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(54) Title: STANDARDIZED NEURONAL CELL ASSAYS FROM PRIMATE SPECIES

(57) Abstract: This patent application relates to a method to generate standardized and robust neuronal cell culture assays from primate species, for example suitable for high-throughput screening of drug candidates. The method includes differentiating human and/or non-human primate neuronal precursor cells (NPCs) to neuronal cells (NCs) and producing uniform NC cultures based on a dissociation and reseeding step performed on the differentiated NCs resulting in robust cultures suitable for high-throughput drug screening assays, in particular to screen antisense oligonucleotides.

Standardized neuronal cell assays from primate species

FIELD OF THE INVENTION

This patent application relates to a method to generate standardized and robust neuronal cell culture assays from primate species, for example suitable for high-throughput screening of drug candidates. The method includes differentiating human and/or non-human primate neuronal precursor cells (NPCs) to neuronal cells (NCs) and producing uniform NC cultures based on a dissociation and reseeding step performed on the differentiated NCs resulting in robust cultures suitable for high-throughput drug screening assays, in particular to screen antisense oligonucleotides.

BACKGROUND OF THE INVENTION

High-throughput screening assays based on diverse cell types or cell culture models displaying the pathophysiology of various diseases, including diseases of the central nervous system (CNS), are widely employed to assess efficacy and toxicity profiles of drug candidates early in development. Restrictions to limited types of cells or to cells derived from embryonic stem cell have been overcome in the past decade by establishing protocols to generate pluripotent cell from somatic cells. Since Yamanaka and colleagues (Takahashi, K. & Yamanaka, S. Cell. 2006; 126:663–676) demonstrated that somatic cells can be reprogrammed to induced pluripotent stem cells (IPSCs) it became possible to generate pluripotent cells from a variety of cell sources. Different types of somatic cells including fibroblasts, keratinocytes, adipocytes and blood cells have been reprogrammed to an IPSC pluripotent state. More recently, specific somatic cell types could be transdifferentiated to a completely different somatic cell type such as a neuron. Vierbuchen and colleagues demonstrated the direct conversion of mouse fibroblasts to functional neurons by transduction of three crucial genes: Mash1, Brn2 and Myt1l (Vierbuchen et al. Nature. 2010; 463:1035-41). Notably, US2010/0021437 discloses a method for generating induced pluripotent stem cells from fibroblasts and inducing those cells to differentiate into NPCs. More recently direct conversion of differentiated somatic cells to NPCs has been described (WO2012/022725). Such neuronal cells are thought to be a valuable tool for modelling the pathophysiology of various CNS diseases.

NPCs are multipotent stem cells and propagate under specific conditions. They can grow as a monolayer adherent culture or as floating neurospheres in non-adherent cell culture plates. The two types of NPC cultures (neurospheres, adherent cultures) seem to be completely inter-convertible. NPCs can be grown indefinitely and still remain truly multipotent. Upon special conditions they differentiate into the neuronal cell types that compose the adult brain, including differentiated NCs. Differentiated NC cultures are a valuable disease model to screen effective and safe drugs. Indeed, NC cultures are important to assess toxicity and efficacy of drug candidates in a drug development setting.

Before administered in human patients for the first time, drug candidates need to be evaluated thoroughly in *in vitro* cell culture systems followed by *in vivo* testing in rodent and non-human primate (NHP) species. Today, the toxicity and efficacy assessment of novel drug candidates is performed in different assay formats using different protocols for different species. While the *in vitro* testing of drug candidates allows to reduce the number of laboratory animals sacrificed for drug testing, it also posed challenges of outcome translatability from *in vitro* to *in vivo* and to the human physiology. Indeed, the translatability of the efficacy and toxicity profiles of drug candidates from rodent species to humans can be challenging, due to non-close genetic relation between the species. Notably, the protein-coding regions of the mouse and human genome are about 85% identical. The difference in genetic makeup may account for different physiological reactions to drugs and also to different tolerance against toxins or mutagens. Translatability between species becomes even more important to date with novel drug classes targeting specific DNA or RNA sequences, as e.g. antisense oligonucleotides, or genetic variants of a gene or gene product. Therefore, in view of the close genetic relatedness (>90%), NHP species represent the most meaningful model system to generate efficacy and toxicity data to predict response and adverse event rate in humans. Without being bound to theory it is assumed that *in vivo* tests in NHP species, e.g. cynomolgus monkey, are preferable prior to first human trials for all drug classes, but especially for drug classes targeting a defined human genetic makeup. On the other hand, the use of NHP species in drug discovery remains a controversial matter. The very fact that NHP species are genetically close to humans raises ethical questions. Therefore, the number of NHP laboratory animals, e.g. cynomolgus monkey, should be reduced as much as possible.

A comprehensible setup for efficacy and toxicity screening of a novel drug candidate should, therefore, comprise both *in vitro* and *in vivo* tests on NHP species, and parallel *in vitro* tests using IPSC derived human cells. The sequence of testing should include parallel *in vitro* tests both on a NHP species and human cells preceding *in vivo* tests in the NHP species, e.g. cynomolgus monkey. Drug candidates with poor efficacy and/or toxicity profile should already

be rejected at an early stage upon *in vitro* assessment on NHP and/or human cells before starting *in vivo* tests in an NHP species. Indeed, this sequence ensures that the number of NHP laboratory animals can be kept as low as possible.

However, there are formidable hurdles for gaining such primate inter-species transferable efficacy and toxicity data *in vitro* from stem cell-derived differentiated NCs: first, species specific cell culture protocols and non-transferability of cell culture conditions between primate species and, second, inhomogeneous distribution of the differentiating NPCs leading to non-optimal survival conditions or hampered differentiation effects due to local concentration of cells and autocrine and paracrine signalling leading to challenges in phenotypic assessment of drug effects. This becomes most pronounced when the cultures have to be differentiated for a long period of time to obtain the desired differentiation state of the cells. Most notably, differentiated primate NCs are innate sensitive to cell culture conditions and do not tolerate harsh treatment which is an inherent obstacle to producing standardized assays with these cells.

Hence, there remains a need for an easy accessible and reproducible technology for the generation of uniform NC assays from different primate species including humans.

SUMMARY OF THE INVENTION

Provided herein is an *in vitro* method for producing standardized cell cultures of uniformly distributed differentiated neuronal cells (NCs) from different primate species, the method comprising the steps of

a) providing neuronal precursor cells (NPCs);
b) differentiating the NPCs to NCs comprising the steps of
   (i) dissociating the differentiated NCs from its support after about 20 days to about 45 days of differentiation; and
   (ii) reseeding the cells in a suitable cell culture format and continuing differentiation of the NCs for about 4 days to about 15 days.

In one embodiment, the primate species are selected from the group consisting of human (Homo sapiens), Cynomolgus monkey (Macaca fascicularis) and Rhesus monkey (Macaca mulatta).

