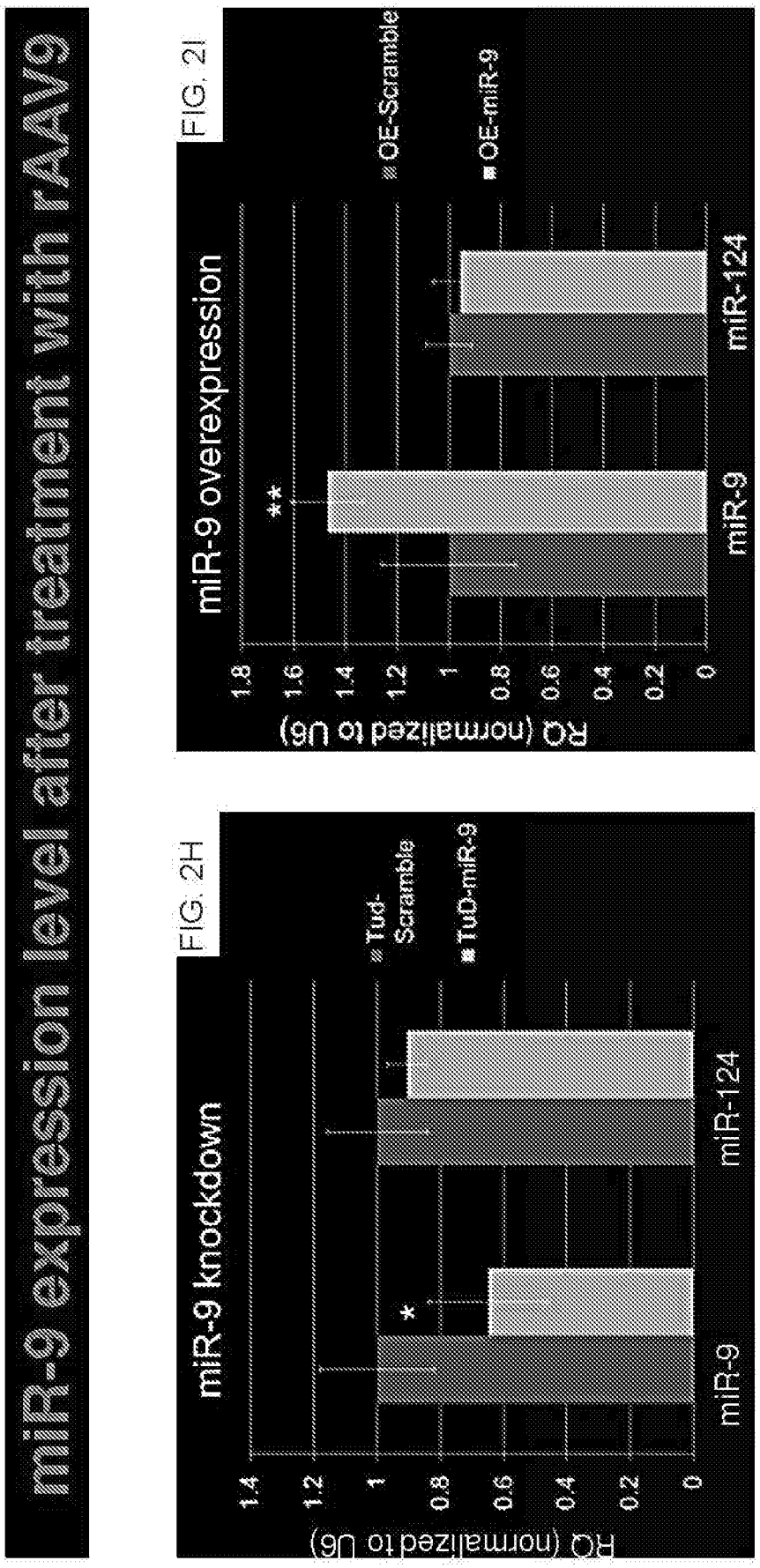


FIG. 2A


miR-9 expression level after treatment with rAAV9
FIG. 2H
miR-9 knockdown
RQ (normalized to U6)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Tud-Scramble
TuD-miR-9
*
miR-9
miR-124
FIG. 2I
miR-9 overexpression
RQ (normalized to U6)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OE-Scramble
OE-miR-9
**
miR-9
miR-124

