(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 December 2010 (16.12.2010)

PCT

(10) International Publication Number
WO 2010/144698 A2

(51) International Patent Classification:
A61K 48/00 (2006.01)

(21) International Application Number:
PCT/US2010/038168

(22) International Filing Date:
10 June 2010 (10.06.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/185,773 10 June 2009 (10.06.2009) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

(54) **Title**: METHODS, SYSTEMS, AND COMPOSITIONS FOR NEURONAL DIFFERENTIATION OF MULTIPOTENT STROMAL CELLS

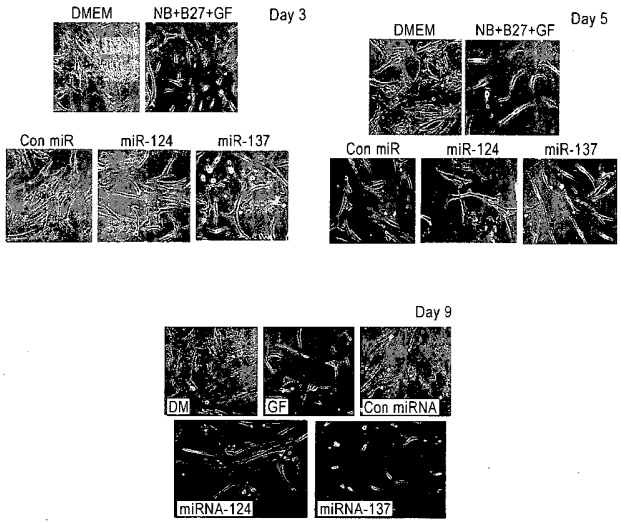

FIG. 1

(57) **Abstract**: Some embodiments of the invention comprise methods, systems, and compositions to selectively induce, whether in vitro or in vivo, the neuronal differentiation of multipotent stromal cells through the application of microRNAs, including but not limited to miRNA- 124, miRNA-137 and/or miRNA-9* expression products of those miRNAs, and molecules and compositions containing functional elements of those miRNAs. Some embodiments of the invention also comprise the therapeutic administration and use of such induced cells to treat mammalian injuries and diseases, including but not limited to, nervous system injuries or diseases that may otherwise result in decreased cell or system function.

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METHODS, SYSTEMS, AND COMPOSITIONS FOR NEURONAL DIFFERENTIATION OF MULTIPOTENT STROMAL CELLS

TECHNICAL FIELD

[0001] Without limitation, certain embodiments of the invention relate to induction and application of cell types for the treatment of mammalian nervous system injuries and diseases.

BACKGROUND

[0002] Certain nervous system injuries, autoimmune diseases affecting the central or peripheral nervous system, and neurodegenerative diseases are characterized by loss of specific cells, or abnormal functions of existing nerve cells, which cause the patient to present with different neurological signs and symptoms and potentially irreversible loss of neurological functions. As one example only, some patients suffering stroke, spinal cord injury, or other neural injury and degeneration experience loss of functioning cell types, or neurological conditions like Parkinson's disease and Alzheimer's disease which in turn results in loss of or abnormal function of system function. Currently therapeutic options for treating and restoring such cell and system functions are limited. Thus, a need remains for methods, systems, and compositions to promote additional therapies, including therapies addressed to replacement of missing or damaged nervous system cells, tissues, and functions.

BRIEF SUMMARY

[0003] Without limitation to only those embodiments described herein and without disclaimer, some embodiments of the invention comprise methods, systems, and compositions to selectively induce, whether *in vitro* or *in vivo*, the neuronal differentiation of multipotent stromal cells through the application of microRNAs, including but not limited to miRNA-124, miRNA-137 and/or miRNA-9* expression products of those miRNAs, and molecules and compositions containing functional elements of those miRNAs. Some embodiments of the invention also comprise the therapeutic administration and use of such induced cells to treat mammalian injuries and diseases, including but not limited to, nervous system injuries or diseases that may otherwise result in decreased cell or system function.

BRIEF DESCRIPTION OF THE DRAWINGS

[0004] Some embodiments of the present invention will now be described, by way of example only and without disclaimer of other embodiments, with reference to the accompanying drawings, in which:

[0005] FIG. 1 shows bright field images of MSCs treated with growth factors or transfected with control miRNA and miRNA-124 or miRNA-137 for 3, 5 and 9 days.

[0006] FIG. 2 is a data representation showing that miRNA-124, miRNA-137 and miRNA-9* induce neuronal markers in MSCs.

[0007] FIG. 3 shows Western Blot results following transfection of cells with tested miRNAs or treatment with DMEM.

[0008] FIG. 4 shows results of transfecting adipose and cord derived MSCs with control miRNA, miRNA-124, or miRNA-137.

DETAILED DESCRIPTION

[0009] Without limitation to only those embodiments expressly disclosed herein and without disclaiming any embodiments, some embodiments of the invention comprise methods, systems, and composition to selectively induce, whether *in vitro* or *in vivo*, the neuronal differentiation of multipotent stromal cells ("MSCs") through the application of microRNAs ("miRNA(s)" or "miR(s)"), including but not limited to, miRNA-124 and/or miRNA-137, and/or miRNA-9*, expression products of those miRNAs, and molecules and compositions containing functional elements of those miRNAs. Some embodiments of the invention also comprise the therapeutic administration and use of such induced cells to treat mammalian injuries and diseases, including but not limited to, nervous system injuries or diseases that may otherwise result in decreased cell or system function. In some embodiments, such induction of differentiated MSCs, and/or the resulting cells, may be used to treat cell, tissue, or organ damage in a patient by administering to said patient a therapeutically effective amount of an miRNA of interest, or of differentiated MSCs induced by such miRNAs.

[00010] We have discovered unexpectedly that certain miRNAs are capable of inducing long-term neuronal differentiation of MSCs for the use of cell-based therapies in subjects presenting with nervous system injury and disease, including but not limited to, neurodegenerative disorders and spinal injury. Such subjects may include mammals, including but not limited to, humans. Thus, we have discovered novel applications for such miRNAs and resulting induced MSCs which, among other possible uses, can reduce or alleviate the effects of certain nervous system injuries or diseases in mammals.

[00011] Without limitation, some embodiments of the invention comprise methods, systems, and/or compositions for inducing neuronal differentiation of MSCs through the use and expression of miRNA-124, miRNA-137, and/or miRNA-9*. MSCs are mesoderm-derived cells that typically reside in adult bone marrow, typically at very low concentration (about 1 in 10,000 nucleated cells). MSCs can differentiate to generate cells such as bone marrow stroma, blood vessels, fat, bone and cartilage. These cells may also have the potential to differentiate into neurons\ or glia-like cells depending on the environmental signals. Moreover, these cells may be further induced to express or maintained specific neuronal or glial phenotypes by incubation with different combinations of growth factors and hormones.

[00012] MSCs have been shown to exert therapeutic effects in a variety of neurological diseases and dysfunctions in experimental animal models and more recently in pilot clinical trials. Their effects have been mainly attributed to immunosuppressive and neuroprotective functions. In experimental autoimmune encephalitis ("EAE"), an animal model of multiple sclerosis ("MS"), treatment of mice with bone marrow derived MSCs resulted in significant suppression of disease manifestations. Some studies demonstrated that in addition to down regulation of autoimmunity neural differentiation of these cells increased their therapeutic effect in various instances such as the ischemic brain.

[00013] In our work, we tested the effect of three neuronal-associated miRNAs, miRNA-l24, miRNA-137, and miRNA-9*, on the differentiation of human MSCs. These miRNAs are not normally expressed in MSCs. We discovered that the expression of miRNA-124, miRNA137, or miRNA-9* induced neuronal differentiation of MSCs, as indicated by the morphology of the cells and by the increased expression of βIII-tubulin and MAP2. miRNA-124, miRNA-137 and miRNA-9* induced an increase in tyrosine hydroxylase,

suggesting differentiation of the MSCs to dopaminergic phenotype. One of the targets of pre-miRNA124 is the transcription factor REST that represses a large number of neuronal genes. Our results indicate that neuronal-associated miRNAs may induce long-term neuronal differentiation of MSCs for the use of cell-based therapy in neurodegenerative and neuroinflammatory disorders and spinal injury. One advantage of the use of miRNAs over the existing methods is that one can stably express pre-miRNAs in MSCs that will result in long-term neuronal differentiation, as compared with transient differentiation that is induced by treatment with growth factors. As such, easy access to patient's own bone marrow derived MSCs and the feasibility to enrich and expand MSCs in large numbers indicates that neuronal differentiation of such cells can serve as autologous neuronal stem cells that can be available for treatment of a large number of acquired or congenital neurological disorders associated with lack of or damaged neurons. As one example only without limitation, MSCs can be prepared from fat removed by liposuction and from cord blood or the placenta. Reduced immunogenicity of MSCs may facilitate the use of allogeneic neurons off the shelf or from matched or partially mismatched family member for treatment of conditions caused, as nonlimiting examples only, by congenital deficiencies of essential enzymes or other essential products.

[00014] Without limitation to only embodiments expressly disclosed herein, and without disclaiming any embodiments, some embodiments of the invention comprise:

[00015] 1. the neuronal differentiation of MSCs through culture or other exposure to miRNAs, including but not limited to, miRNA124 and/or miRNA 137, and/or miRNA 9*.

[00016] 2. transfection of MSCs with such miRNAs;

[00017] 3. administration of MSCs induced *in vitro* into neuronal differentiation to a subject suffering from nervous system injury or disease; and/or

[00018] 4. administration of MSCs transfected with such miRNAs to a subject suffering from nervous system injury or disease.

[00019] In some embodiments, without limitation, with reproducible transdifferentiation of MSCs to neurons, the therapeutic use of MSCs can be obtained and expanded, whether *in vitro* or *in vivo*, to include, as some examples only, treatment of

cerebrovascular disease, spinal cord injury, treatment of neurodegenerative disorders such as amyotrophic lateral sclerosis ("ALS"), multiple sclerosis ("MS"), and related motor neuron diseases. Ongoing clinical studies already indicate that infusion of MSCs intrathecally and intravenously can improve partially the clinical manifestation of the disease in patients with MS and to a lesser extent in patients with ALS. Such clinical studies provide evidence that both intrathecal and intravenous infusions of MSCs are safe procedures since none of the treated patients has developed any severe side effect. Thus, cell therapy with MSCs represents prophetically an important approach for the treatment of a large number of neurological disorders, especially where MSCs can be induced into neurons or oligodendrocytes and/or secrete factors that can induce neurogenesis of locally residing stem cells.

[00020] **Examples:**

[00021] The following examples of some embodiments of the invention are provided without limiting the invention to only those embodiments described herein and without disclaiming any embodiments.

[00022] **microRNAs**

[00023] microRNAs ("miRNAs") represent a family of endogenous, small (as some nonlimited examples, 19-23 nucleotides) non-coding RNAs that function through the RNA interference ("RNAi") pathway to effect post-transcriptional gene silencing. miRNAs target the mRNAs of specific genes based on complementarity, and mediate either mRNA cleavage (perfect complementarity) or translation repression (partial complementarity). miRNAs have been demonstrated to play important roles in development and may function as fundamental genetic regulatory elements that serve to establish or maintain specific expression profiles determining cell fate.

[00024] Manipulating neuronal differentiation of MSCs may involve regulatory pathways that orchestrate the program of gene expression during the differentiation process. Differentiation often requires shifts in the mRNA and protein constitution of cells. One class of gene regulatory molecules are the microRNAs, a subclass of small RNAs, that are thought to use the elements of the RNA-interference pathway to post transcriptionally down-regulate the expression of protein-coding genes. miRNAs may play an important role in

cell differentiation since they are predicted to individually regulate hundreds of target genes simultaneously.

[00025] **Methods**

[00026] To determine the effect of miRNA-124, miRNA-137, and miRNA-9* on the differentiation of MSCs, we employed three different preparations of these cells in passages 4-12. MSC cells were plated in DMEM + 10% FCS for 24 hr and were then transfected with double-stranded RNA oligonucleotides of the mature sequence of the three miRNAs and with a negative control oligonucleotide. The miRNAs used were as follows:

[00027] Dharmacon mimic products:

MI0000443 / MIMAT0000422 - Human
Selected Precursor/Mature
Mature:
hsa-miR-124 [MIMAT0000422]
Precursor:
hsa-miR-124-1 [MI0000443]
Organism:
Human
Mature Sequence:
UAAGGCACGCGGUGAAUGCC (SEQ ID NO. 1)

[00028] MI0000454 / MIMAT0000429 – Human

Selected Precursor/Mature
Mature:
hsa-miR-137 [MIMAT0000429]
Precursor:
hsa-miR-137 [MI0000454]
Organism:
Human
Mature Sequence:
UUAUUGCUUAAGAAUACGCGUAG (SEQ ID NO. 2)

[00029] miRNA 9* sequence:

[00030] AUAAAGCUAGAUAACCGAAAGU (SEQ ID NO. 3)

[00031] miRNA 9 mimic:

[00032] UCUUUGGUUAUCUAGCUGUAUGA (SEQ ID NO. 4)

[00033] Following 3 days, cells were transferred to Neurobasal Medium (NB) supplemented with B27. Cell morphology was monitored every 24 hr and analysis of neuronal markers by either immunofluorescence staining, Western blot analysis or real-time PCR was performed following 5, 7 and 9 days post-transfection. As a positive control for the induction of neuronal differentiation, we used cells stimulated with combination of Shh, FGF8 and bFGF.