In one embodiment, the primate species are selected from the group consisting of human (Homo sapiens) and Cynomolgus monkey (Macaca fascicularis).

In one embodiment, standardized cell cultures of differentiated NCs are individually produced for 2 primate species, wherein the first primate species is human (Homo sapiens) and the second primate species is Cynomolgus monkey (Macaca fascicularis).

In one embodiment, the NPCs are derived from induced pluripotent stem cells (IPSCs).

In one embodiment, step b) comprises cultivating the NPCs with one or more differentiating agents selected from the group consisting of Shh, FGF8, BDNF, GDNF, cAMP and ascorbic acid phosphate.

In one embodiment, step b)(i) comprises cultivating the cells in basal medium comprising Shh, FGF8 and ascorbic acid phosphate for a period of about 5 to about 10 days followed by cultivating the cells in basal medium comprising BDNF, GDNF, cAMP and ascorbic acid phosphate for a further period of about 15 days to about 35 days prior to dissociating the differentiated NCs from its support.

In one embodiment, the concentration of the one or more differentiating agent in basal medium is 50-500 ng/ml for Shh, 25-250 ng/ml for FGF8, 1-50 ng/ml for BDNF, 1 50 ng/ml for GDNF, 0.1-10 mM for cAMP and 20-200 µM for ascorbic acid phosphate.

In one embodiment, the concentration of the one or more differentiating agent in basal medium is 200 ng/ml for Shh, 100 ng/ml for FGF8, 20 ng/ml for BDNF, 10 ng/ml for GDNF, 0.5 mM for cAMP and 100 µM for ascorbic acid phosphate.

In one embodiment, the cultivation period with Shh, FGF8 and ascorbic acid phosphate is about 7 days and the cultivation period with BDNF, GDNF, cAMP and ascorbic acid phosphate is about 20 to about 40 days.

In one embodiment, the NPCs are provided on a Laminin521 support.

In one embodiment, step b)(i) comprises dissociating the cells with a cell detachment solution.

In one embodiment, the cell detachment solution is Accutase.

In one embodiment, step b)(ii) comprises reseeding the cells at 200000 cells/cm^2 on a Laminin521 support.

In one embodiment, the cells are reseeded in 96 well or 384 well plates.

In one embodiment, step b)(ii) comprises reseeding the cells in the presence of a ROCK inhibitor.

In one embodiment, the ROCK inhibitor is Y-27632.

In one embodiment, step b)(ii) comprises continuing differentiation by cultivating the cells in basal medium supplemented with BDNF, GDNF, cAMP and ascorbic acid after reseeding the cells.

In one embodiment, the differentiated NCs express MAP2.

In one embodiment, the differentiated NCs comprise MAP2 positive neurites.

In one embodiment, the NPCs are derived from an individual with a neuronal disorder.

In one embodiment, the NPCs are derived from a healthy individual.

In one embodiment, the cell cultures are produced sequentially for different species.

In one embodiment, the cells are essentially uniformly distributed over the cell culture area as assessed by cell nucleus staining.

In one embodiment, the distribution of the cells is assessed by DNA staining, in particular by Hoechst staining.

In one embodiment, provided is a cell culture system obtained by a method as described herein.

In one embodiment, provided is the use of the cell cultures obtained according to a method as described herein for *in vitro* testing of the toxicity of a drug candidate.

In one embodiment, provided is the use of the cell cultures obtained according to a method as described herein for *in vitro* testing of the efficacy of a drug candidate.

In one embodiment, provided is the use of the cell cultures obtained according to a method as described herein for *in vitro* testing of the efficacy of a drug candidate, wherein the efficacy is tested in a cell culture derived from an individual with a neuronal disorder and a cell culture derived from a healthy individual.

In one embodiment, provided is the use of the cell cultures obtained according to a method as described herein for selecting a drug candidate, in particular for selecting a drug candidate for further development.

In one embodiment, provided is the cell cultures for use as described herein, wherein the drug candidate comprises a polynucleotide or targets a specific sequence of a polynucleotide.

In one embodiment, provided is the cell cultures for use as described herein, wherein the drug candidate comprises at least one nucleic acid molecule such as a RNAi agent or an antisense oligonucleotide.

In one embodiment, provided is the cell cultures for use as described herein, wherein the nucleic acid molecule comprise one or more 2' sugar modified nucleosides, independently selected from the group consisting of 2'-O-alkyl-RNA, 2'-O-methyl-RNA, 2'-alkoxy-RNA, 2'-O-methoxyethyl-RNA, 2'-amino-DNA, 2'-fluoro-DNA, arabino nucleic acid (ANA), 2'-fluoro-ANA and LNA nucleosides.

In one embodiment, provided is the cell cultures for use as described herein, wherein the one or more 2' sugar modified nucleoside is a LNA nucleoside.

In one embodiment, provided is the cell cultures for use as described herein, wherein the LNA nucleoside is selected from beta-D-oxy-LNA, alpha-L-oxy-LNA, beta-D-amino-LNA, alpha-L-amino-LNA, beta-D-thio-LNA, alpha-L-thio-LNA, (S)cET, (R)cET, beta-D-ENA or alpha-L-ENA.

In one embodiment, provided is the cell cultures for use as described herein, wherein the nucleic acid molecule comprises at least one modified internucleoside linkage.

In one embodiment, provided is the cell cultures for use as described herein, wherein the internucleoside linkages within the contiguous nucleotide sequence are phosphorothioate internucleoside linkages.

In one embodiment, provided is the cell cultures for use as described herein, wherein the antisense oligonucleotide is capable of recruiting RNase H.

In one embodiment, provided is the cell cultures for use as described herein, wherein the antisense oligonucleotide is a gapmer.

In one embodiment, provided is the cell cultures for use as described herein, wherein the oligonucleotide is a gapmer of formula 5'-F-G-F'-3', where region F and F' independently comprise 1 to 7 modified nucleosides and G is a region between 6 and 16 nucleosides which are capable of recruiting RNaseH.

In one embodiment, provided is the methods and uses essentially as described herein.

SHORT DESCRIPTION OF THE FIGURES

Figure 1: The upper strand illustrates the region of the SNHG14 transcript downstream of SNORD109B (UBE3A-ATS) where the black boxes indicate the location of the tested mouse oligonucleotides. The lower strand illustrates the UBE3A coding region, where the black boxes indicate exons. Exon 1 is located around 160kb. The oligonucleotides are placed in the antisense region of Exon 9 (positioned at ~97kb), Exon 10 (positioned at ~92kb), Exon 13 (positioned at ~77kb) and the 5‘ end of Exon 16 (positioned at ~60kb).