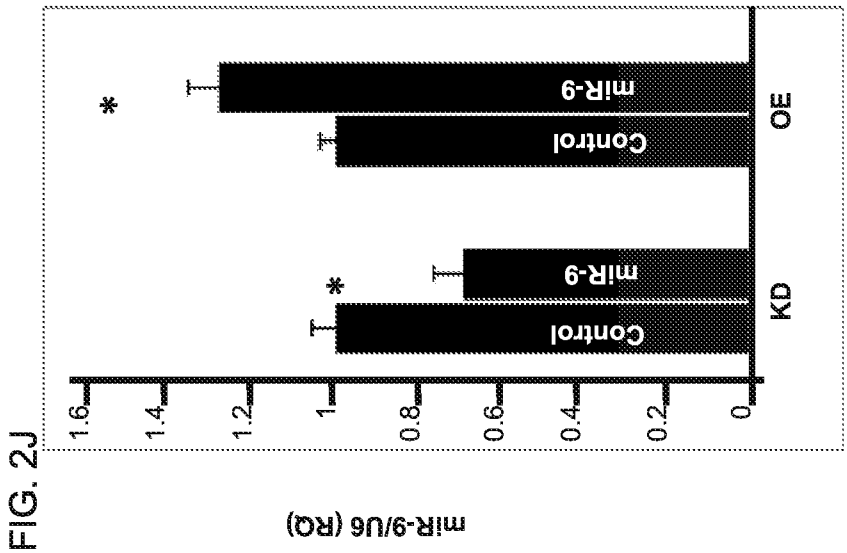

miR-9/U6 (RQ)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
KD
OE
Control
miR-9
Control
miR-9
*
*

FIG. 2J

FIG. 3A
Overexpression
Knock-down
Control
miR-9

FIG. 3B

FIG. 3E
# of motor neuron/ ventral horn
35
30
25
20
15
10
5
0
OE-Ctrl
OE-miR-9
KD-Ctrl
KD-miR-9