[00034] **Results**

[00035] *miRNA-124, miRNA-137, and miRNA-9* promote neuronal differentiation of MSCs.* The pictures of FIG. 1 are representative of six separate experiments that gave similar results. As presented in FIG. 1, transfection of the cells with miRNA-137, miRNA-124 or miRNA-9* decreased cell proliferation and induced morphological differentiation in the cells already after 72 hr of transfection. Transfection of the MSCs with miRNA-137 induced rapid and robust morphological changes and the cells acquired a typical neuronal phenotype with compact cell bodies and elongated processes with varicosities. miRNA-124-transfected cells exhibited a strong decrease in cell proliferation followed by the generation of a number of cell types; elongated cells with long processes, small cells with multiple shorter processes and flat star-like cells. Cells transfected with the control miRNA resembled the control untreated cells. Interestingly, the effect of miRNA-137 was more rapid and stronger than that of the GF. About 90% of the miRNA-137 transfected cells exhibited neuronal morphology.

[00036] *miRNA-124, miRNA-137, and miRNA-9* increase the expression of neuronal markers in MSCs.* To further examine the effect of miRNA-124, miRNA-137 and miRNA-9* on neuronal differentiation, we examined the expression of the neural stem cell marker, nestin, the astrocytic marker GFAP and the neuronal markers beta III–tubulin and tyrosine hydroxylase. Cells were transfected with the appropriate miRNA or treated with DMEM or with neurobasal medium+B27. Following 5 days, the expression of nestin mRNA was determined using real-time PCR and the expression of nestin, GFAP, beta III-tubulin and

tyrosine hydroxylase was examined after 9 days of treatment by Western blot analysis. The results represent five different experiments that gave similar results. FIG. 2 shows that after 5 days of transfection, there was a large increase in nestin mRNA as determined by real-time PCR. In contrast, after 9 days of transfection with the different miRNAs we found an increase in the expression of beta III-tubulin, whereas no expression of nestin or GFAP was observed. In addition we found that miRNA-137 and miRNA-9* induced a large increase in the expression of tyrosine hydroxylase, whereas a smaller increase was observed in miRNA-124 transfected cells. The expression of all these markers in the control miRNA transfected cells was absent or negligible.

[00037] *miR-9* induced the dopaminergic marker, tyrosine hydroxylase, in MSCs.* FIG. 3 shows Western Blot results following MSC transfection with the appropriate miRNAs or treatment with DMEM. Following 9 days, the expression tyrosine hydroxylase was examined by Western blot analysis. The results represent five different experiments that gave similar results. miRNA-9* induced the dopaminergic marker, tyrosine hydroxylase, in MSCs.

[00038] Our results demonstrate that miRNA-124, miRNA-137 and miRNA-9* induce neuronal differentiation of MSCs, albeit to respectively different degrees in our test model. miRNA-137 induces a more rapid and robust effect resulting in a homogenous population of neuronal cells. The high level of tyrosine hydroxylase expressed in these cells suggests that these cells display a dopaminergic phenotype.

[00039] miRNA-124 also induces neuronal differentiation as determined by the high level of βIII- tubulin compared to the control miRNA-treated cells. In our work, this treatment resulted in a mixed population of cells which expressed lower level of tyrosine hydroxylase. None of the treatments induced astrocytic differentiation as determined by the lack of GFAP expression.

[00040] Moreover, following 5 days of treatment, both miRNAs induced a large transient increase in nestin expression, indicating generation of neural stem cell-like or neuronal progenitor-like cells. A controlled differentiation of MSCs to NSC or NPC-like cells may be further exploited to differentiate these cells to different neuronal lineages or to neurons with different phenotypes using specific transcription factors or specific combination of growth factors.

[00041] miRNA-124 and miRNA-9* have been reported to be involved in neuronal differentiation and neurite outgrowth. Similarly, there is one report demonstrating the effect of miRNA137 on neuronal differentiation of glioma stem cells and NSCs. However, no effects of miRNAs have been reported on the neuronal differentiation of MSCs and no effect of miRNA124 and miRNA137 has been shown on the generation of neurons with a specific phenotype. Moreover, none of these miRNAs has been reported to induce cells with a NSC/NPC phenotype.

[00042] *miRNA-124 and miRNA-137 induced neuronal differentiation in the Adipose and cord derived MSCs*. Adipose and cord derived MSCs were transfected with control miRNA, miRNA-124 and miRNA-137 similar to the bone marrow MSCs (FIG. 4).

[00043] Preparation of adipose-derived MSCs: Adipose-derived MSCs were obtained from liposuction from the thighs or abdominal walls. 100-200 ml aspirates were processed in a special designed Cytori separator that separates the MSCs from the fats cells and debris. The cells were further processed and maintained as described for the bone-marrow derived MSCs.

[00044] Preparation of human umbilical (cord) MSCs: Fresh human umbilical cords were obtained after birth (with parental consent) and collected in DMEM at 4°C. The umbilical cord vessels were removed and the mesenchymal tissue (Wharton's jelly) was minced into small pieces. Following centrifugation, at 250×g for 5 min the tissue was washed with serum-free DMEM was treated with collagenase at 37°C for 18 h followed by digestion with 2.5% trypsin at 37°C for 30 min. The dissociated MSCs were further dispersed and maintained in conditions similar to those described for bone marrow-derived MSCs.

[00045] After 12 days, mRNA was extracted and the levels of b3-tubulin and the house keeping gene S12 were determined using real-time PCR.

[00046] Our results (FIG. 4) demonstrate that miRNA-124 and miRNA-137 induce neuronal differentiation not only in bone-marrow derived MSCs but also in adipose-tissue and cord blood-derived cells. The neuronal marker beta III tubulin was induced in these cells following miRNA treatment. Each of these cell sources has its own advantages. Bone-marrow derived MSCs are very well characterized and have been used for over 20 years successfully with no oncogenic potential. Adipose-derived MSCs are less characterized but

can be obtained in larger numbers and cord blood cells can be easily obtained in a non-invasive manner they do not require complete genetic compatibility between the donor and the patient and therefore are more accessible.

[00047] *Construction of a plasmid containing pre-miRNA and GDNF.* Since GDNF has been implicated in the survival of dopaminergic neurons, we constructed plasmids that co-express pre-miRNA-124 or pre-miRNA-137 together with GDNF under separate promoters.

[00048] Without limitation to only embodiments described herein, and without disclaiming any embodiments, steps for sequence and procedure of cloning the pre-miRNA GDNF vectors and that of the miRNAs are described with respect to step by step cloning of GDNF into premir vectors (CD-511_1 or PCDH-CMV-MCS-EF1-copGFP from System Biosciences).

[00049] cDNA of Homo sapiens glial cell derived neurotrophic factor ("GDNF") template was obtained from Origene. For cloning GDNF into premir 124 and 137 vectors (System Biosciences), primers with Xho1 and Sal1 restriction enzyme digestion sites for GDNF ORF were designed as follows:

[00050] Forward: cacc ctcgag(Xho1) atg aag tta tgg gat gtc gtg gct gtc tgc (SEQ ID NO. 5)

[00051] Reverse: aaa gtcgac(Sal1) tca gat aca tcc aca cct ttt agc gga atg (SEQ ID NO. 6)

[00052] After PCR, the GDNF DNA product was cleaned, then digested with Xho1 and Sal1, and the DNA was cleaned again, resulting in DNA of GDNF now ready for cloning.

[00053] Xho1 restriction site was added into the vector of premir 124 and premir 137 by using primers:

[00054] Forward: gac gcc acc atg gag agc ctc gag (Xho1) agc ggc ctg ccc gcc (SEQ ID NO. 7)

[00055] Reverse: ggc ggg cag gcc gct ctc gag (Xho1) gct ctc cat ggt ggc gtc (SEQ ID NO. 8)

[00056] The GFP gene was removed from premir 124 and 137 vector by using restriction enzymes Xho1 and Sal1, then the vector was cleaned.

[00057] Ligation of GDNF and premir 124 and 137 vectors. The ligated plasmids were transformed into One shot Top10 chemical competent cell. Plasmids with premir 124 and 137 were selected by culturing the clones, following by processing with mini prep.

[00058] The plasmids were digested by using Xho1 and Sal1 to detect the insert of GDNF. The plasmids were then sequenced. The sequence of miR-124 and 137 and the backbone of the premir vector:

[00059] MiR-124:

[00060] GAACAAAGAGCCTTTGGAAGACGTCGCTGTTATCTCATTGTCTGTG
TGATTGGGGGAGCTGCGGCGGGGAGGATGCTGTGGTCCCTTCCTCCGGCGTTCCC
CACCCCCATCCCTCTCCCCGCTGTCAGTGCGCACGCACACGCGCCGCTTTTTATTT
CTTTTTCCTGGTTTTCTTATTCCATCTTCTACCCACCCCTCTTCCTTTCTTTCACCTT
TCCTTCCTTCCTTCCTCCTTTCCTTCCTCAGGAGAAAGGCCTCTCTCTCCGTGTTCA
CAGCGGACCTTGATTTAAATGTCCATACAATTAAGGCACGCGGTGAATGCCAAG
AATGGGGCTGGCTGAGCACCGTGGGTCGGCGAGGGCCCGCCAAGGAAGGAGCG
ACCGACCGAGCCAGGCGCCCTCCGCAGACCTCCGCGCAGCGGCCGCGGGCGCGA
GGGGAGGGGTCTGGAGCTCCCTCCGGCTGCCTGTCCCGCACCGGAGCCCGTGGG
GTGGGGAGGTGTGCAGCCTGTGACAGACAGGGGCTTAGAGATGC (SEQ ID NO. 9)

[00061] MiR-137:

[00062] CAGCACTCTTCTGTGTTAAGTATTTGATTTTGTGATTTGTCTTTCAG
AATTGGAAATAGAGCGGCCATTTGGATTTGGGCAGGAAGCAGCCGAGCACAGCT
TTGGATCCTTCTTTAGGGAAATCGAGTTATGGATTTATGGTCCCGGTCAAGCTCA
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CGGCAGCTTGGTCCTCTGACTCTCTTCGGTGACGGGTATTCTTGGGTGGATAATA
CGGATTACGTTGTTATTGCTTAAGAATACGCGTAGTCGAGGAGAGTACCAGCGGC

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[00063] The sequence of backbone vector (CD-511_1) was attached:

[00064] LOCUS CD511B_1_pCDH_CMV_ 7544 bp ds-DNA circular 16-DEC-2008:

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ACCESSION
VERSION
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ORGANISM
COMMENT
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7381 attcgccatt caggctgcgc aactgttggg aagggcgatc ggtgcgggcc tcttcgctat
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7501 tttcccagtc acgacgttgt aaaacgacgg ccagtgccaa gctg (SEQ ID NO. 11)

[00065] We found that MSCs transfected with these plasmids secrete GDNF and express the respective miRNAs. Thus, the GDNF secreted by the differentiated dopaminergic

neurons is expected to provide survival signals to the differentiated cells and to endogenous dopaminergic neurons.

[00066] *Construction of inducible miRNAs.* Implanted MSCs have been reported to migrate to damaged tissues in the central nervous systems and to exert neurotrophic and immunomodulatory effects. Specifically, in Parkinson's animal models, implanted MSCs have been shown to engraft in the lesioned striatum. In some embodiments, without limitation, inducible pre-miRNA expression vectors might be used that will allow the induction of the specific pre-miRNA expression at desired time points. Thus, MSCs would be transfected with the specific pre-miRNA and its expression would be induced at different time points prior or following the engraftment of the MSCs in the lesioned striatum. For such studies we have employed the inducible miRNA and living color, fluorescent protein reporters using the Tet-on system (Clontech). This system allows the induction of the specific miRNA by the addition of a promoter, as one example, only, by doxycyline, and the identification of cells in which the miRNA is produced.

[00067] In summary, we have demonstrated the ability of miRNA124, miRNA137 and miRNA-9* to induce transdifferentiation of MSCs to NSC/NPC and neurons with a specific neuronal phenotype (miRNA137). Additional neuronal miRNAs such as miRNA-9 and miR218 may also effect transdifferentiation of MSCs and induce neuronal differentiation.

[00068] An advantage of using miRNAs over the existing methods is that one can stably express pre-miRNAs in the MSCs which will result in long-term neuronal differentiation as compared with transient differentiation that is induced by treatment with growth factors.

[00069] Our work indicates that neuronal-associated miRNAs may be employed to induce long-term neuronal differentiation of MSCs for the use of cell-based therapy in neurodegenerative disorders and spinal injury, as some examples only, as shown by:

[00070] 1. Neuronal differentiation of MSCs by microRNAs (miRNA-124, miRNA-137, miRNA-9*);

[00071] 2. Specific dopaminergic differentiation of MSCs by miRNA-137, miRNA-124 and miRNA-9*; and

[00072] 3. Induction by microRNAs of transient differentiation of MSCs to neural stem cell like- or neural progenitor-like cells. Transfection with the miRNAs provides a window of opportunity where cells can be differentiated to the different lineages of the central nervous system (neurons, astrocytes and oligodendrocytes) or to a specific neuronal phenotype using transcription factors or a specific combination of growth factors. This window can be controlled by level of miRNA expression or by a specific time point post-transfection.