Figure 2: Representation of the ability of the oligonucleotides, tested in Example 2, to induce re-expression of UBE3A in human neuronal cell cultures. Oligonucleotides complementary to the region of human SNHG14 long non-coding RNA between SNORD109B and the region upstream of the UBE3A coding region (position 1 to 55318 of SEQ ID NO: 1) are indicated with • nonoverlap. Oligonucleotides complementary to the region of human SNHG14 long non-coding RNA which is antisense to the UBE3A pre-mRNA (position 55319 to 141053 of SEQ ID NO: 1) are indicated with ▲ overlap. Oligonucleotides from Table 3 with conservation to human and rhesus monkey are indicated at the bottom of each plot as ▒ . Conservation between human:rhesus:mouse is indicated by ■ . The oligonucleotide concentrations were 0.2, 1 and 5 microM as indicated in the right hand side each plot.

Figure 3: Schematic representation of a screening strategy to assess efficacy of a drug candidate on primate *in vitro* cell culture models followed by *in vivo* studies on a primate species before assessing drug efficacy on humans. Cynomolgus monkey and human IPSC-derived neurons are used as *in vitro* model to assess target engagement and efficacy of oligonucleotides targeting UBE3A antisense. Candidates with favorable efficacy profile are prioritized prior to *in vivo* studies.

Figure 4: Overview of the different steps carried out for the derivation of neural progenitor cells from cynomolgus IPSCs. A modified version of the dual SMAD inhibition protocol is used for neuralization of primate IPSC lines. MT refers to MT medium; N2B27+SB+LDN refers to N2B27 medium supplemented with SB-431542 and LDN-193189; N2B27+FEB refers to N2B27 medium supplemented with FGF, EGF and BDNF.

Figure 5: Cyno and human neural precursors display a similar staining positive for Sox2 and Nestin and respective differentiated neurons stain positive for Map2.

Figure 6: The applied directed-differentiation method induces a comparable cross-species neuronal differentiation. Figure 5A) Cyno IPSCs were differentiated in basal medium

supplemented with BDNF, GDNF, cAMP and ascorbic acid phosphate (BGAA) for 14 days. RNA from cyno IPSCs and day 14 NCs was isolated and the expression of the indicated markers was analyzed by qPCR. Data are normalized to the housekeeping gene GAPDH and are presented relative to expression levels in cyno IPSCs. Figure 5B) RNA was isolated from human PSCs and human NCs differentiated for 14 days in basal medium supplemented with BGAA and the expression of the indicated markers was analyzed by RNA sequencing. Data are presented as RPKM values. Figure 5C) RNA was isolated from human PSCs and human NCs differentiated for 14 days in basal medium supplemented with BGAA and the expression of the indicated markers was analyzed by qPCR. Data are normalized to the housekeeping gene GAPDH and are presented relative to expression levels in human PSCs. Figure 5D) RNA from cyno (left panel) and human (right panel) NCs differentiated in basal medium supplemented with BGAA for 14 days has been isolated and expression of UBE3A and UBE3A-ATS transcripts has been analyzed by qPCR. Left panel: Data are normalized to the housekeeping gene TBP and presented relative to cyno IPSCs. Right panel: Data are normalized to the housekeeping gene GAPDH and presented relative to human PSCs.

Figure 7: Schematic depiction of experimental layout for comparison of 4 differentiation methods (A-D) to derive robust neuronal cultures from cyno neural precursors, which is amenable for compound screening. MI refers to Mitotic inhibitor.

Figure 8: Image-based comparison of the 4 neuronal differentiation methods as described in Figure 7. NCs were fixed at day 35 of differentiation and stained for Sox2 (marker for Glia and NSCs) depicted in magenta and Map2 (marker for NCs) depicted in green.

Figure 9: To test the feasibility of dissociating and reseeding human iPSC-derived differentiated NCs, two cell lines were differentiated in parallel. For each, cells were differentiated directly (i.e. without replating) for 6 weeks, or dissociated and reseeded at day 21 and then cultured for a further 3 weeks. Immunofluorescent staining for neuronal (MAP2) and glial (GFAP) markers shows that the dissociating and reseeding (replating) does not hinder the ability of the cells to differentiate.

Figure 10: Quantification of the extend of differentiation in two different iPSC-derived cell lines with and without dissociation and reseeding (replating). HuC/D is a marker expressed by neurons and was detected by immunofluorescence staining in cultures differentiated for a total of 6 weeks, and quantified by high content imaging. The data show that replating does not significantly change the extent of neuronal differentiation.

Figure 11: The expression of the microtubule associated protein Tau and two of its phosphorylated forms was analyzed. The constant expression and extent of phosphorylation in two cell lines with and without replating proves that replating (dissociating and reseeding) does not disrupt the cytoskeletal characteristics of human iPSC-derived NCs, suggesting that physiological features will not be altered by dissociating and reseeding the NCs.

Figure 12: Schematic illustration of the workflow enabling a constant supply of robust primate neuronal cultures for screening in a high-throughput format. SFA refers to basal medium supplemented with Shh, FGF8 and ascorbic acid phosphate; BGAA refers to basal medium supplemented with BDNF, GDNF, cAMP and ascorbic acid phosphate; arrows signify dissociation and reseeding (replating).

Figure 13: Target engagement of tested compounds on standardized cell cultures of uniformly distributed neural cells (NCs) from human and Cynomolgus monkey. Two concentrations (low: 0.02 μM; high: 2 μM) of antisense oligonucleotides directed against UBE3A-antisense transcript (antisense) with known target engagement in human were tested. The NC cultures derived from Cynomolgus monkey in A and B display a comparable target engagement as compared to the NC cultures derived from human in C and D. Treatment of cyno and human NCs at indicated concentration results in a reduction of the UBE3A-antisense transcript accompanied with an upregulation of the UBE3A transcript (sense). The cell cultures were produced according to the workflow as included in Figure 12.

DETAILED DESCRIPTION

The term “antisense oligonucleotide” as used herein is defined as oligonucleotides capable of modulating expression of a target gene by hybridizing to a target nucleic acid, in particular to a contiguous sequence on a target nucleic acid. The antisense oligonucleotides are not essentially double stranded and are therefore not siRNAs. Preferably, the antisense oligonucleotides of the present invention are single stranded.

As used herein, the term “basal medium” refers to a defined medium composed of equal volumes of DMEM:F12 Glutamax medium and Neurobasal medium (Gibco, Invitrogen), supplemented with 1x B27 (Gibco, Invitrogen), 1x N2 (Gibco, Invitrogen), 0.1 mM beta-mercaptoethanol (Gibco, Invitrogen). The term “BGAA” refers to basal medium supplemented with 20 ng/ml BDNF (Peprotech), 10 ng/ml GDNF (Peprotech), 0.5 mM cAMP (BIOLOG Life Science), and 100 µM ascorbic acid phosphate (Sigma). The term “SFA” refers to basal medium supplemented with 200 ng/ml Shh, 100 ng/ml FGF8 and 100 µM ascorbic acid phosphate.