FIG. 3C
Overexpression
Control
miR-9
10x
20x

FIG. 3D
Knock-down
10x
20x

FIG. 4A

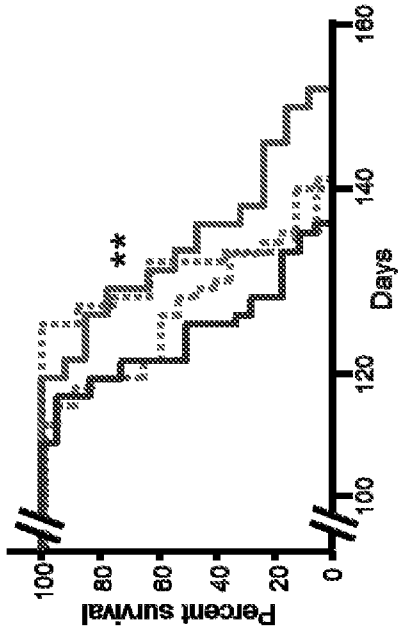


FIG. 4B

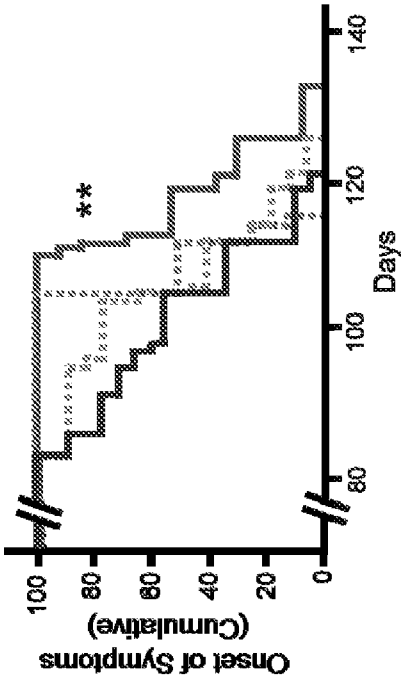


FIG. 4C

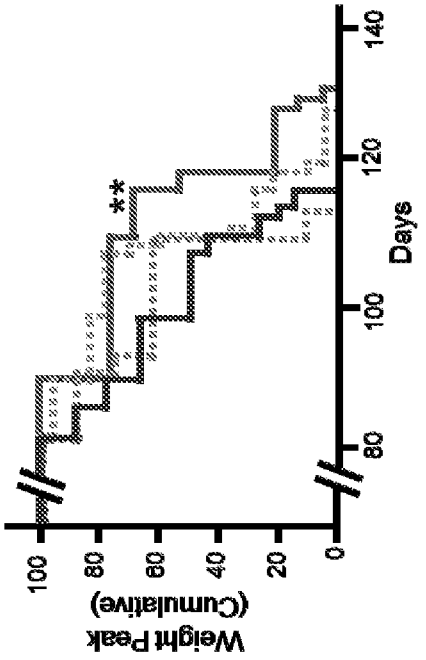
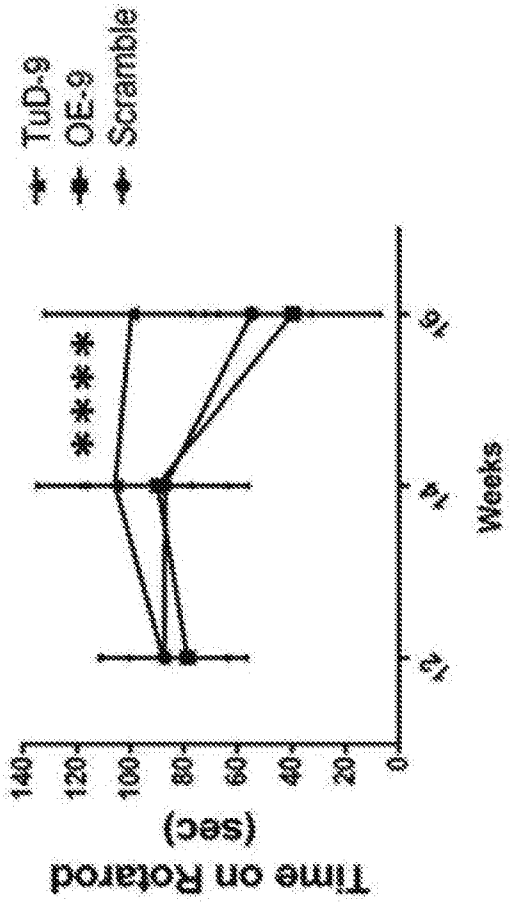