[00073] The ability of miRNAs to transdifferentiate MSCs to uncommitted progenitor cells and to different neuronal cell subsets makes it possible to use these cells for treatment of a large variety of neurological diseases, including spinal cord and peripheral nerve injuries, damage to the central nervous system caused by hemorrhage or obstructive lesions ("CVA") or to traumatic central or peripheral nerve injury. In addition, transdifferentiated MSCs may be employed in the case of neurodegenerative diseases caused by idiopathic autoimmune diseases ("EAE") or diseases such as Parkinson's disease or Alzheimer/s disease or diseases with unknown etiology such as ALS. Moreover, improvement of neurological functions by transdifferentiated MSCs may also be used in various degenerative disorders caused by drug-induced neuronal damage and /or toxicity.

[00074] Thus, in our work, miRNA-124, miRNA-137 and miRNA-9* promote neural differentiation of MSC's, with accompanying morphological changes and expression of phenotypic markers.

[00075] The inducing miRNA(s) of some embodiments would be administered and dosed in accordance with good medical practice, taking into account the techniques of use to accomplish the desired effect of target MSCs, the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners. The "pharmaceutically effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve improvement, including but not limited to, the desired differentiation of MSCs *in vivo* and/or *in vitro*, decreased damage or injury, or improvement or elimination of symptoms and other indicators as are selected as appropriate measures by those skilled in the art.

[00076] Embodiments of the invention may expand the therapeutic window for treatment of nervous system injury and diseases and could be applied to treatment of a large patient population which suffers such injury and diseases each year in the United States. Thus, in some embodiments, the invention comprises novel methods to prevent, control, or alleviate mammalian nervous system injury and disease, including without limitation, brain damage, neural degeneration, or spinal cord injury, through the selective application of inducing miRNAs comprising embodiments of the invention. In accordance with some embodiments, without limitation, one may effect such therapeutic intervention through the use and/or administration of one or more such miRNAs to induce differentiation in target cells *in vivo* or *in vitro* for use in treatment to limit the effects of such injury or disease. Thus, without limitation and without disclaimer of subject matter, some embodiments comprise novel compositions and methods to prevent, control, or alleviate mammalian injury, including without limitation, brain damage, through the selective application and/or induction of transdifferentiated MSCs.

[00077] This application may reference various publications by author, citation, and/or by patent number, including without limitation, articles, presentations, and United States patents. The disclosures of each of any such references in their entireties are hereby incorporated by reference into this application.

[00078] While the present invention has been particularly shown and described with reference to the foregoing preferred and alternative embodiments, it should be understood by those skilled in the art that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention without departing from the spirit and scope of the invention as defined in the following claims. It is intended that the following claims define the scope of the invention and that the method and apparatus within the scope of these claims and their equivalents be covered thereby. This description of the invention should be understood to include all novel and non-obvious combinations of elements described herein, and claims may be presented in this or a later application to any novel and non-obvious combination of these elements. The foregoing embodiments are illustrative, and no single feature or element is essential to all possible combinations that may be claimed in this or a later application. Where the claims recite "a" or "a first" element of the equivalent thereof, such claims should be understood to include incorporation of one or more such elements, neither requiring nor excluding two or more such elements.

CLAIMS

What is claimed is:

1. A method of stimulating neuronal differentiation of multipotent stromal cells from a mammal, comprising the steps of:

providing a composition comprised of microRNA-124, and

administering to a mammalian population of multipotent stromal cells a pharmaceutically effective amount of said composition in order to stimulate neuronal differentiation in the population.

2. The method of claim 1, wherein the mammal is a human.

3. A method of stimulating neuronal differentiation of multipotent stromal cells from a mammal, comprising the steps of:

providing a composition comprised of microRNA-137, and

administering to a mammalian population of multipotent stromal cells a pharmaceutically effective amount of said composition in order to stimulate neuronal differentiation in the population.

4. The method of claim 3, wherein the mammal is a human.

5. A method of stimulating neuronal differentiation of multipotent stromal cells from a mammal, comprising the steps of:

providing a composition comprised of microRNA-9*, and

administering to a mammalian population of multipotent stromal cells a pharmaceutically effective amount of said composition in order to stimulate neuronal differentiation in the population.

6. The method of claim 5, wherein the mammal is a human.

7. Use of microRNA-124 in a medicament for stimulating neuronal differentiation of multipotent stromal stem cells of a mammal.

8. The use of claim 7, wherein the mammal is a human.

9. Use of microRNA-137 in a medicament for stimulating neuronal differentiation of mesenchymal stem cells of a mammal.

10. The use of claim 9, wherein the mammal is a human.

11. Use of microRNA-9* in a medicament for stimulating neuronal differentiation of multipotent stromal cells of a mammal.

12. The use of claim 11, wherein the mammal is a human.

13. A method of treating a mammal suffering from nervous system injury or disease, comprising the steps of:

stimulating neuronal differentiation of multipotent stromal cells by exposing such cells *in vitro* to microRNA-124, microRNA-137, or microRNA-9*, individually or in any combination thereof; and

administering such neuronally differentiated cells to the mammal.

14. The method of claim 13, wherein the mammal is a human.

15. A method of treating a mammal suffering from nervous system injury or disease, comprising the steps of:

transfecting multipotent stromal cells *in vitro* with microRNA-124, microRNA-137, or microRNA-9*, individually or in any combination thereof; and

administering such transfected cells to the mammal.

16. The method of claim 15, wherein the mammal is a human.

17. A method of treating a mammal suffering from nervous system injury or disease, comprising the steps of:

transfecting multipotent stromal cells with a vector comprised of one or more nucleotides coding for microRNA-124, microRNA-137, or microRNA-9*, individually or in any combination thereof, wherein expression of such nucleotide(s) in the vector is inducible by a promoter,

administering such transfected cells to the mammal, and

inducing expression of the nucleotide(s) within the mammal to produce microRNA-124, microRNA-137, or microRNA-9* by administering the promoter to the mammal.

18. The method of claim 15, wherein the mammal is a human.

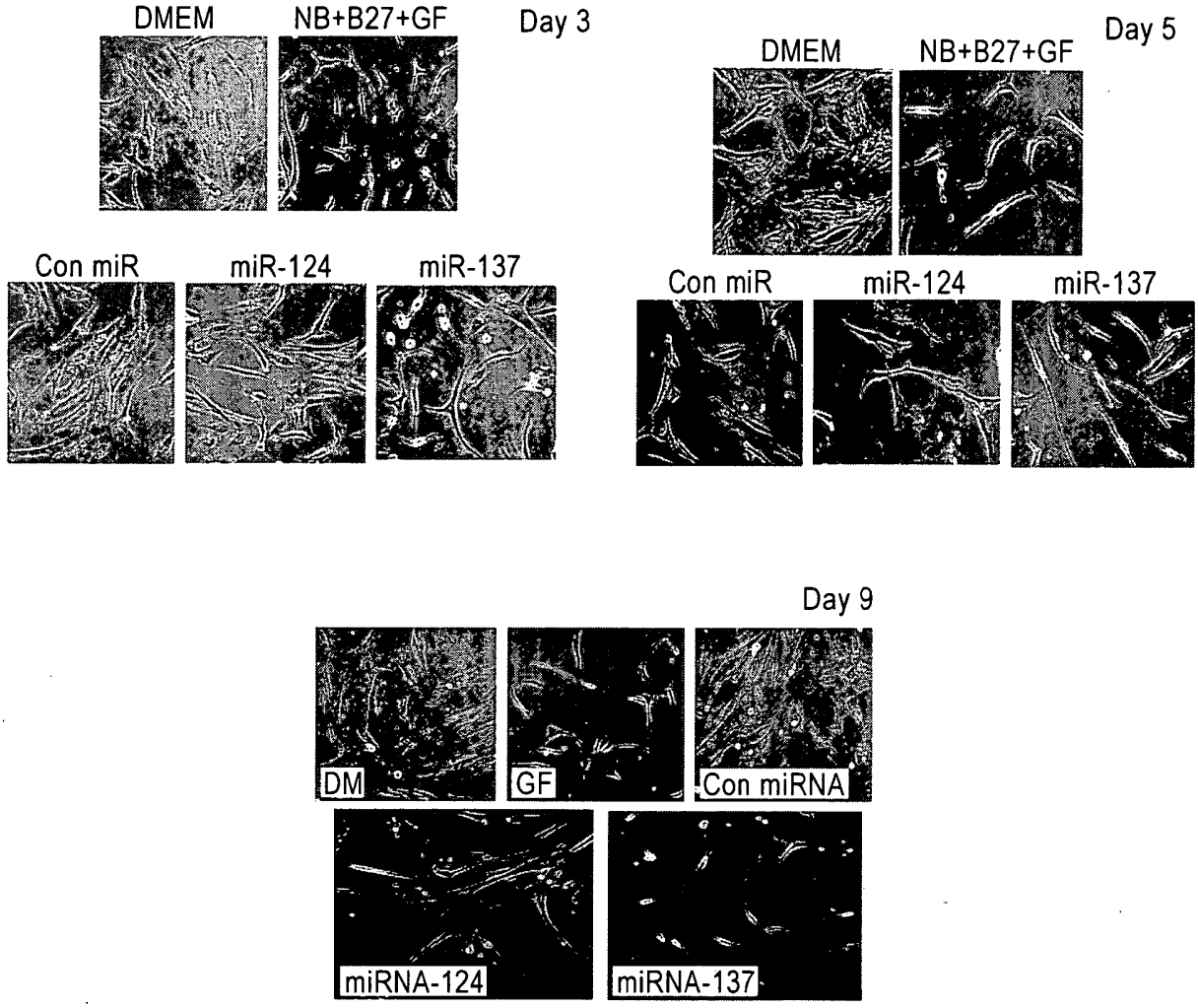
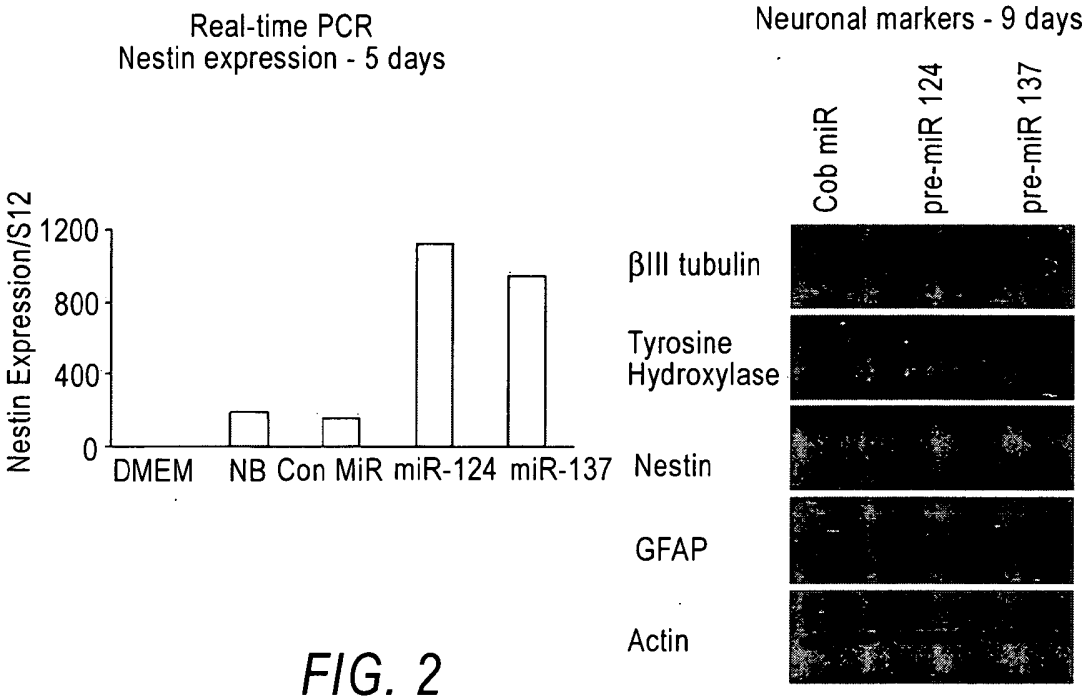

DMEM
NB+B27+GF
Day 3
Con miR
miR-124
miR-137
Day 5
DMEM
NB+B27+GF
Con miR
miR-124
miR-137
Day 9
DM
GF
Con miRNA
miRNA-124
miRNA-137

FIG. 1


Real-time PCR
Nestin expression - 5 days
Nestin Expression/S12
1200
800
400
0
DMEM
NB Con MiR
miR-124
miR-137
Neuronal markers - 9 days
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pre-miR 124
pre-miR 137
βIII tubulin
Tyrosine Hydroxylase
Nestin
GFAP
Actin