The term “contiguous nucleotide sequence” as used herein refers to the region of the oligonucleotide which is complementary to the target nucleic acid. The term is used interchangeably herein with the term “contiguous nucleobase sequence” and the term “oligonucleotide motif sequence”. In some embodiments all the nucleotides of the oligonucleotide are present in the contiguous nucleotide sequence. In some embodiments the oligonucleotide comprise the contiguous nucleotide sequence and may, optionally comprise further nucleotide(s), for example a nucleotide linker region which may be used to attach a functional group to the contiguous nucleotide sequence. The nucleotide linker region may or may not be complementary to the target nucleic acid.

As used herein, the term “defined medium” or “chemically defined medium” refers to a cell culture medium in which all individual constituents and their respective concentrations are known. Defined media may contain recombinant and chemically defined constituents.

As used herein the terms “differentiating” and “differentiation” refers to one or more steps to convert a less-differentiated cell into a more-differentiated cell, in particular a postmitotic tissue-specific cell type, e.g., to convert a NPC into a NC. Differentiation of NPCs to NCs can be induced *inter alia* by adding one or several differentiating agents to the cell culture medium.

As used herein the term “efficacy profile” or “efficacy” is defined as generally understood by the skilled person to comprise an assessment of the efficacy of a drug candidate based on exposure of a test system, e.g., a cell culture or an organism, with the drug candidate, in

particular in different concentrations and/or with different routes of administration, followed by the determination of resulting cellular and/or physiological effects correlating to the desired effect of the drug candidate. Parameters to determine a cellular and/or physiological effect are defined in context with the respective drug candidate, comprising parameters correlating with the desired phenotypic effect of the drug candidate on the test system or an organism. Preferably, more than one parameter including but not limited to survival, cell viability, morphology, expression and/or expression level of specific genes and protein synthesis is recorded to establish an efficacy profile of a drug candidate. In one aspect of the invention, establishing the efficacy profile includes assessing target engagement.

"Expression markers" or "markers" can be used to determine the identity of a cell type. A certain informative DNA sequence of a cell specific gene is transcribed into mRNA and usually is subsequently translated into a protein (its gene product) which exerts a certain function in a cell. The expression of a marker can be detected and quantified on the RNA level or on the protein level by methods known in the art. IPSC cell markers are known in the art and include but are not limited to TRA-1-60, TRA-1-81, Ecat1, Nanog, Oct4/POU5F1, Sox2, Rex1/Zfp-42 and UTF1, or any combinations thereof. NPC cell markers are known in the art and include but are not limited to Sox2, Nestin, Sox1, Pax6, Dach1. NC cell markers are known in the art and include but are not limited to MAP2, β-III-Tubulin, DCX/Doublecortin, SYN 1/Synapsin 1 and GPHN/ Gephyrin.

As used herein, the term "genetic distance" shall be understood as a measure of the genetic divergence between two species, two genomes or two populations. The genetic distance, e.g., between different species, can be determined by methods known in the art including but not limited to determining the Nei's standard distance, the Goldstein distance or the Rynolds/Weir/Cockerham's genetic distance. Genetic distance can be calculated using software known to the art including but not limited to POPTREE2 or DISPAN. The "genetic similarity" is high when the genetic distance is low.

As used herein, the following abbreviations are used; fibroblast growth factor (FGF), basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), sonic hedgehog (shh), fibroblast growth factor 8 (FGF8), brain-derived neurotrophic factor (BDNF), glial cell-derived neurotrophic factor (GDNF), cyclic adenosine monophosphate (cAMP), Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK).

As used herein, the term "growth factor" means a biologically active polypeptide or a small molecule compound which causes cell proliferation, and includes both growth factors and their analogs.

”High-throughput screening” as used herein shall be understood to signify that a relatively large number of different disease model conditions and/or chemical compounds can be analyzed and compared with the novel assay described herein. Typical, such high-throughput screening is performed in multi-well microtiter plates, e.g., in a 96 well plate or a 384 well plate or plates with 1536 or 3456 wells.

“LNA nucleosides” are modified nucleosides which comprise a linker group (referred to as a biradicle or a bridge) between C2’ and C4’ of the ribose sugar ring of a nucleotide. These nucleosides are also termed bridged nucleic acid or bicyclic nucleic acid (BNA) in the literature.

As used herein, the terms “uniformly distributed”, “uniform distribution” or “homogenous distribution” refers as generally understood by the skilled person to the distribution of an entity in a 1-, 2- or multidimensional space, in particular to the distribution of cells on the 2-dimensional surface of a cell culture support. A uniform distribution is established on a 2-dimensional cell culture surface if the mean cell count per area is essentially constant over the whole cell culture surface. The distribution of cells can be assessed, e.g., by nuclear staining and determining the number of cell nuclei per area using fluorescence microscopy. Indicators for non-homogenous distribution of cells include, e.g., clumps of cells, a significant number of overlapping cell nuclei, or a significant portion of the cell culture area devoid of cells. A cell culture with uniformly distributed cells, of one or more cell types, is referred to as “standardized” as used herein. A “standardized cell culture” or “standardized NC culture” refers to a cell culture produced according to the present invention wherein the distribution of the cells is essentially uniform, i.e., the cells are uniformly distributed and the cell cultures are characterized by uniform distribution of the cells, wherein the cell culture may include one or more cell types. Accordingly, a cell culture is considered to be standardized if the cells display a homogenous distribution as assessed, e.g., by nuclear staining and determining the number of cell nuclei per area.

The term “modified internucleoside linkage” as used herein is defined as generally understood by the skilled person as linkages other than phosphodiester (PO) linkages that covalently couple two nucleosides together. Nucleotides with modified internucleoside linkage are also termed “modified nucleotides”. The modified internucleoside linkage increases the nuclease resistance of the oligonucleotide compared to a phosphodiester linkage. For naturally occurring oligonucleotides, the internucleoside linkage includes phosphate groups creating a phosphodiester bond between adjacent nucleosides. Modified internucleoside linkages are particularly useful in stabilizing oligonucleotides for *in vivo* use, and may serve to protect against nuclease cleavage at regions of DNA or RNA nucleosides in the oligonucleotide of the invention, for example within the gap region of a gapmer oligonucleotide, as well as in regions of modified nucleosides.

As used herein, the term “MT medium” refers to a defined medium that contains Dulbecco’s Modified Eagle Medium with Ham’s F12 Nutrient Mixture (DMEM/F12) with 2.5 mM GlutaMAX™, 7 µg/ml insulin, 450 µM monothioglycerole, 1x Lipid concentrate, 5 mg/ml BSA, 14 ng/ml sodium selenite, 1x non-essential amino acids, 2 mg/ml heparin, 15 µg/ml transferrin, and 220 µM ascorbic acid-2-phosphate.