FIG. 4D



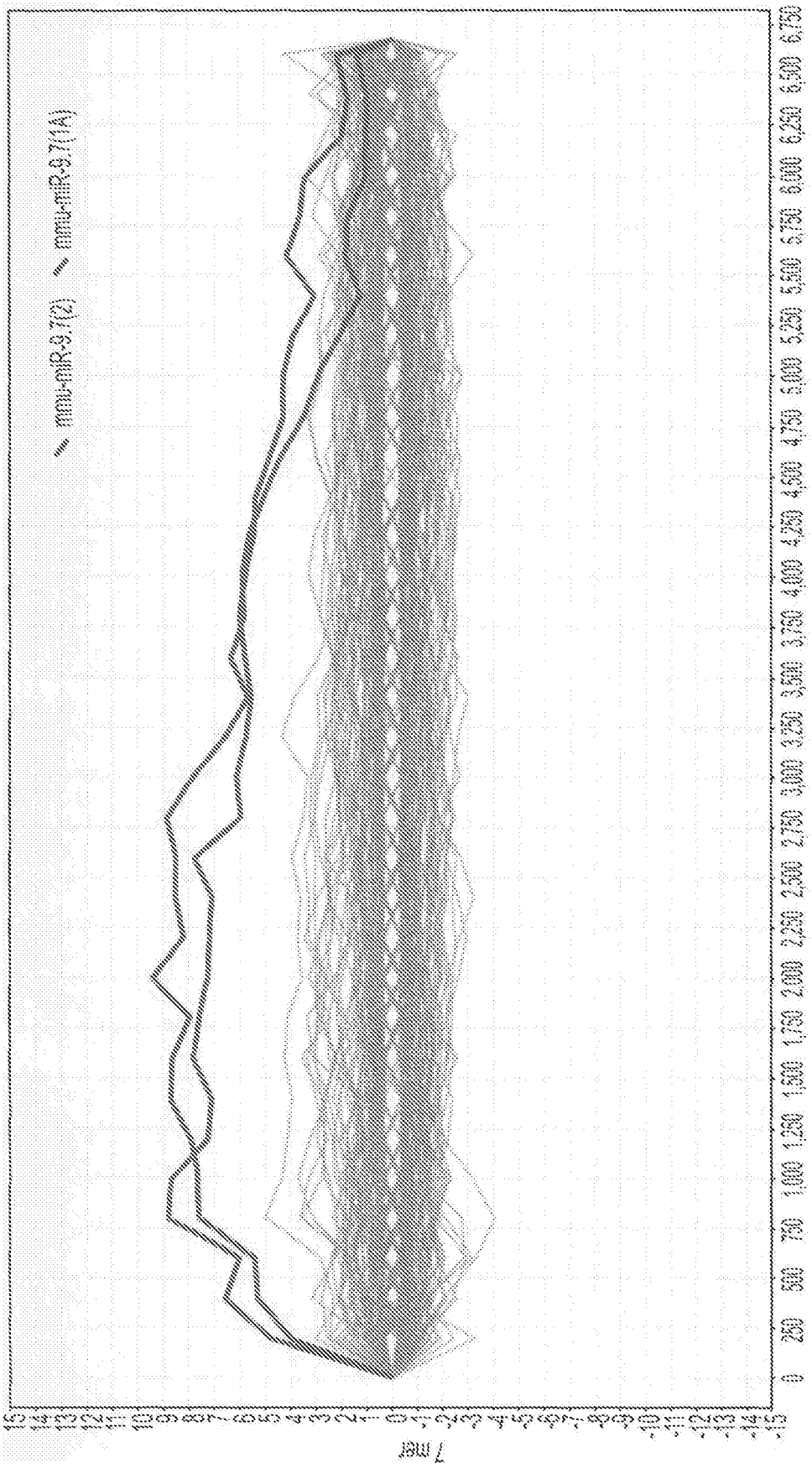

mmu-miR-9.7(2)
mmu-miR-9.7(1A)
7 mer
0 250 500 750 1,000 1,250 1,500 1,750 2,000 2,250 2,500 2,750 3,000 3,250 3,500 3,750 4,000 4,250 4,500 4,750 5,000 5,250 5,500 5,750 6,000 6,250 6,500 6,750

FIG. 5

INTERNATIONAL SEARCH REPORT

International application No
PCT/IL2015/050867

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/113 A61K31/7088
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal , BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	Fenghua Zhou ET AL.: "miRNA-9 expression is upregulated in the spinal cord of G93A-S0D1 transgenic mice", International Journal of Clinical and Experimental Pathology, 1 September 2013 (2013-09-01), pages 1826-1838, XP055230499, Retrieved from the Internet: URL: http://www.ncbi.nlm.nih.gov/pmc/articles/ PMC3759489/pdf/ijcep0006-1826.pdf the whole document --- WO 2014/020608 Al (YEDA RESEARCH AND DEVELOPMENT CO. LTD. [IL]; HORNSTEIN ERAN; EMDE A.M.) 6 February 2014 (2014-02-06) claims page 47 the whole document --- -/- ·	1-26 1-26

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" documentwhich may throw doubts on priority claim(s) orwhich is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
2 December 2015	11/01/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Macchia, Giovanni

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2010/064248 A2 (YEDA RESEARCH AND DEVELOPMENT CO. LTD. [IL]; HORNSTEIN CHEN HARAMATI) 10 June 2010 (2010-06-10) claims page 5 the whole document	1-26
A	WO 2010/144698 A2 (HENRY FORD HEALTH SYSTEM [US]; BRODIE CHAYA [US]; SLAVIN SHIMON [IL]) 16 December 2010 (2010-12-16) claims paragraph [[0019]] the whole document	1-26
A	ZHIJUN ZHANG ET AL.: "Downregulation of microRNA-9 in iPSC-derived neurons of FTD/ALS patients with TDP-43 mutations", PLOS ONE, vol. 8, no. 10, E76055, 15 October 2013 (2013-10-15), XP055230323, DOI: 10.1371/journal.pone.0076055 the whole document	1-26
A	-& Zhijun Zhang et al.: "supporting information to Downregulation of microRNA-9 in iPSC-derived neurons of FTD/ALS patients with TDP-43 mutations", PLOS ONE , October 2013 (2013-10), XP002751630, Retrieved from the Internet: URLr http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0076055#s5 [retrieved on 2015-11-30] the whole document	1-26
A	KATIE NOLAN ET AL.: "Increased expression of microRNA-29a in ALS mice: functional analysis of its inhibition", JOURNAL OF MOLECULAR NEUROSCIENCE, vol. 53, no. 2, 4 April 2014 (2014-04-04), pages 231-241, XP055230288, US ISSN: 0895-8696, DOI: 10.1007/sl2031-014-0290-y the whole document	1-26