FIG. 2

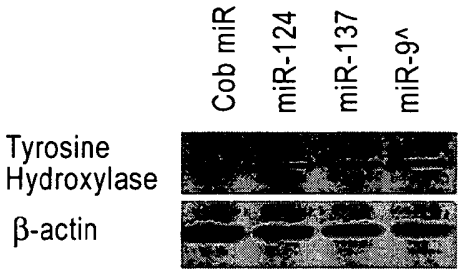

FIG. 3

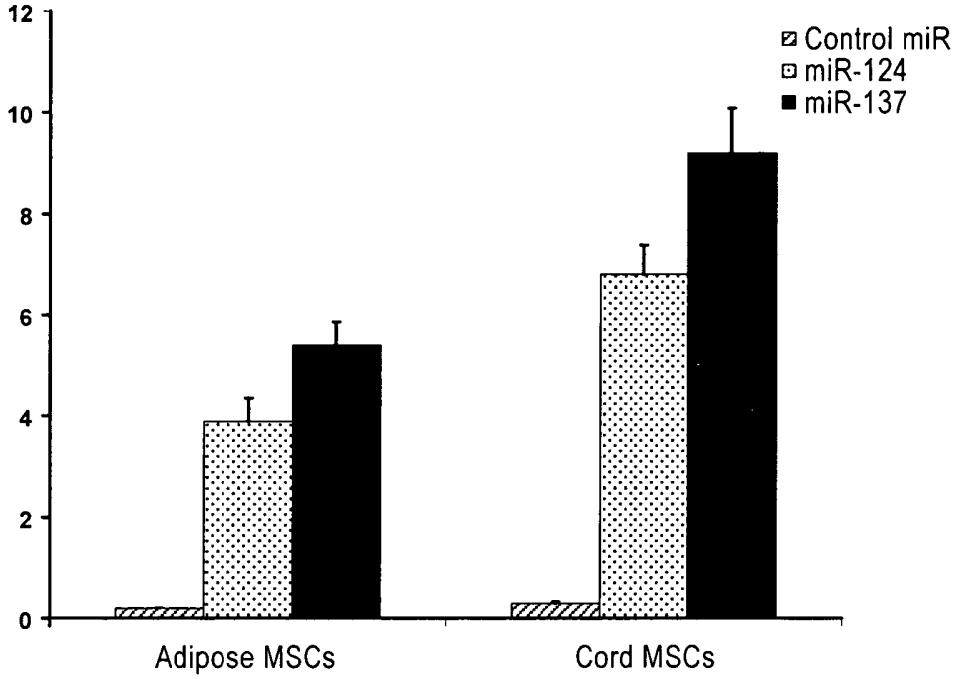

FIG. 4

(y-axis = beta III tubulin mRNA/S12 mRNA)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 December 2010 (16.12.2010)

PCT

(10) International Publication Number
WO 2010/144698 A3

(51) International Patent Classification:
C12N 15/11 (2006.01) *C12N 5/00* (2006.01)
C07H 21/04 (2006.01) *C12N 5/071* (2010.01)

(21) International Application Number:
PCT/US2010/038168

(22) International Filing Date:
10 June 2010 (10.06.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/185,773 10 June 2009 (10.06.2009) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(88) Date of publication of the international search report:
3 February 2011

WO 2010/144698 A3

(54) Title: METHODS, SYSTEMS, AND COMPOSITIONS FOR NEURONAL DIFFERENTIATION OF MULTIPOTENT STROMAL CELLS

(57) Abstract: Some embodiments of the invention comprise methods, systems, and compositions to selectively induce, whether in vitro or in vivo, the neuronal differentiation of multipotent stromal cells through the application of microRNAs, including but not limited to miRNA- 124, miRNA-137 and/or miRNA-9* expression products of those miRNAs, and molecules and compositions containing functional elements of those miRNAs. Some embodiments of the invention also comprise the therapeutic administration and use of such induced cells to treat mammalian injuries and diseases, including but not limited to, nervous system injuries or diseases that may otherwise result in decreased cell or system function.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/38168

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/11;C07H 21/04;C12N 5/00;C12N 5/071(2010.01)
USPC - 514/44A; 536/24.5; 435/375; 435/368
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)
IPC(8) - C12N 15/11;C07H 21/04;C12N 5/00;C12N 5/071(2010.01)
USPC - 514/44A; 536/24.5; 435/375; 435/368

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 435/325; 435/366; 435/354; 435/366; 435/6; 536/23.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB,USPT,USOC,EPAB,JPAB); Google Scholar: mir-124, mirna-124, microrna-124, pluirpot$, multipot$, stem, progenitor, neuroblast, totipot$, cell, neuro$, nerv$, differentia$, growth, heal, healing, regrowth, rna, mirna, mrna, microrna, mir, ribonucleoligonucl$, transplan$, graft, graft$, implan$, $graft

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X ----- Y	US 2008/0171715 A1 (BROWN et al.) 17 July 2008 (17.07.2008) para [0013]; [0038]; [0040]-[0042]; [0051]-[0053]; [0076]-[0078]; [0200]; [0209]-[0211]; [0282]-[0285]; [0290]; [0295]-[0300]; [0304]; [0308]	1-2, 7-8 ---------- 13-18
Y	US 2008/0241207 A1 (TANG et al.) 02 October 2008 (02.10.2008) para [0038]-[0040]; [0091]-[0092]; [0212]-[0213]; [0299]	13-18

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"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" document which may throw doubts on priority claim(s) or which 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
29 November 2010 (29.11.2010)	02 DEC 2010
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/38168

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I: claims 1-2, 7-8, 13-18, drawn to a method of stimulating neuronal differentiation of multipotent stromal cells from a mammal, comprising the steps of:
providing a composition comprised of microRNA-124, and
administering to a mammalian population of multipotent stromal cells a pharmaceutically effective amount of said composition in order to stimulate neuronal differentiation in the population.

----------continued on first blank sheet attached hereto----------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-2, 7-8, 13-18 limited to microRNA-124

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/38168

********** Supplemental Box **********

continuation of Box No. III: Observations where unity of invention is lacking

Group II, claims 3-4, 9-10, 13-18, drawn to a method of stimulating neuronal differentiation of multipotent stromal cells from a mammal, comprising the steps of:
providing a composition comprised of microRNA-137, and
administering to a mammalian population of multipotent stromal cells a pharmaceutically effective amount of said composition in order to stimulate neuronal differentiation in the population.

Group III, claims 5-6, 11-18, drawn to a method of stimulating neuronal differentiation of multipotent stromal cells from a mammal, comprising the steps of:
providing a composition comprised of microRNA-9*, and administering to a mammalian population of multipotent stromal cells a pharmaceutically effective amount of said composition in order to stimulate neuronal differentiation in the population.

The inventions listed as Groups I-III do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The inventions of Group I+ share the technical feature of a method of stimulating neuronal differentiation of mammal multipotent stromal cells by providing a composition comprised of a microRNA, and administering to a mammalian population of multipotent stromal cells a pharmaceutically effective amount of said composition in order to stimulate neuronal differentiation in the population. However, this shared technical feature would have been obvious to one of ordinary skill in the art. Specifically, a paper titled "Concise Review. Micro RNA Expression in Multipotent Mesenchymal Stromal Cells" by Lakshmipathy et al. (Stem Cells 2008, 26(2): 356-363) (hereinafter 'Lakshmipathy') discloses that microRNA-124 is expressed in neuronal cells as well as during neuronal differentiation of embryonic stem cells (pg 4, first full para) and that microRNA-124 is involved in developing neural tube (pg 17, Table 2).

In addition, a paper titled "The MicroRNA miR-124 promotes neuronal differentiation by triggering brain-specific alternative pre-mRNA splicing" by Makeyev, et al. (Mol Cell. 2007, 27(3):435-448) further elucidates the role of miR-124 in neuronal differentiation: "Both microRNAs and alternative pre-mRNA splicing have been implicated in the development of the nervous system (NS), but functional interactions between these two pathways are poorly understood. We demonstrate that the neuron-specific microRNA miR-124 directly targets PTBP1 (PTB/hnRNP I) mRNA, which encodes a global repressor of alternative pre-mRNA splicing in nonneuronal cells. Among the targets of PTBP1 is a critical cassette exon in the pre-mRNA of PTBP2 (nPTB/brPTB/PTBLP), an NS-enriched PTBP1 homolog. When this exon is skipped, PTBP2 mRNA is subject to nonsense-mediated decay (NMD). During neuronal differentiation, miR-124 reduces PTBP1 levels, leading to the accumulation of correctly spliced PTBP2 mRNA and a dramatic increase in PTBP2 protein. These events culminate in the transition from non-NS to NS-specific alternative splicing patterns. We also present evidence that miR-124 plays a key role in the differentiation of progenitor cells to mature neurons. Thus, miR-124 promotes NS development, at least in part by regulating an intricate network of NS-specific alternative splicing" (Abstract).

Finally, a paper titled "Specific MicroRNAs Modulate Embryonic Stem Cell-Derived Neurogenesis" by Krichevsky et al. (Stem Cells 2006, 24(4): 857-864) teaches that brain-specific miR-124a and miR-9 molecules affect neural lineage differentiation in the ES cell-derived cultures (Abstract). Thus, one of ordinary skill in the art would have been motivated to provide a composition comprising microRNA-124 to a culture of multipotent stromal cells from a mammal to induce differentiation of said cells into neuronal cells, because prior art discloses that microRNA-124 is stimulates neuronal differentiation of multipotent stromal cells. As said method would have been obvious to one of ordinary skill in the art at the time of the invention, this cannot be considered a special technical feature that would otherwise unify the groups.

Another technical feature of the inventions listed as Groups I-III is the specific microRNA recited therein. As said microRNAs were known in the art at the time of the invention and thus no significant structural similarities can readily be ascertained among the microRNAs, the inventions do not share a special technical feature. Without a shared special technical feature, the inventions lack unity with one another.

Groups I-III therefore lack unity under PCT Rule 13 because they do not share a same or corresponding special technical feature.

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 102666855 A
(43)申请公布日 2012.09.12

(21)申请号 201080035364.3
(22)申请日 2010.06.10
(30)优先权数据
61/185,773 2009.06.10 US
(85)PCT申请进入国家阶段日
2012.02.09
(86)PCT申请的申请数据
PCT/US2010/038168 2010.06.10
(87)PCT申请的公布数据
WO2010/144698 EN 2010.12.16
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代理人 李丙林 张英
(51)Int.Cl.
C12N 15/11(2006.01)
C07H 21/04(2006.01)
C12N 5/00(2006.01)
C12N 5/071(2006.01)

权利要求书 2 页 说明书 13 页 附图 3 页

(54)发明名称

用于多能性基质细胞的神经元分化的方法、系统和组合物

(57)摘要

本发明的一些实施方式包括通过微小RNA的应用而在体外或体内选择性诱导多能性基质细胞的神经元分化的方法、系统和组合物，该微小RNA包括但不限于miRNA-124、miRNA-137和/或miRNA-9*，这些miRNA的表达产物、以及含有这些miRNA的功能元件的分子和组合物。本发明的一些实施方式还包括这样的诱导的细胞用于治疗哺乳动物损伤和疾病的治疗给药和用途，包括但不限于，可能在其它方面导致细胞或系统功能降低的神经系统损伤或疾病。

CN 102666855 A

1. 一种刺激来自哺乳动物的多能性基质细胞的神经元分化的方法，包括以下步骤：

提供包括微小 RNA-124 的组合物，以及

向哺乳动物的多能性基质细胞群给予药学有效量的所述组合物以在所述群中刺激神经元分化。

2. 根据权利要求 1 所述的方法，其中，所述哺乳动物是人。

3. 一种刺激来自哺乳动物的多能性基质细胞的神经元分化的方法，包括以下步骤：

提供包括微小 RNA-137 的组合物，以及

向哺乳动物的多能性基质细胞群给予药学有效量的所述组合物以在所述群中刺激神经元分化。

4. 根据权利要求 3 所述的方法，其中，所述哺乳动物是人。

5. 一种刺激来自哺乳动物的多能性基质细胞的神经元分化的方法，包括以下步骤：

提供包括微小 RNA-9* 的组合物，以及

向哺乳动物的多能性基质细胞群给予药学有效量的所述组合物以在所述群中刺激神经元分化。

6. 根据权利要求 5 所述的方法，其中，所述哺乳动物是人。

7. 微小 RNA-124 在用于刺激哺乳动物的多能性基质干细胞的神经元分化的药物中的用途。

8. 根据权利要求 7 所述的用途，其中，所述哺乳动物是人。

9. 微小 RNA-137 在用于刺激哺乳动物的间充质干细胞的神经元分化的药物中的用途。

10. 根据权利要求 9 所述的用途，其中，所述哺乳动物是人。

11. 微小 RNA-9* 在用于刺激哺乳动物的多能性基质细胞的神经元分化的药物中的用途。

12. 根据权利要求 11 所述的用途，其中，所述哺乳动物是人。

13. 一种治疗患有神经系统损伤或疾病的哺乳动物的方法，包括以下步骤：

通过在体外使多能性基质细胞暴露于单独的或者以任意组合的微小 RNA-124、微小 RNA-137、或微小 RNA-9*，从而刺激所述细胞的神经元分化；以及

将这样的神经元分化的细胞给予所述哺乳动物。

14. 根据权利要求 13 所述的方法，其中，所述哺乳动物是人。

15. 一种治疗患有神经系统损伤或疾病的哺乳动物的方法，包括以下步骤：

在体外用单独的或者以任意组合的微小 RNA-124、微小 RNA-137、或微小 RNA-9* 转染多能性基质细胞；以及

将这样的经转染的细胞给予所述哺乳动物。

16. 根据权利要求 15 所述的方法，其中，所述哺乳动物是人。

17. 一种治疗患有神经系统损伤或疾病的哺乳动物的方法，包括以下步骤：

用包括一种或多种编码单独的或者以任意组合的微小 RNA-124、微小 RNA-137 或微小 RNA-9* 的核苷酸的载体来转染多能性基质细胞，其中，这样的核苷酸在所述载体中的表达可由启动子诱导，