As used herein, “neuronal precursor cells” or “NPCs” refers to a subset of multipotent cells, which were derived from IPSCs and express some neural progenitor cell markers including, for example, Nestin. NPCs can be produced, *inter alia*, according to the methods as described in Costa et al. Cell Rep 2016; 15:86-95 and Dunkley et al. Proteomics Clin Appl 2015; 7-8:684-94 which are incorporated herein by reference in their entirety or according to the methods as described herein. NPCs can be expanded indefinitely and may differentiate into neurons or glial cells (e.g., astrocytes and oligodendrocytes). The term “patient specific NPCs” refers to NPCs obtained from patient IPSCs that have been reprogrammed from somatic cells of a patient. “NPCs obtained from a healthy individual” as used herein refers to NPCs differentiated from IPSCs obtained from somatic cells of an individual that is apparently healthy and not suspected to suffer from any disorder or disease.

As used herein, “neuronal cells” or “NCs” refer to tissue-spedific cells from the neuronal lineage. NCs can be differentiated *in vitro* from NPCs using specific cell culture conditions, e.g., by withdrawal of growth factors or by addition of one or more differentiating agents, as described herein.

The term “non-human primate” or “NHP” as used herein refers to species belonging to the order of primates with the exception of Homo sapiens. In particular, NHP species according to the methods disclosed in the present invention include but are not limited to Pan troglodytes, Pan paniscus, Hylobates lar, Gorilla gorilla, Pongo abelii, Pongo pygmaeus, Cercopithecus mitis, Cercopithicus neglectus, Chlorocebus aethiops, Chlorocebus sabaeus, Colobus guereza, Lophocebus aterrimus, Macaca arctoides, Macaca assamensis, Macaca fascicularis (Cynomolgus monkey), Macaca fuscata, Macaca mulatta (Rhesus monkey), Macaca nemestrina, Macaca silenus, Mandrillus leucophaeus, Mandrillus sphinx, Macaca thibetana, Papio anubis, Papio cynocephalus, Papio hamadryas, Papio papio, Papio ursinus, Presbytis entellus, Theropithecus gelada, Aotus azarae, Aotus nancymaae, Aotus nigriceps, Aotus trivirgatus, Aotus vociferans, Ateles belzebuth, Ateles fusciceps, Callithrix jacchus, Callicebus moloch, Cebuella pygmaea, Cebus apella, Leontopithecus rosalia, Pithecia pithecia, Saguinus fuscicollis, Saguinus geoffroyi, Saguinus labiatus, Saguinus mystax, Saguinus Oedipus and Saimiri sciureus.

The term “cyno” as used herein is an abbreviation for Cynomolgus monkey and/or refers to material derived from Cynomolgus monkeys including but not limited to cells, tissues, organs, blood or cells derived therefrom.

“Nucleotides” are the building blocks of oligonucleotides and polynucleotides, and for the purposes of the present invention include both naturally occurring and non-naturally occurring nucleotides. In nature, nucleotides, such as DNA and RNA nucleotides comprise a ribose sugar moiety, a nucleobase moiety and one or more phosphate groups (which is absent in nucleosides). Nucleosides and nucleotides may also interchangeably be referred to as “units” or “monomers”.

As used herein, the term “nucleotide sequence derived from a human genome” means that the respective nucleotide sequence is derived from a human genome reference, i.e. at least a subpopulation of the global human population comprises the respective nucleotide sequence in the genome. Furthermore, as used herein the term “nucleotide sequence derived from a human genome” is used for sequences assigned to a human genome with highest maximal score using the NCBI/Blast database and algorithm (Zheng Zhang, Scott Schwartz, Lukas Wagner, and Webb Miller (2000), "A greedy algorithm for aligning DNA sequences", J Comput Biol 2000; 7(1-2):203-14.). Without being bound to theory it is assumed that the alignment Score of a query sequence is higher for a human reference sequence compared to a non-human reference sequence if the query sequence is derived from a human genome.

The term “modified nucleoside” or “nucleoside modification” as used herein refers to nucleosides modified as compared to the equivalent DNA or RNA nucleoside by the introduction of one or more modifications of the sugar moiety or the (nucleo)base moiety. In a preferred embodiment the modified nucleoside comprises a modified sugar moiety. The term modified nucleoside may also be used herein interchangeably with the term “nucleoside analogue” or modified “units” or modified “monomers”.

The term “modified internucleoside linkage” is defined as generally understood by the skilled person as linkages other than phosphodiester (PO) linkages, that covalently couples two nucleosides together. Nucleotides with modified internucleoside linkage are also termed “modified nucleotides”. In some embodiments, the modified internucleoside linkage increases the nuclease resistance of the oligonucleotide compared to a phosphodiester linkage. For naturally occurring oligonucleotides, the internucleoside linkage includes phosphate groups creating a phosphodiester bond between adjacent nucleosides. Modified internucleoside linkages are particularly useful in stabilizing oligonucleotides for *in vivo* use, and may serve to protect against nuclease cleavage at regions of DNA or RNA nucleosides in the oligonucleotide

of the invention, for example within the gap region of a gapmer oligonucleotide, as well as in regions of modified nucleosides.

As used herein, the term “N2B27” refers to a defined medium composed of equal volumes of DMEM:F12 (Gibco, Invitrogen) supplemented with N2 and B27 (both from Gibco, Invitrogen).

The term “oligonucleotide” as used herein is defined as it is generally understood by the skilled person as a molecule comprising two or more covalently linked nucleosides. Such covalently bound nucleosides may also be referred to as nucleic acid molecules or oligomers. Oligonucleotides are commonly made in the laboratory by solid-phase chemical synthesis followed by purification. When referring to a sequence of the oligonucleotide, reference is made to the sequence or order of nucleobase moieties, or modifications thereof, of the covalently linked nucleotides or nucleosides. The oligonucleotide may comprise one or more modified nucleosides or nucleotides. The term “antisense oligonucleotide” as used herein is defined as oligonucleotides capable of modulating expression of a target gene by hybridizing to a target nucleic acid, in particular to a contiguous sequence on a target nucleic acid. The antisense oligonucleotides are not essentially double stranded and are therefore not siRNAs. Preferably, the antisense oligonucleotides are single stranded.

As used herein, the term “reprogramming” refers to one or more steps needed to convert a somatic cell to a less-differentiated cell, for example for converting a fibroblast cell, adipocytes, keratinocytes or leucocyte into a NPC. “Reprogrammed” cells refer to cells derived by reprogramming somatic cells as described herein.

The term “small molecule”, or “small compound”, or “small molecule compound” as used herein, refers to organic or inorganic molecules either synthesized or found in nature, generally having a molecular weight less than 10,000 grams per mole, optionally less than 5,000 grams per mole, and optionally less than 2,000 grams per mole.

The term “somatic cell” as used herein refers to any cell forming the body of an organism that are not germline cells (e.g., sperm and ova, the cells from which they are made (gametocytes)) and undifferentiated stem cells.