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	STEFANIA MARCUZZO ET AL. : "Al tered mi RNA expressi on i s associ ated wi t h neuronal fate i n G93A-S0D1 ependymal stem progeni tor cel l s", EXPERIMENTAL NEUROLOGY, vol . 253 , 1 March 2014 (2014-03-01) , pages 91-101 , XP055230309 , US ISSN : 0014-4886, D0I : 10. 1016/j .expneurol .2013 . 12 .007 the whol e document	1-26
A	-& MARCUZZO S. et al . : "Suppl emental Informati on for Al tered mi RNA expressi on i s associ ated wi t h neuronal fate i n G93A-S0D1 ependymal stem progeni tor cel l s", EXPERIMENTAL NEUROLOGY , 1 March 2014 (2014-03-01) , XP055232337 , Retri eved from the Internet: URL: http ://www. sci encedi rect . com/sci ence/M i amiMul tiMedi aURL/l-s2 .0-S0014488613003737 /l-s2 .0-S0014488613003737-mmcl . doc/272536/ html /S0014488613003737/d3372385653040fca68 a9e2bada3c9e5/mmcl . doc [retri eved on 2015-11-30] the whol e document	1-26
A	E. D. K0VAL ET AL. : "Method for wi despread mi croRNA-155 i nhi bi t i on prol ongs survi val i n ALS-model mi ce" , HUMAN MOLECULAR GENETICS, vol . 22 , no. 20, 15 October 2013 (2013-10-15) , pages 4127-4135 , XP055165068, ISSN : 0964-6906, D0I : 10. 1093/hmg/ddt261 the whol e document	1-26
A	C00LEN MARION ET AL. : "mi R-9 : a versati l e regul ator of neurogenesi s", FRONTI ERS I N CELLULAR NEUROSCI ENCE, vol . 7 , 220, 2013 , pages 1-11 , XP002751726, ISSN : 1662-5102 the whol e document	1-26

-/--

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	STEFANIA MARCUZZO ET AL.: "Up-regulation of neural and cell cycle-related microRNAs in brain of amyotrophic lateral sclerosis mice at late disease stage", MOLECULAR BRAIN, vol. 8, no. 1, 28 January 2015 (2015-01-28), page 5, XP021213302, BIOMED CENTRAL LTD, LONDON UK ISSN: 1756-6606, DOI: 10.1186/S13041-015-0095-0 the whole document	
T	-& MARCUZZO S. et al.: "additional files to Up-regulation of neural and cell cycle-related microRNAs in brain of amyotrophic lateral sclerosis mice at late disease stage", MOLECULAR BRAIN , 28 January 2015 (2015-01-28), XP002751727, Retrieved from the Internet: URL: http://molecularbrain.biomedcentral.com/articles/10.1186/sl3041-015-0095-0#Secl9 [retrieved on 2015-11-30] the whole document	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL201 5/050867

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13fer1 (a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13fer1 (a)).

      [ ] on paper or in the form of an image file (Rule 13fer1 (b) and Administrative Instructions, Section 713).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IL2015/050867

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014020608 Al	06-02-2014	CN 104684558 A	03-06-2015
		EP 2879678 Al	10-06-2015
		HK 1205453 Al	18-12-2015
		JP 2015523410 A	13-08-2015
		US 2015164891 Al	18-06-2015
		WO 2014020608 Al	06-02-2014
WO 2010064248 A2	10-06-2010	EP 2373301 A2	12-10-2011
		EP 2633854 Al	04-09-2013
		ES 2442168 T3	10-02-2014
		US 2011239312 Al	29-09-2011
		US 2014243393 Al	28-08-2014
		WO 2010064248 A2	10-06-2010
WO 2010144698 A2	16-12-2010	CA 2772869 Al	16-12-2010
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		KR 20120098573 A	05-09-2012
		US 2012148550 Al	14-06-2012
		WO 2010144698 A2	16-12-2010