将这样的经转染的细胞给予所述哺乳动物，以及

通过将所述启动子给予所述哺乳动物，在所述哺乳动物中诱导所述核苷酸的表达以产

生微小 RNA-124、微小 RNA-137、或微小 RNA-9*。

18. 根据权利要求 15 所述的方法，其中，所述哺乳动物是人。

用于多能性基质细胞的神经元分化的方法、系统和组合物

技术领域

[0001] 本发明的一些实施方式涉及用于治疗哺乳动物神经系统损伤和疾病的细胞类型的诱导和应用，但不限于此。

背景技术

[0002] 某些神经系统损伤、影响中枢或周围神经系统的自身免疫性疾病、以及神经退行性疾病的特征在于特定细胞的损失、或存在神经细胞的功能异常，这些会引起患者出现不同的神经病学体征和症状以及可能不可逆转的神经功能丧失。仅作为一个实例，一些患有中风、脊椎损伤或其它神经损伤和变性的患者经受功能细胞类型的损失，或如帕金森氏症和阿尔茨海默氏症这样的反过来导致系统功能的丧失或异常的神经病症。目前，用于治疗和修复这样的细胞和系统功能的治疗选择是有限的。因此，仍然需要方法、系统和组合物来促进其它治疗，包括致力于替换缺失或受损的神经系统细胞、组织和功能的疗法。

发明内容

[0003] 本发明的一些实施方式包括通过微小 RNA(microRNA) 的应用而在体外或体内选择性地诱导多能性基质细胞的神经元分化的方法、系统和组合物，包括但不限于 miRNA-124、miRNA-137 和 / 或 miRNA-9*，这些 miRNA 的表达产物，和含有这些 miRNA 的功能元件 (functional elements) 的分子和组合物，但本发明并不局限于本文所描述的那些实施方式，且不存在具体放弃。本发明的一些实施方式也包含这样的诱导细胞的治疗给予和应用以治疗哺乳动物损伤和疾病，包括但不限于，可能在其它方面导致细胞或系统功能降低的神经系统损伤或疾病。

附图说明

[0004] 现在将参照附图仅通过举例方式描述本发明的一些实施方式，对其它实施方式不存在具体的放弃，在附图中 ：

[0005] 图 1 显示用生长因子处理或用对照 miRNA 和 miRAN-124 或 miRNA-137 转染 3、5 和 9 天的 MSC 的亮场图像 (明场像，bright fieldimage)。

[0006] 图 2 是显示 miRAN-124、miRNA-137 和 miRNA-9* 在 MSC 中诱导神经元标记的数据表示。

[0007] 图 3 显示用测试的 miRNA 转染细胞或由 DMEM 处理后的蛋白质印迹结果。

[0008] 图 4 显示用对照 miRNA、miRAN-124 或 miRNA-137 转染 (动物) 脂肪和脐带来源的 MSC(cord derived MSC) 的结果。

具体实施方式

[0009] 本发明的一些实施方式包括通过微小 RNA(microRNA)(“miRNA”或“miR”) 的应用而在体外或体内选择性诱导多能性基质细胞 (“MSC”) 的神经元分化的方法、系统和组合

物，包括但不限于，miRNA-124 和 / 或 miRNA-137、和 / 或 miRNA-9*，这些 miRNA 的表达产物，以及含有这些 miRNA 的功能元件的分子和组合物，但本发明并不局限于本文所描述的那些实施方式，且对任何实施方式不存在具体放弃。本发明的一些实施方式也包含这样的经诱导的细胞用于治疗治疗哺乳动物损伤和疾病的治疗给药和用途，包括但不限于，可能在其它方面导致细胞或系统功能降低的神经系统损伤或疾病。在一些实施方式中，分化的 MSC 的这种诱导，和 / 或所产生的细胞，可以通过给予所述患者治疗有效量的感兴趣的 miRNA、或由这样的 miRNA 诱导的分化 MSC，而用于治疗患者体内的细胞、组织或器官损伤。

[0010] 我们已经出乎意料地发现某些 miRNA 能够诱导受试者中 MSC 的长期神经元分化用于基于细胞疗法的用途，这些受试者呈现神经系统损伤和疾病，包括但不限于，神经退行性紊乱和脊髓损伤。这样的受试者可以包括哺乳动物，包括但不限于人。因此，我们已经发现这样的 miRNA 以及所产生的经诱导的 MSC 的新应用，除了其它可能的用途，它们能够减少或减轻哺乳动物中某些神经系统损伤或疾病的影响。

[0011] 本发明的一些实施方式包括通过 miRNA-124、miRNA-137、和 / 或 miRNA-9* 的应用和表达而诱导 MSC 的神经元分化的方法、系统、和 / 或组合物，但不限于此。MSC 是典型地以非常低的浓度 (10000 个有核细胞中约 1 个) 典型地存在于成年骨髓中的中胚层来源的细胞 (mesoderm-derived cells)。MSC 能够分化产生细胞，如骨髓基质、血管、脂肪、骨骼和软骨。这些细胞可能也具有分化为神经元 \ 或神经胶质 - 样细胞的潜能，这取决于环境信号。此外，这些细胞可能被进一步诱导表达或通过与生长因子和激素的不同组合进行培养而保持特定神经元或神经胶质表型。

[0012] 在实验动物模型中以及最近在试验性临床实验中，MSC 已经显示出在各种神经疾病和功能异常中发挥治疗作用。它们的作用主要归因于免疫抑制和神经保护功能。在实验性自身免疫性脑炎 (“EAE”) 中，多发性硬化症 (“MS”) 的动物模型，用骨髓来源的 MSC 处理小鼠引起了疾病表现的显著抑制。一些研究证明，除了自身免疫调节降低之外，这些细胞的神经分化还在诸如缺血性脑的各种实例中增强了它们的治疗作用。

[0013] 在我们的研究中，我们检测了三种神经元 - 相关性 miRNA，即 miRNA-124、miRNA-137 和 miRNA-9* 对人 MSC 分化的影响。这些 miRNA 没有在 MSC 中正常表达。我们发现 miRNA-124、miRNA-137 或 miRNA-9* 的表达诱导了 MSC 的神经元分化，正如细胞形态学以及 βIII- 微管蛋白和 MAP2 的表达提高所指示的。miRNA-124、miRNA-137 和 miRNA-9* 引起酪氨酸羟化酶的增加，这暗示着 MSC 向多巴胺能表型的分化。miRNA124 前体 (前体 miRNA124，pre-miRNA124) 的靶标之一是抑制大量神经元基因的转录因子 REST。我们的结果显示神经元 - 相关性 miRNA 可以诱导 MSC 的长期神经元分化，用于在神经退行性和神经炎性紊乱以及脊髓损伤中基于细胞疗法的用途。与通过生长因子处理而诱导的瞬时分化 (transient differentiation) 相比，miRNA 的用途优于现有方法的一个优点在于 :能够在将引起长期神经元分化的 MSC 中稳定地表达 miRNA 前体。就这点而论，容易获得患者自己的骨髓来源的 MSC、以及大量富集和扩增 MSC 的可行性表明 :这样的细胞的神经元分化能够用作自体神经干细胞，其可用于治疗大量与神经元缺乏或受损有关的获得性或先天性神经性紊乱。仅作为一个实例，MSC 能够由通过吸脂去除的脂肪进行制备，以及由脐带血 (cord blood) 或胎盘进行制备，但不限于此。MSC 降低的免疫原性可以促进现成的 (off the shelf) 或者来自匹配或部分错配家族成员的异体神经元的使用，而用于治疗 (仅作为非限制性实例) 由

必需酶 (essential enzymes) 或其它必需产物 (essential products) 的先天性缺失而引起的病症。

[0014] （本发明）不限于仅在本文中明确公开的实施方式，且不存在对任何实施方式的具体放弃，本发明的一些实施方式包括：

[0015] 1.MSC 的神经元分化，其是通过培养或其它暴露于 miRNA，包括但不限于，miRNA124 和 / 或 miRNA137、和 / 或 miRNA-9*。

[0016] 2. 用这样的 miRNA 对 MSC 的转染；

[0017] 3. 将在体外诱导神经元分化的 MSC 给予患有神经系统损伤或疾病的受试者；和 / 或

[0018] 4. 将用这样的 miRNA 转染的 MSC 给予患有神经系统损伤或疾病的受试者。

[0019] 在一些实施方式中，随着 MSC 向神经元的可再现的转分化 (reproducible transdifferentiation)，MSC 的治疗用途能够在体外或体内获得和扩展，从而包括（仅作为某些实例）：对脑血管疾病、脊髓损伤的治疗，对神经退行性紊乱如肌萎缩性脊髓侧索硬化症（“ALS”）、多发性硬化症（“MS”）以及相关的运动神经元疾病的治疗，但不限于此。正在进行的临床研究已经表明 MSC 鞘内注射和静脉输注能够部分地改善 MS 患者中疾病的临床表现，且使 ALS 患者达到较轻程度。这样的临床研究提供了以下证据，即 MSC 鞘内注射和静脉输注都是安全的过程，因为被处理的患者中没有一个产生任何严重的副作用。因此，用 MSC 的细胞疗法预示性地代表用于治疗大量神经紊乱的一个重要途径，其中，特别地，MSC 能够被诱导为神经元或少突细胞和 / 或能够诱导局部存在的干细胞神经发生的分泌因子。

[0020] 实施例

[0021] 提供本发明的一些实施方式的以下实施例，但并不是要将本发明限于仅在此处描述的那些实施方式且不存在对任何实施方式的具体放弃。

[0022] 微小 RNA

[0023] 微小 RNA（“miRNA”）代表一类内源性、小的（作为一些非限制性的实例，19-23 个核苷酸）非编码 RNA，其通过 RNA 干扰（“RNAi”）途径起作用而影响转录后基因沉默。miRNA 基于互补靶向特定基因的 mRNA，且介导 mRNA 断裂 (完全互补 (perfect complementarity)) 或翻译阻抑 (translation repression)（部分互补）。miRNA 已经被证实在发育中起重要作用，且可以作为用于建立或保持决定细胞命运的特定表达谱的基本的基因调控元件而起作用。

[0024] MSC 的操纵性 (manipulating) 神经元分化可能涉及在分化过程中协调基因表达程序的调控途径。分化通常需要细胞的 mRNA 和蛋白质构成的改变。一类基因调控分子是微小 RNA，一个小的（小分子的）RNA 的亚类，其被认为使用 RNA 干扰途径中的元件 (elements) 在转录后下调蛋白编码基因的表达。miRNA 可能在细胞分化中起重要作用，因为它们被预测同时单独调控数百个靶基因。

[0025] 方法

[0026] 为了确定 miRNA-124、miRNA-137 和 miRNA-9* 对 MSC 分化的影响，我们在 4-12 段 (passages) 中采用这些细胞的三种不同制剂。将 MSC 细胞涂布于 DMEM+10％ FCS 上 24hr，然后用所述三种 miRNA 的成熟序列的双链 RNA 寡核苷酸和阴性对照寡核苷酸转染。所使用的 miRNA 如下：

[0027] Dharmacon 模拟产物：

[0028] MI0000443/MIMAT0000422- 人

[0029] 选定的前体 / 成熟

[0030] 成熟：

[0031] hsa-miR-124[MIMAT0000422]

[0032] 前体：

[0033] hsa-miR-124-1[MI0000443]

[0034] 有机体：

[0035] 人

[0036] 成熟序列：

[0037] UAAGGCACGCGGUGAAUGCC(SEQ ID NO.1)

[0038] MI0000454/MIMAT0000429- 人

[0039] 选定的前体 / 成熟

[0040] 成熟：

[0041] hsa-miR-137[MIMAT0000429]

[0042] 前体：

[0043] hsa-miR-137[MI0000454]

[0044] 有机体：

[0045] 人

[0046] 成熟序列：

[0047] UUAUUGCUUAAGAAUACGCGUAG(SEQ ID NO.2)

[0048] miRNA 9* 序列：

[0049] AUAAAGCUAGAUAACCGAAAGU(SEQ ID NO.3)

[0050] miRNA 9 模拟

[0051] UCUUUGGUUAUCUAGCUGUAUGA(SEQ ID NO.4)

[0052] 3 天后，将细胞转移到补充有 B27 的神经基质培养基 (NeurobasalMedium，NB) 中。细胞形态每 24 小时监测一次，且在转染 5、7 和 9 天后通过免疫荧光染色、蛋白质印迹分析或实时 PCR 对神经元标记进行分析。作为神经元分化诱导的阳性对照，我们使用通过 Shh、FGF8 和 bFGF 的组合刺激的细胞。