The term “stem cell” as used herein refers to a cell that has the ability for self-renewal and differentiation. An “undifferentiated stem cell” as used herein refers to a stem cell that has not undergone differentiation. As used herein, “pluripotent stem cells” or “PSCs” refers to stem cells that can give rise to cell types of the three germlayers (endoderm, ectoderm, mesoderm) as well as the germline. Pluripotent stem cells (PSCs) include but are not limited to “embryonic stem cells” (“ESCs”) and “induced pluripotent stem cells” (“IPSCs”). The terms “hIPSCs” and

"cIPSCs" refer to IPSCs derived from human cells and to IPSCS derived from Cynomolgus monkey cells, respectively.

As used herein the term "substantial loss of cell viability" refers to a reduction of cell viability upon application of distinct cell culture conditions or manipulating the cells in a defined process, in particular in connection to dissociating cells from the cell culture support. In one embodiment, substantial loss of cell viability means that more than 5% of the cells become non-viable and/or undergo apoptosis. In further embodiments, substantial loss of cell viability means that more than 10%, more than 15%, more than 20% or more than 25% of the cells become non-viable and/or undergo apoptosis. Accordingly, in one embodiment, the term "essentially remain viable" means that more of 95% of the cells remain viable. In further embodiments, essentially remain viable means that more than 90%, more than 85%, more than 80% or more than 75% of the cells remain viable.

A "suitable medium for differentiation", also depicted as "differentiation medium", as used herein refers to any chemically defined medium useful for differentiation of NPCs to NCs. A differentiation medium as described herein contains at least one "differentiating agent". Differentiating agents include but are not limited to biologically active polypeptides or a small molecule compounds which cause cell differentiation.

The "target" refers to the protein which it is desired to modulate. A "target nucleic acid" is the intended target which the oligonucleotide of the invention hybridizes to, and may for example be a gene, a RNA, a non-coding RNA, a long non-coding RNA, a mRNA, and pre-mRNA, a mature mRNA or a cDNA sequence. In some embodiments the target nucleic acid is a non-coding RNA or a long non-coding RNA, or a subsequence thereof. For particular *in vivo* or *in vitro* application, the oligonucleotide of the invention is capable of decreasing the level of the SNHG14 transcript downstream of SNORD109B of and thereby relieving the suppression of the paternal UBE3A transcript in the intended target cell. The contiguous sequence of nucleobases of the oligonucleotide of the invention is complementary to the target nucleic acid, as measured across the length of the oligonucleotide, optionally, with the exception of one or two mismatches, and, optionally, excluding nucleotide based linker regions which may link the oligonucleotide to an optional functional group such as a conjugate. The oligonucleotide comprises a contiguous nucleotide sequence which is complementary to or hybridizes to a sub-sequence of the target nucleic acid molecule.

The term "target sequence" as used herein refers to a sequence of nucleotides present in the target nucleic acid which comprises the nucleobase sequence which is complementary to the oligonucleotide of the invention. In some embodiments, the target sequence consists of a region

on the target nucleic acid which is complementary to the contiguous nucleotide sequence of the oligonucleotide of the invention. In some embodiments the target sequence is longer than the complementary sequence of a single oligonucleotide, and may, for example represent a preferred region of the target nucleic acid which may be targeted by several oligonucleotides of the invention. The oligonucleotide of the invention comprises a contiguous nucleotide sequence which is complementary to the target nucleic acid, such as a target sequence. The oligonucleotide comprises a contiguous nucleotide sequence of at least 8 nucleotides which is complementary to or hybridizes to a target sequence present in the target nucleic acid molecule. The contiguous nucleotide sequence (and therefore the target sequence) comprises of at least 8 contiguous nucleotides, such as 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 contiguous nucleotides, such as from 12-25, such as from 14-18 contiguous nucleotides.

The term a “target cell” as used herein refers to a cell which is expressing the target nucleic acid. In some embodiments the target cell may be *in vivo* or *in vitro*. In some embodiments the target cell is a mammalian cell such as a rodent cell, such as a mouse cell or a rat cell, or a primate cell such as a monkey cell or a human cell. In one embodiment the target cell is a neuronal cell (NC).

As used herein the term “toxicity profile” or “toxicity” is defined as generally understood in the art to comprise a toxicological assessment of a potential harmful or non-harmful substance based on exposure of a test system, e.g., a cell culture or an organisms, with the substance, in particular in different concentrations and/or with different routes of administration, followed by the determination of resulting cellular and/or physiological effects, e.g., cell survival or health. Parameters to determine cellular and/or physiological effects are well known in the art including but not limited to survival, cell viability, morphology, expression and/or expression level of certain genes and protein synthesis. Preferably, more than one parameter is recorded to establish a toxicity profile of a substance with unknown toxicity. The toxicity profile of a drug candidate is used in the art to select drug candidates for further development, e.g., *in vivo* testing on primates or humans.

The present invention provides a novel method for producing reproducible and standardised differentiated NC cultures from different primate species, including human, which can be used for *in vitro* high-throughput testing of drug candidates. The method comprises providing primate NPCs, differentiating the NPCs to NCs, and capacitating the cell cultures for high-throughput screening of drug candidates by dissociating the differentiated primate NCs and reseeding the cells in a suitable cell culture format without significant loss of cell viability, and pursuing differentiation of the NCs. The primate NPCs can be derived from IPSCs or transdifferentiated

cells, which both can be generated from somatic cells. Preferably said somatic cells are primate cells including human somatic cells.

In one embodiment provided is an *in vitro* method for producing cell cultures of uniformly distributed differentiated neuronal cells (NCs) from different primate species, the method comprising the steps of

a) providing neuronal precursor cells (NPCs); and

b) differentiating the NPCs to NCs comprising the steps of

(i) dissociating the differentiated NCs from its support between day 20 and 45 of differentiation; and

(ii) reseeding the cells in a suitable cell culture format and continuing differentiation of the NCs for 4 to 15 days.

To achieve the inventive method described here, it was necessary to bypass some of the existing limitations of NC cultures. It is widely accepted that in contrast to NPCs, which can be expanded indefinitely either as adherent or floating cultures, differentiating NCs are critically dependent on interaction with a matrix usually consisting of a biopolymer coating of a cell culture plate. Furthermore, differentiated NCs become sensitive to cellular stress and, therefore, passaging of differentiated cells is considered to be detrimental to the cells. The innovative method according to the present invention discloses cell culture conditions whereby uniform NC cultures can be produced from different primate species wherein the NCs are dissociated from its support between day 10 and 40 of differentiation without substantial loss of viability. The NCs can be reseeded in a suitable cell culture format and the differentiation continued. The dissociation and reseeding results in a homogenous distribution of the differentiated NCs over the cell culture area. Furthermore, the method of the present invention allows changing the cell culture format at a late stage of NC differentiation. In further embodiments, step b)(i) comprises dissociating the differentiated NCs from its support between about day 25 and about day 40, between about day 28 and about day 30, at about day 28 or at about day 30 of differentiation.

Accordingly, one aspect of the present invention is a method as described herein to produce uniform differentiated NC cultures. In one embodiment, step b) comprises differentiating the NPCs to neural cells (NCs) comprising the steps of (i) dissociating the differentiated NCs from its support between day 20 and 45 of differentiation; and (ii) reseeding the cells in a suitable cell culture format and continuing differentiation of the NCs for 4 to 10 days, wherein the NCs essentially remain viable. Said dissociation and reseeding step is critical to obtain a uniform distribution of differentiated primate NCs across the area of a cell culture surface, e.g., a cell culture well and/or for harvesting differentiated primate NCs without substantial loss of viability. Consequently, differentiated primate NC culture assays produced according to the present

invention display more evenly distributed cells and are better suited for high-throughput assays compared to cell cultures produced without dissociation and reseeding. Importantly, the cell culture format can be changed at a late stage of NC differentiation. This is in contrast to methods of the art, wherein the final cell culture format has to be employed at an early time point. Indeed, being able to change the cell culture format allows for considerable logistic flexibility.

The highly reproducible cell cultures of primate NCs are well standardised and can be used in compound screening assays including but not limited to *in vitro* efficacy assessment of drug candidates. Furthermore, the cell cultures as described herein can be used for selecting drug candidates, in particular for selecting a drug candidate for further development as described herein. In one embodiment, the cell cultures according to the invention are used to determine the *in vitro* efficacy profile of a drug candidate as described herein. In a further embodiment the *in vitro* efficacy profile of a drug candidate is determined prior to the determination of an *in vivo* efficacy profile as described herein. In a further embodiment, the cell cultures according to the invention are used to determine the *in vitro* toxicity profile of a drug candidate as described herein.

Accordingly, in one embodiment, differentiated primate NCs are dissociated from the cell culture vessel, wherein the NCs essentially remain viable. The dissociated NCs can be reseeded in a desired cell culture format at a cell density optimized to the needs of a given cell culture assay. In one embodiment, the dissociation and reseeding conditions as described in the present invention can be applied to different primate species. In one embodiment, the method according to the present invention can be used for inter-species comparison of the efficacy profile of a drug candidate, wherein the cell cultures are produced individually from cells of at least two species, wherein essentially the same conditions are applied to the cultures for all species and wherein the efficacy profile is determined and compared for all species. It is within the scope of the present invention to produce standardised cell culture assays deriving from at least one primate species, at least two primate species or at least three primate species using essentially the same cell culturing conditions and to compare and integrate the results as determined by the assay readout to determine a comprehensive efficacy profile of at least one drug candidate.

In one embodiment the NPCs are generated from IPSCs derived from reprogrammed somatic cells. Reprogramming of somatic cells to IPSCs can be achieved by introducing specific genes involved in the maintenance of IPSC properties. Genes suitable for reprogramming of somatic cells to IPSCs include, but are not limited to Oct4, Sox2, Klf4 and C-Myc and combinations thereof. In one embodiment the genes for reprogramming are Oct4, Sox2, Klf4 and C-Myc.

Combinations of genes for transdifferentiating somatic cells to NPCs are described in WO2012/022725 which is herein included by reference.

Internal organs, skin, bones, blood and connective tissue are all made up of somatic cells. Somatic cells used to generate IPSCs include but are not limited to fibroblast cells, adipocytes and keratinocytes and can be obtained from skin biopsy. Other suitable somatic cells are leucocytes, erythroblasts cells obtained from blood samples or epithelial cells or other cells obtained from blood or urine samples and reprogrammed to IPSCs by the methods known in the art and as described herein. The somatic cells can be obtained from a healthy individual or from a diseased individual. The genes for reprogramming as described herein are introduced into somatic cells by methods known in the art, either by delivery into the cell via reprogramming vectors or by activation of said genes via small molecules. Methods for reprogramming comprise, *inter alia,* retroviruses, lentiviruses, adenoviruses, plasmids and transposons, microRNAs, small molecules, modified RNAs and recombinant proteins. In one embodiment, a lentivirus is used for the delivery of genes as described herein. In another embodiment, Oct4, Sox2, Klf4 and C-Myc are delivered to the somatic cells using Sendai virus particles. In addition the somatic cells can be cultured in the presence of at least one small molecule. In one embodiment, said small molecule comprises an inhibitor of the Rho-associated coiled-coil forming protein serine/threonine kinase (ROCK) family of protein kinases. Non-limiting examples of ROCK inhibitors comprise fasudil (1-(5-Isoquinolinesulfonyl) homopiperazine), Thiazovivin (N-Benzyl-2-(pyrimidin-4-ylamino) thiazole-4-carboxamide) and Y-27632 ((+)-(*R*)-*trans*-4-(1-aminoethyl)-*N*-(4-pyridyl) cyclo-hexanecarboxamide dihydrochloride). Resulting IPSCs can be induced to differentiate into NPCs. In one embodiment, the IPSCs are induced to differentiate into NPCs.

In one embodiment, primate NPCs are generated from IPSCs by dual SMAD inhibition. In one embodiment NPCs are generated from IPSCs by contacting the cells with SB-431542 (Calbiochem) and LDN-193189 (Calbiochem). In a particular embodiment NPCs are generated from IPSCs by contacting the cells with 5 ng/ml FGF (Peprotech), 10 µM SB-431542 (Calbiochem) and 100 nM LDN-193189 (Calbiochem). Resulting primate NPCs can be expanded in basal medium supplemented with FGF, EGF and BDNF. In one embodiment, NPCs are expanded in basal medium supplemented with 10 ng/ml FGF (Peprotech), 10 ng/ml EGF (RnD), and 20 ng/ml BDNF (Peprotech). Continued passaging in basal medium supplemented with FGF, EGF and BDNF leads to a stable neural progenitor cell line (NPC line). A stable NPC line is defined by its capacity to self-renew and by the expression of the developmental stage-specific markers Sox2 and Nestin. Accordingly, in one embodiment, the primate NPCs express Sox2 and Nestin.

For propagating proliferation of NPCs the cells are grown in an expansion medium comprising a serum free medium supplemented with growth factors. In one embodiment, said growth factors comprise FGF, BDNF and EGF. Accordingly, in one embodiment, the method additionally comprises incubating the cells of step a) under conditions suitable for proliferation of NPCs, e.g., until a defined number of cells per area is reached. Non-limiting examples of expansion media are described herein. In one embodiment, the expansion medium is supplemented with 10-50 ng/ml FGF, 10-50 ng/ml EGF and 1-20 ng/ml BDNF. In a particular embodiment, the NPC expansion medium is basal medium supplemented with 10 ng/ml FGF2, 10 ng/ml EGF and 20 ng/ml BDNF. NPCs can be produced in unrestricted quantities and are therefore most suitable for high-throughput cell culture assays requiring large numbers of assay plates. Culturing is within the capabilities of the person skilled in the art.