[0053] 结果

[0054] miRNA-124、miRNA-137、和 miRNA-9* 促进 MSC 的神经元分化。图 1 是代表产生相似结果的六个单独实验的图示。如图 1 所显示的，用 miRNA-137、miRNA-124 或 miRNA-9* 对细胞的转染在转染 72 小时后就已经减少细胞增殖且诱导细胞中形态分化。用 miRNA-137 对 MSC 的转染诱导了快速和稳健的形态变化，且所述细胞获得了有致密细胞体 (compactcell bodies) 的典型神经元表型和有静脉曲张的长突起 (elongatedprocesses)。miRNA-124- 转染的细胞在细胞增殖以及随后产生若干细胞类型方面显示出强烈的下降；有长突起的伸长细胞 (elongated cell)、具有多个较短突起的小细胞和扁平的星状细胞。对照 miRNA 转染的细胞与对照未处理的细胞类似。有趣地，miRNA-137 的作用比 GF 的作用更快速且更强烈。约 90％ miRNA-137 转染的细胞显示神经元形态。

[0055] miRNA-124、miRNA-137 和 miRNA-9* 增加 MSC 中神经元标记的表达。为了进一步检测 miRNA-124、miRNA-137 和 miRNA-9* 对神经元分化的影响，我们检测了神经干细胞标记、巢蛋白 (nestin)、星形细胞标记 GFAP 和神经元标记 βIII- 微管蛋白以及酪氨酸羟化酶的表达。细胞用合适的 miRNA 转染或用 DMEM 或神经基质培养基 +B27 处理。5 天后，巢蛋白 mRNA 的表达使用实时 PCR 测定，且巢蛋白、GFAP、βIII- 微管蛋白和酪氨酸羟化酶的表达在处理 9 天后通过蛋白质印迹分析检测。结果表示为产生相似结果的五个不同实验。图 2 显示转染 5 天后，如实时 PCR 测定的，巢蛋白 mRNA 大量增加。相反，用不同的 miRNA 转染 9 天后，我们发现 βIII- 微管蛋白的表达增加，然而没有观察到巢蛋白或 GFAP 的表达。此外，我们发现 miRNA-137 和 miRNA-9* 诱导酪氨酸羟化酶表达的大量增加，然而在 miRNA-124 转染的细胞中观察到较小的增加。在对照 miRNA 转染的细胞中，所有这些标记物的表达不存在的或者可忽略不计。

[0056] miR-9* 诱导 MSC 中多巴胺能标记物，酪氨酸羟化酶。图 3 显示 MSC 用合适的 miRNA 转染或用 DMEM 处理后的蛋白质印迹结果。9 天后，通过蛋白质印迹分析检测酪氨酸羟化酶表达。结果表示为产生相似结果的五个不同实验。在 MSC 中，miRNA-9* 诱导多巴胺能标记物，酪氨酸羟化酶。

[0057] 我们的结果证实 miRNA-124、miRNA-137 和 miRNA-9* 诱导 MSC 的神经元分化，尽管在我们的测试模型中分别以不同程度（进行诱导）。miRNA-137 诱导更快速更稳健的效果，产生神经元细胞的同质群体（同源群体，homogenous population）。酪氨酸羟化酶在这些细胞中高水平的表达暗示这些细胞显示多巴胺能表型。

[0058] 与对照 miRNA- 处理的细胞相比，miRNA-124 也诱导神经元分化，如通过高水平的 βIII- 微管蛋白所测定的。在我们的研究中，这种处理引起了表达较低水平酪氨酸羟化酶的细胞的混合群体。没有处理诱导星形细胞分化，如通过 GFAP 表达缺失所测定的。

[0059] 此外，处理 5 天后，两种 miRNA 都引起了巢蛋白表达的大量瞬时增加，表明神经干细胞样或神经元祖细胞样细胞的生成。MSC 向 NSC 或 NPC 样细胞的受控分化可被进一步利用，以通过使用特定转录因子或生长因子的特定组合将这些细胞分化为不同的神经元细胞系或具有不同表型的神经元。

[0060] miRNA-124 和 miRNA-9* 已经被报道参与神经元分化和神经突生长。相似地，有一项报道证实了 miRNA137 对神经胶质瘤干细胞和 NSC 的神经元分化的影响。然而，没有报道 miRNA 对 MSC 的神经元分化的作用，且没有示出 miRNA124 和 miRNA137 对具有特定表型的神经元生成的影响。此外，没有报道这些 miRNA 中的任一个可诱导具有 NSC/NPC 表型的细胞。

[0061] miRNA-124 和 miRNA-137 诱导脂肪和脐带来源的 MSC(cord derivedMSC) 中神经元分化。脂肪和脐带来源的 MSC 用对照 miRNA、miRNA-124 和 miRNA-137 转染，这类似于骨髓 MSC（图 4）。

[0062] 脂肪来源的 MSC 的制备：脂肪来源的 MSC 通过从大腿或腹壁吸脂而获得。将 100-200ml 吸出物在可从脂肪细胞和碎片中分离 MSC 的特别设计的 Cytori 分离器中处理。所述细胞被进一步处理和保持，如对骨髓来源的 MSC 描述的那样。

[0063] 人脐带 MSC 的制备：新鲜的人脐带在出生后获得（经父母同意）且在 4℃在 DMEM 中收集。去除脐带血管且将间充质组织（华通氏胶 (Wharton's jelly)）切成小块。250×g 离心 5min 后，所述组织用无血清 DMEM 清洗，在 37℃用胶原酶处理 18h，随后在 37℃用 2.5%

胰蛋白酶消化 30min。所述经分离的 MSC 在与对骨髓来源的 MSC 所描述的相似条件下进一步分散和保持。

[0064] 12 天后，提取 mRNA，且使用实时 PCR 测定 b3- 微管蛋白和看家基因 (house keeping gene)S12 的水平。

[0065] 我们的结果 (图 4) 证实 miRNA-124 和 miRNA-137 不仅在骨髓来源的 MSC 中还在脂肪组织和脐带血来源的细胞中诱导神经元分化。神经元标记 βIII 微管蛋白在 miRNA 处理后在这些细胞中被诱导。这些细胞来源中的每一个都具有自己的优点。骨髓来源的 MSC 已得到很好的特征描述，且已经成功地使用超过 20 年而没有致癌性。脂肪来源的 MSC 被较少的描述，但是能被大量获得，且脐带血细胞能够以非侵入方式容易地获得，它们不需要捐赠者和患者之间完全的基因相容性 (genetic compatibility)，因而更容易得到。

[0066] 含有 miRNA 前体和 GDNF 的质粒的构建。由于 GDNF 已牵涉多巴胺能神经元的存活，因而我们构建了在分开的启动子下与 GDNF 一起共表达 miRNA-124 前体或 miRNA-137 前体的质粒。

[0067] 关于分步克隆 (step by step cloning)GDNF 于 premir 载体 (来自 SystemBiosciences 的 CD-511_1 或 PCDH-CMV-MCS-EF1-copGFP)，描述了测序步骤和克隆所述 miRNA 前体 GDNF 载体的程序以及克隆 miRNA 的程序，但并不限于仅在此处描述的实施方式，且不存在对任何实施方式的具体放弃。

[0068] 人胶质细胞来源的神经营养因子 (“GDNF”) 模板的 cDNA 从 Origene 获得。为了将 GDNF 克隆至 premir 124 和 137 载体 (System Biosciences) 中，针对 GDNF ORF 的具有 Xho1 和 Sal1 限制性酶消化位点的引物设计如下 :

[0069] 正向 :cacc ctcgag(Xho1)atg aag tta tgg gat gtc gtg gct gtc tgc(SEQID NO.5)

[0070] 反 向 :aaa gtcgac(Sal1)tca gat aca tcc aca cct ttt agc gga atg(SEQ IDNO.6)

[0071] PCR 之后，清洗 GDNF DNA 产物，然后用 Xho1 和 Sal1 消化，且再次清洗 DNA，产生可立即进行克隆的 GDNF 的 DNA。

[0072] 通过使用以下引物将 Xho1 限制性位点添加到 premir 124 和 premir137 载体中 :

[0073] 正向 :gac gcc acc atg gag agc ctc gag(Xho1)agc ggc ctg ccc gcc(SEQID NO.7)

[0074] 反向 :ggc ggg cag gcc gct ctc gag(Xho1)gct ctc cat ggt ggc gtc(SEQID NO.8)

[0075] GFP 基因通过使用限制性酶 Xho1 和 Sal1 而从 premir 124 和 137 载体中去除，然后清洗所述载体。

[0076] GDNF 与 premir 124 和 137 载体的连接 (ligation)。将连接的质粒转入 One Shot Top 10 化学感受态细胞中。用小量抽提法 (mini prep) 处理后，通过培养克隆而选择具有 premir 124 和 137 的质粒。

[0077] 通过使用 Xho1 和 Sal1 消化质粒从而检测 GDNF 的插入。然后测序所述质粒。miR-124 和 137 的序列以及 premir 载体的骨架 (backbone) :

[0078] MiR-124 :

[0079] GAACAAAGAGCCTTTGGAAGACGTCGCTGTTATCTCATTGTCTGTGTGATTGGGGGAGCTGCGGCGGGG AGGATGCTGTGGTCCCTTCCTCCGGCGTTCCCCACCCCCATCCCTCTCCCCGCTGTCAGTGCGCACGCACACGCGCC GCTTTTTATTTCTTTTTCCTGGTTTTCTTATTCCATCTTCTACCCACCCCTCTTCCTTTCTTTCACCTTTCCTTCCT TCCTTCCTCCTTTCCTTCCTCAGGAGAAAGGCCTCTCTCTCCGTGTTCACAGCGGACCTTGATTTAAATGTCCATAC AATTAAGGCACGCGGTGAATGCCAAGAATGGGGCTGGCTGAGCACCGTGGGTCGGCGAGGGCCCGCCAAGGAAGGAG CGACCGACCGAGCCAGGCGCCCTCCGCAGACCTCCGCGCAGCGGCCGCGGGCGCGAGGGGAGGGGTCTGGAGCTCCC TCCGGCTGCCTGTCCCGCACCGGAGCCCGTGGGGTGGGGAGGTGTGCAGCCTGTGACAGACAGGGGCTTAGAGATGC (SEQ ID NO.9)

[0080] MiR-137：

[0081] CAGCACTCTTCTGTGTTAAGTATTTGATTTTGTGATTTGTCTTTCAGAATTGGAAATAGAGCGGCCATT TGGATTTGGGCAGGAAGCAGCCGAGCACAGCTTTGGATCCTTCTTTAGGGAAATCGAGTTATGGATTTATGGTCCCG GTCAAGCTCAGCCCATCCCCAGGCAGGGGCGGGCTCAGCGAGCAGCAAGAGTTCTGGTGGCGGCGGCGGCGGCAGTA GCAGCGGCAGCGGTAGCAGCGGCAGCGGTAGCAGCGGCAGCGGCAGCTTGGTCCTCTGACTCTCTTCGGTGACGGGT ATTCTTGGGTGGATAATACGGATTACGTTGTTATTGCTTAAGAATACGCGTAGTCGAGGAGAGTACCAGCGGCAGGG GGGCAGCGGCCGCCCTCCCCAGCCCACCAGCTGGCCACTAAACGCCCGTGGTTGCCAAGGTAGCACTTTCTTGTTCT TTTCATTTCCTCGGGTGTTTTCGCACTGGTTCCACCGGAAAGGCTGTGCGCTGCGCCTCTGGTGACCAGGACTGGA(SEQ ID NO.10)

[0082] 所附为骨架载体 (CD-511_1) 的序列：

[0083] 位点 (LOCUS) CD511B_1_pCDH_CMV_7544bp ds-DNA 环形 16-DEC-2008：

[0084] 定义

[0085] 登录号

[0086] 版本

[0087] 来源

[0088] 有机体

[0089] 注释

[0090] 注释 ApEinfo：甲基化：1

[0091] 特征 位点 / 修饰 (Qualifiers)

[0092] 尚未归类的特征 (misc_feature)2315..2764

[0093] / 标记＝ EF1 启动子

[0094] /ApEinfo_fwd 颜色＝蓝绿色

[0095] /ApEinfo_rev 颜色＝绿色

[0096] 尚未归类的特征 2765..2789

[0097] / 标记＝ EF1 启动子 (1)

[0098] /ApEinfo_ 标记＝ EF1 启动子

[0099] /ApEinfo_fwd 颜色＝蓝绿色

[0100] /ApEinfo_rev 颜色＝绿色

[0101] 尚未归类的特征 2874..3629

[0102] / 标记＝ copGFP

[0103] /ApEinfo_fwd 颜色＝ #00ff00

[0104] /ApEinfo_rev 颜色＝绿色

[0105] 尚未归类的特征 3639..4229

[0106] / 标记＝ WPRE

[0107] /ApEinfo_fwd 颜色＝蓝绿色

[0108] /ApEinfo_rev 颜色＝绿色

[0109] 尚未归类的特征 2790..2860

[0110] / 标记＝ EF1 启动子 (2)

[0111] /ApEinfo_ 标记＝ EF1 启动子

[0112] /ApEinfo_fwd 颜色＝蓝绿色

[0113] /ApEinfo_rev 颜色＝绿色

[0114] 尚未归类的特征 2765..2789

[0115] / 标记＝ EFfwd 引物

[0116] /ApEinfo_fwd 颜色＝ #ff80ff

[0117] /ApEinfo_rev 颜色＝绿色

[0118] 尚未归类的特征 1922..2183

[0119] / 标记＝ CMV

[0120] /ApEinfo_fwd 颜色＝ #ff80ff

[0121] /ApEinfo_rev 颜色＝绿色

[0122] 尚未归类的特征 2272..2314

[0123] / 标记＝ MCS

[0124] /ApEinfo_fwd 颜色＝ #80ff00

[0125] /ApEinfo_rev 颜色＝绿色

[0126] 尚未归类的特征 2205..2271

[0127] / 标记＝ CMV(1)

[0128] /ApEinfo_ 标记＝ CMV

[0129] /ApEinfo_fwd 颜色＝ #ff80ff

[0130] /ApEinfo_rev 颜色＝绿色

[0131] 尚未归类的特征 2184..2204

[0132] / 标记＝ DAB 90 正向引物

[0133] /ApEinfo_fwd 颜色＝蓝绿色

[0134] /ApEinfo_rev 颜色＝绿色

[0135] 源

[0136] 1 acgcgtgtag tcttatgcaa tactcttgta gtcttgcaac atggtaacga tgagttagca

[0137] 61 acatgcctta caaggagaga aaaagcaccg tgcatgccga ttggtggaag taaggtggta

[0138] 121 cgatcgtgcc ttattaggaa ggcaacagac gggtctgaca tggattggac gaaccactga

[0139] 181 attgccgcat tgcagagata ttgtatttaa gtgcctagct cgatacaata aacgggtctc

[0140] 241 tctggttaga ccagatctga gcctgggagc tctctggcta actagggaac ccactgctta

[0141] 301 agcctcaata aagcttgcct tgagtgcttc aagtagtgtg tgcccgtctg ttgtgtgact

[0142] 361 ctggtaacta gagatccctc agacccttt agtcagtgtg gaaaatctct agcagtggcg

[0143] 421 cccgaacagg gacctgaaag cgaaagggaa accagagctc tctcgacgca ggactcggct

[0144] 481 tgctgaagcg cgcacggcaa gaggcgaggg gcggcgactg gtgagtacgc caaaaatttt

[0145] 541 gactagcgga ggctagaagg agagagatgg gtgcgagagc gtcagtatta agcgggggag

[0146] 601 aattagatcg cgatgggaaa aaattcggtt aaggccaggg ggaaagaaaa aatataaatt

[0147] 661 aaaacatata gtatgggcaa gcagggagct agaacgattc gcagttaatc ctggcctgtt

[0148] 721 agaaacatca gaaggctgta gacaaatact gggacagcta caaccatccc ttcagacagg

[0149] 781 atcagaagaa cttagatcat tatataatac agtagcaacc ctctattgtg tgcatcaaag

[0150] 841 gatagagata aaagacacca aggaagcttt agacaagata gaggaagagc aaaacaaaag

[0151] 901 taagaccacc gcacagcaag cggccactga tcttcagacc tggaggagga gatatgaggg

[0152] 961 acaattggag aagtgaatta tataaatata aagtagtaaa aattgaacca ttaggagtag

[0153] 1021 cacccaccaa ggcaaagaga agagtggtgc agagagaaaa aagagcagtg ggaataggag

[0154] 1081 ctttgttcct tgggttcttg ggagcagcag gaagcactat gggcgcagcc tcaatgacgc

[0155] 1141 tgacggtaca ggccagacaa ttattgtctg gtatagtgca gcagcagaac aatttgctga

[0156] 1201 gggctattga ggcgcaacag catctgttgc aactcacagt ctggggcatc aagcagctcc

[0157] 1261 aggcaagaat cctggctgtg gaaagatacc taaaggatca acagctcctg gggatttggg

[0158] 1321 gttgctctgg aaaactcatt tgcaccactg ctgtgccttg gaatgctagt tggagtaata

[0159] 1381 aatctctgga acagattgga atcacacgac ctggatggag tgggacagag aaattaacaa

[0160] 1441 ttacacaagc ttaatacact ccttaattga agaatcgcaa aaccagcaag aaaagaatga

[0161] 1501 acaagaatta ttggaattag ataaatgggc aagtttgtgg aattggttta acataacaaa

[0162] 1561 ttggctgtgg tatataaaat tattcataat gatagtagga ggcttggtag gtttaagaat

[0163] 1621 agtttttgct gtactttcta tagtgaatag agttaggcag ggatattcac cattatcgtt

[0164] 1681 tcagacccac ctcccaaccc cgaggggacc cgacaggccc gaaggaatag aagaagaagg

[0165] 1741 tggagagaga gacagagaca gatccattcg attagtgaac ggatctcgac ggttaacttt

[0166] 1801 taaaagaaaa gggggggattg gggggtacag tgcaggggaa agaatagtag acataatagc

[0167] 1861 aacagacata caaactaaag aattacaaaa acaaattaca aaaattcaaa attttatcga

[0168] 1921 tactagtatt atgcccagta catgacctta tgggactttc ctacttggca gtacatctac

[0169] 1981 gtattagtca tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga

[0170] 2041 tagcggtttg actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg

[0171] 2101 ttttggcacc aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg

[0172] 2161 caaatgggcg gtaggcgtgt acggtgggag gtctatataa gcagagctcg tttagtgaac

[0173] 2221 cgtcagatcg cctggagacg ccatccacgc tgttttgacc tccatagaag attctagagc

[0174] 2281 tagcgaattc gaatttaaat ggatccgcgg ccgcaaggat ctgcgatcgc tccggtgccc

[0175] 2341 gtcagtgggc agagcgcaca tcgcccacag tccccgagaa gttgggggga ggggtcggca

[0176] 2401 attgaacggg tgcctagaga aggtggcgcg gggtaaactg ggaaagtgat gtcgtgtact

[0177] 2461 ggctccgcct ttttcccgag ggtgggggag aaccgtatat aagtgcagta gtcgccgtga

[0178] 2521 acgttctttt tcgcaacggg tttgccgcca gaacacagct gaagcttcga ggggctcgca

[0179] 2581 tctctccttc acgcgcccgc cgccctacct gaggccgcca tccacgccgg ttgagtcgcg

[0180] 2641 ttctgccgcc tcccgcctgt ggtgcctcct gaactgcgtc cgccgtctag gtaagtttaa

[0181] 2701 agctcaggtc gagaccgggc ctttgtccgg cgctcccttg gagcctacct agactcagcc

[0182] 2761 ggctctccac gctttgcctg accctgcttg ctcaactcta cgtctttgtt tcgttttctg

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[0184] 2881 gcgacgagag cggcctgccc gccatggaga tcgagtgccg catcaccggc accctgaacg

[0185] 2941 gcgtggagtt cgagctggtg ggcggcggag agggcacccc caagcagggc cgcatgacca

[0186] 3001 acaagatgaa gagcaccaaa ggcgccctga ccttcagccc ctacctgctg agccacgtga

[0187] 3061 tgggctacgg cttctaccac ttcggcacct accccagcgg ctacgagaac cccttcctgc

[0188] 3121 acgccatcaa caacggcggc tacaccaaca cccgcatcga gaagtacgag gacggcggcg

[0189] 3181 tgctgcacgt gagcttcagc taccgctacg aggccggccg cgtgatcggc gacttcaagg

[0190] 3241 tggtgggcac cggcttcccc gaggacagcg tgatcttcac cgacaagatc atccgcagca

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[0201] 3901 gtcagctcct ttccgggact ttcgctttcc ccctccctat tgccacggcg gaactcatcg

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[0213] 4621 gcttataatg gttacaaata aagcaatagc atcacaaatt tcacaaataa agcatttttt

[0214] 4681 tcactgcatt ctagttgtgg tttgtccaaa ctcatcaatg tatcttatca tgtctggctc

[0215] 4741 tagctatccc gcccctaact ccgcccagtt ccgcccattc tccgccccat ggctgactaa

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[0226] 5401 aaagatacca ggcgtttccc cctggaagct ccctcgtgcg ctctcctgtt ccgaccctgc

[0227] 5461 cgcttaccgg atacctgtcc gcctttctcc cttcgggaag cgtggcgctt tctcatagct

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[0232] 5761 ggacagtatt tggtatctgc gctctgctga agccagttac cttcggaaaa agagttggta

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[0240] 6241 cagatttatc agcaataaac cagccagccg gaagggccga gcgcagaagt ggtcctgcaa

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[0242] 6361 cagttaatag tttgcgcaac gttgttgcca ttgctacagg catcgtggtg tcacgctcgt

[0243] 6421 cgtttggtat ggcttcattc agctccggtt cccaacgatc aaggcgagtt acatgatccc

[0244] 6481 ccatgttgtg caaaaaagcg gttagctcct tcggtcctcc gatcgttgtc agaagtaagt

[0245] 6541 tggccgcagt gttatcactc atggttatgg cagcactgca taattctctt actgtcatgc

[0246] 6601 catccgtaag atgcttttct gtgactggtg agtactcaac caagtcattc tgagaatagt

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[0248] 6721 gcagaacttt aaaagtgctc atcattggaa aacgttcttc ggggcgaaaa ctctcaagga

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[0250] 6841 catcttttac tttcaccagc gtttctgggt gagcaaaaac aggaaggcaa aatgccgcaa

[0251] 6901 aaaagggaat aagggcgaca cggaaatgtt gaatactcat actcttcctt tttcaatatt

[0252] 6961 attgaagcat ttatcagggt tattgtctca tgagcggata catatttgaa tgtatttaga

[0253] 7021 aaaataaaca aatagggttt ccgcgcacat ttccccgaaa agtgccacct gacgtctaag

[0254] 7081 aaaccattat tatcatgaca ttaacctata aaaataggcg tatcacgagg ccctttcgtc

[0255] 7141 tcgcgcgttt cggtgatgac ggtgaaaacc tctgacacat gcagctcccg gagacggtca

[0256] 7201 cagcttgtct gtaagcggat gccgggagca gacaagcccg tcagggcgcg tcagcgggtg

[0257] 7261 ttggcgggtg tcggggctgg cttaactatg cggcatcaga gcagattgta ctgagagtgc

[0258] 7321 accatatgcg gtgtgaaata ccgcacagat gcgtaaggag aaaataccgc atcaggcgcc

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[0260] 7441 tacgccagct ggcgaaaggg ggatgtgctg caaggcgatt aagttgggta acgccagggt

[0261] 7501 tttcccagtc acgacgttgt aaaacgacgg ccagtgccaa gctg(SEQ ID NO.11)

[0262] 我们发现用这些质粒转染的MSC分泌GDNF且表达各个miRNA。因此，由分化的多巴胺能神经元分泌的GDNF预期提供存活信号至分化的细胞和内源性多巴胺能神经元。

[0263] 可诱导的miRNA的构建。植入的MSC已被报道迁移至中枢神经系统的受损组织且发挥神经营养和免疫调节的作用。具体地，在帕金森病动物模型中，植入的MSC已显示为移植(engraft)入受损的纹状体(striatum)中。在一些实施方式中，但不限于此，可诱导的miRNA前体表达载体可被使用，其在所需的时间点将允许特定miRNA前体表达的诱导。因此，MSC将被特定miRNA前体转染，且其将在受损纹状体中MSC移植之前或随后的不同时间点被诱导表达。对于这样的研究，我们已采用可诱导的miRNA和染色(living color)，并采用使用Tet-on系统(Clontech)的荧光蛋白报告子(reporter)。这个系统通过启动子的添加而允许特定miRNA的诱导（仅作为实例，可通过强力霉素(doxycyline))，以及其中生成miRNA的细胞的鉴定。

[0264] 概括地说，我们已经证实了miRNA124、miRNA137和miRNA-9*诱导MSC转分化为NSC/NPC以及具有特定神经元表型(miRNA137)的神经元的能力。其它的神经元miRNA，如miRNA-9和miR218，也可影响MSC的转分化和诱导神经元分化。

[0265] 与通过生长因子处理而诱导的瞬时分化相比，使用miRNA124优于现有方法的一个优点是：能够在MSC中稳定地表达miRNA前体，其将引起长期的神经元分化。

[0266] 我们的研究显示神经元-相关性miRNA可被用于诱导MSC的长期神经元分化，用于在神经退行性紊乱和脊髓损伤中的基于细胞的疗法中，仅作为一些实例，其可由以下所显示：

[0267] 1.通过微小RNA(miRNA-124、miRNA-137和miRNA-9*)的MSC的神经元分化；

[0268] 2.通过miRNA-137、miRNA-124和miRNA-9*的MSC的特异性多巴胺能分化；以及

[0269] 3.通过微小RNA诱导MSC向神经干细胞样或神经祖细胞样细胞的瞬时分化。用miRNA转染提供了机会性窗口，其中，细胞能够被分化为中枢神经系统的不同细胞系（神经元、星形细胞和少突细胞）或者使用转录因子或生长因子的特定组合而分化为特定神经元表型。所述窗口能够由miRNA表达的水平或转染后特定时间点所控制。

[0270] miRNA转分化MSC为未决定分化方向的祖细胞(uncommittedprogenitor cells)和不同神经元细胞亚群的能力使利用这些细胞治疗多种神经疾病成为可能，包括脊髓和周围神经损伤、出血或阻塞性病变(obstructive lesion,“CVA”)引起的对中枢神经系统的损害或者外伤性中枢或周围神经损伤。此外，转分化的MSC可以被用于由特发性自身免疫性疾病（“EAE”）引起的神经退行性疾病或者如帕金森氏症或阿尔茨海默病的疾病或者如ALS的病因不明的疾病中。此外，通过转分化的MSC对神经功能的改善也可以被用于药物引起的神经元损伤和/或毒性所造成的各种退行性疾病中。

[0271] 因此，在我们的研究中，miRNA-124、miRNA-137和miRNA-9*促进MSC的神经分化，伴随形态学变化和表型标记的表达。

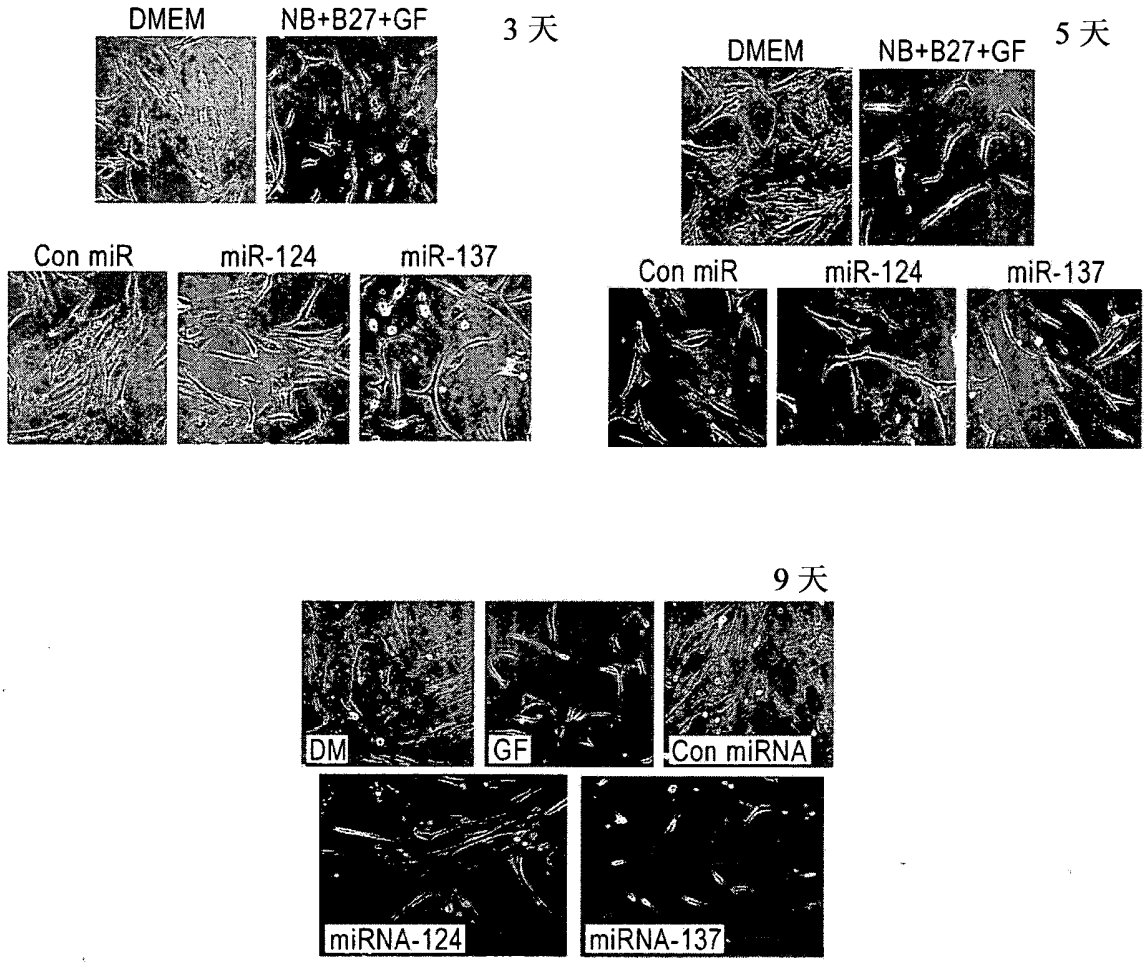
[0272] 一些实施方式的诱导miRNA可按照良好的医疗实践给予和服用，需考虑达到靶MSC预期效果所使用的技术、个体患者的临床情况、给予的部位和方法、给予的时间安排、患者年龄、性别、体重和医生所知的其它因素。用于本文目的的“药学有效量”因此可由本领

域中所知的这些考虑因素所决定。该量必须是对实现改善而有效的，包括但不限于，在体内和 / 或体外 MSC 的所需分化、降低损害和损伤、或者症状的改善或消除、以及作为适当措施被所属领域的技术人员选择的其它指标。

[0273] 本发明的实施方式可以为治疗神经系统损伤和疾病扩大治疗窗口，且能够用于治疗每年在美国患这样的损伤和疾病的庞大患者人群。因此，在一些实施方式中，通过包含本发明实施方式的诱导 miRNA 的选择性应用，本发明包含预防、控制、或减轻哺乳动物神经系统损伤和疾病的新方法，包括但不限于，脑损伤、神经变性、或脊髓损伤。按照一些实施方式，通过一种或多种这样的 miRNA 的使用和 / 或给予而在体内或体外诱导靶细胞中的分化，用于限制这样的损伤或疾病影响的治疗中，可以实现这样的干预治疗，但不限于此。因此，一些实施方式包括通过转分化 MSC 的选择性应用和 / 或诱导而预防、控制、或减轻哺乳动物损伤（包括但不限于脑损伤）的新组合物和方法，不局限于所述主题，也不存在对主题的具体放弃。

[0274] 本申请通过作者、引用、和 / 或通过专利号可参考各种出版物，包括但不限于，文章、报告 (presentations)、和美国专利。每一篇任何这样的文献的公开内容均以其整体并入本申请中供参考。

[0275] 尽管已结合上述优选和可替换实施方式对本发明进行具体示出和描述，但本领域的技术人员应理解的是，在不背离如所附权利要求中限定的本发明的精神和范围的情况下，本文描述的本发明的实施方式的各种替换方案可用于本发明的实施中。所附权利要求旨在限定本发明的范围，并且落在这些权利要求的范围内的方法和设备及其等同物也尤其所涵盖。本发明的描述应当被理解为包括此处所描述的元素的所有新颖性和非显而易见的组合，且对这些元素的任何新颖性和非显而易见组合，权利要求可存在于本申请或以后的申请中。上述实施方式是例示性的，且没有单一的特征或元素对于本申请或以后的申请中可被要求的所有可能组合是必不可少的。在权利要求限定其等同物的“一种”或“第一”元件时，这样的权利要求应当理解为包括一种或多种这样的元素的合并，既不要求也不排除两种或多种这样的元素。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NB+B27+GF
3 天
Con miR
miR-124
miR-137
DMEM
NB+B27+GF
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miR-124
miR-137
9 天
DM
GF
Con miRNA
miRNA-124
miRNA-137

图 1

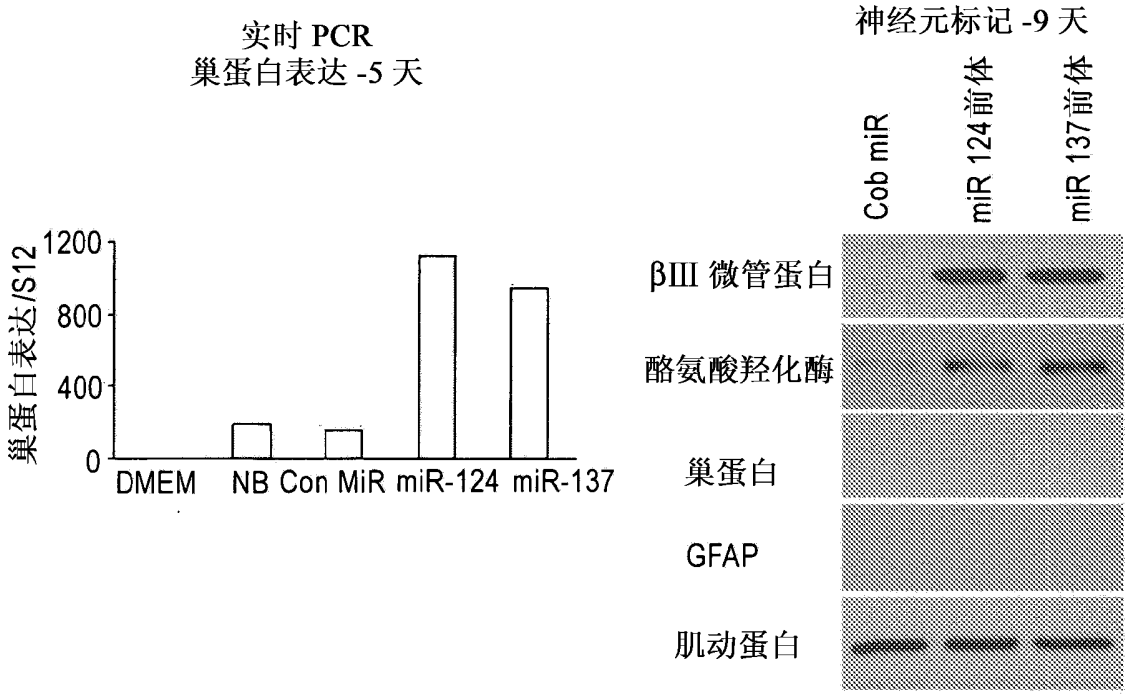
实时 PCR
巢蛋白表达 -5 天
巢蛋白表达/S12
1200
800
400
0
DMEM
NB Con MiR
miR-124
miR-137
神经元标记 -9 天
Cob miR
miR 124前体
miR 137前体
βIII 微管蛋白
酪氨酸羟化酶
巢蛋白
GFAP
肌动蛋白

图 2

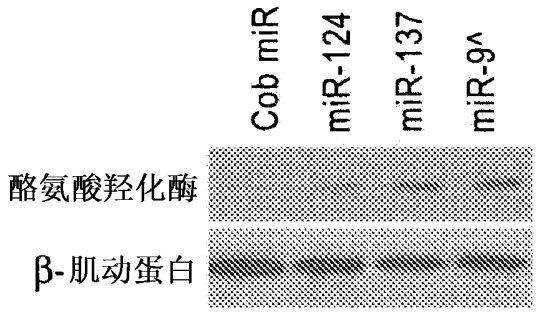
Cob miR
miR-124
miR-137
miR-9^
酪氨酸羟化酶
β-肌动蛋白

图 3

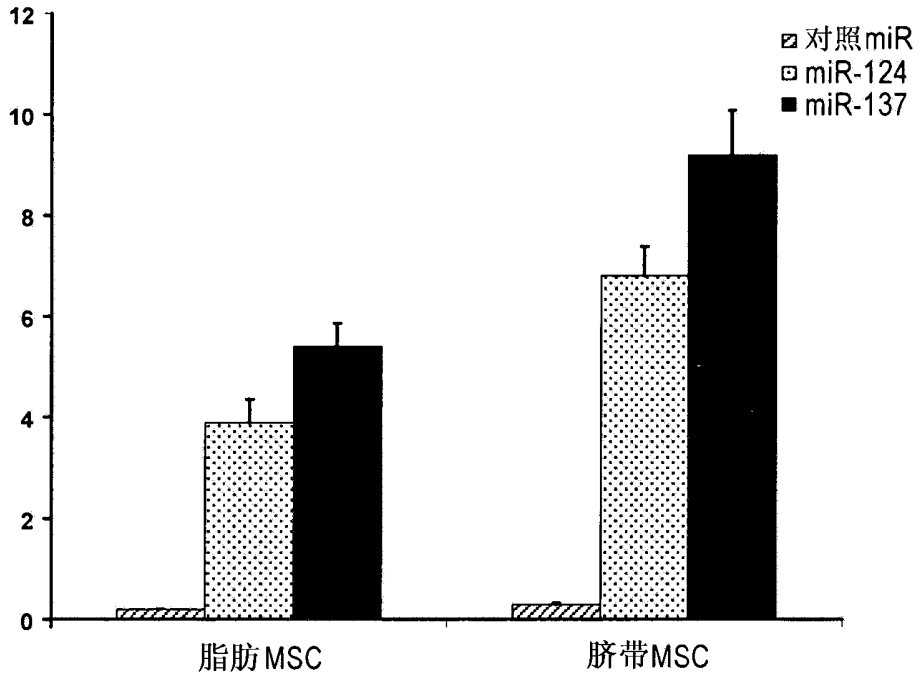
12
10
8
6
4
2
0
对照miR
miR-124
miR-137
脂肪MSC
脐带MSC
(y-轴=βIII 微管蛋白mRNA/S12 mRNA)

图 4