In one embodiment the primate NPCs are washed with a suitable buffer or medium prior to initializing differentiation to remove any dead cells. Preferably the media are changed in between each step of the cell culture protocol, e.g., the medium is removed, by aspiration or centrifuging the cells and discarding the supernatant and then the medium used in the subsequent step is added to the cells. In one embodiment the cells are washed with a suitable buffer or medium prior to adding the medium of the subsequent step to remove any dead cells and any residual medium or growth factors or cytokines applied in the previous step. Buffers or media useful for washing the cells are known in the art. One example of a suitable buffer for washing the cells is phosphate buffered saline (PBS).

In one embodiment, the primate NPC cultures are provided at a density of about 5000 cells/cm^2 to about 100000 cells/cm^2. In further embodiments, the primate NPC cultures are provided at a density of about 10000 cells/cm^2 to about 50000 cells/cm^2. In one embodiment, the adherent primate NPC cultures are provided at a density of about 20000 cells/cm^2 to about 40000 cells/cm^2. In one embodiment the adherent primate NPC cultures are provided at a density of about 30000 cells/cm^2. In one embodiment the adherent primate NPC cultures are provided on a Laminin521 support.

In one embodiment, the primate NPCs obtained by methods known in the art and as described herein are in a next step induced to differentiate to NCs by contacting the cells with Shh (sonic hedgehog), FGF8 (fibroblast growth factor 8) and ascorbic acid phosphate. In one embodiment the NPCs are incubated with a chemically defined medium as described herein comprising Shh, FGF8 and ascorbic acid phosphate. In one embodiment, the medium is supplemented with 50-1000 ng/ml Shh, 25-500 ng/ml FGF8 and 20-200 µM ascorbic acid phosphate. In further embodiments, the cells are contacted with Shh, FGF8 and ascorbic acid phosphate for about 1 day, for about 2 days, for about 3 days, for about 4 days, for about 5 days, for about 6 days, for

about 7 days, for about 8 days, for about 9 days or for about 10 days. In a further embodiment, the cells are contacted with Shh, FGF8 and ascorbic acid phosphate for about 5 days to about 10 days. In a particular embodiment, the primate NPCs are cultivated in basal medium supplemented with 200 ng/ml Shh, 100 ng/ml FGF8 and 100 µM ascorbic acid phosphate for about 7 days.

In one embodiment the cells are replated after induction of neuronal differentiation at a density of about 10000 cells/cm^2 to about 80000 cells/cm^2, about 20000 cells/cm^2 to about 70000 cells/cm^2, about 30000 cells/cm^2 to about 60000 cells/cm^2 or about 40000 cells/cm^2 to about 50000 cells/cm^2. In a particular embodiment the cells are replated after induction of neuronal differentiation at a density of about 45000 cells/cm^2.

In one embodiment, the cells induced to neuronal differentiation are in a next step differentiated in basal medium supplemented with BDNF, GDNF, cAMP and ascorbic acid phosphate for about further 15 days, for about further 16 days, for about further 17 days, for about further 18 days, for about further 19 days, for about further 20 days, for about further 21 days, for about further 22 days, for about further 23 days, for about further 24 days, for about further 25 days, for about further 26 days, for about further 27 days, for about further 28 days, for about further 29 days, for about further 30 days, for about further 31 days, for about further 32 days, for about further 33 days, for about further 34 days or for about further 35 days. In a particular embodiment, the cells are cultivated in basal medium supplemented with 20 ng/ml BDNF (Peprotech), 10 ng/ml GDNF (Peprotech), 0.5 mM cAMP (BIOLOG Life Science), and 100 µM ascorbic acid phosphate (Sigma) for further about 19 days to about 35 days.

In one embodiment, according to the invention, the differentiated NCs are dissociated from its support and reseeded on a suitable cell culture format as described herein. In further embodiments the NCs, are differentiated in basal medium supplemented with BDNF, GDNF, cAMP and ascorbic acid for at least about 10 days, for at least about 11 days, for at least about 12 days, for at least about 13 days, for at least about 14 days, for at least about 15 days, for at least about 16 days, for at least about 17 days, for at least about 18 days, for at least about 19 days, for at least about 20 days, for at least about 21 days, for at least about 22 days, for at least about 23 days, for at least about 24 days, for at least about 25 days, for at least about 26 days, for at least about 27 days or for at least about 28 days before being dissociated and reseeded on a suitable cell culture assay format. In further embodiments, the primate NCs are differentiated in basal medium supplemented with BDNF, GDNF, cAMP and ascorbic acid for about 15 to about 30 days, for about 20 to about 25 days, for about 21 to about 23 days before being dissociated and reseeded on a suitable cell culture assay format.

In one aspect of the invention, the differentiated NCs obtained by the method as described herein are dissociated from the cell culture substrate without substantial loss of cell viability. Accordingly, in one embodiment the differentiated NCs are harvested without substantial loss of cell viability. Cell number can be determined according to conventional methods used in the art including but not limited to counting cell numbers in a haemocytometer or using flow cytometry. Cell viability can be determined according to conventional methods including but not limited to trypan blue and Erythrosin B staining. After dissociation the cells can be reseeded in suitable cell culture wells at a suitable cell density according to the specific need or experimental parameters of the desired assay based on NCs.

Accordingly, in one embodiment, the differentiated NCs are separated from the cell culture surface by use of a cell detachment solution. In one embodiment, the differentiated NCs are dissociated from its support and reseeded in a suitable cell culture format. In a further embodiment the differentiated NCs are dissociated from its support between about day 20 and about day 45 of differentiation. The number of days of differentiation is counted from the day of initiation of differentiation, e.g., incubating with Shh, FGF8 and ascorbic acid phosphate, wherein the day of addition of Shh, FGF8 and ascorbic acid is counted as day 0. In further embodiments the differentiated NCs are dissociated from its support between about day 15 and about day 50, between about day 28 and about day 30, at about day 25 of differentiation, at about day 26 of differentiation, at about day 27 of differentiation, at about day 28 of differentiation, at about day 29 of differentiation, at about day 30 of differentiation, at about day 31 of differentiation, at about day 32 of differentiation, at about day 33 of differentiation or at about day 34 of differentiation. In one embodiment, the differentiated NCs are dissociated from its support by incubating the cells with a cell detachment solution. In one embodiment the cell detachment solution is Accutase. Accutase is a marine-origin enzyme with proteolytic and collagenolytic activity. In one embodiment the cell detachment solution is added to the differentiated NC cultures and incubated for 1 to 5 minutes, for 2 to 4 minutes, preferentially for 3 minutes. After completion of the incubation time, the Accutase solution is diluted with medium, in particular basal medium. Before reseeding, Accutase containing medium is removed and replenished with fresh basal medium supplemented with growth factors as described herein.

After detachment the primate NCs can be reseeded in new cell culture containments as, e.g., cell culture wells in a suitable plate format. NCs can be reseeded at any desired density, including low density, medium density and high density. Accordingly, cultures of uniformly distributed differentiated NCs can be generated at a defined and desired density, including low density, medium density and high density. This is in contrast to conventional cell culture

methods wherein the cell density is fixed at the beginning of culture and wherein the cell density may change considerably in time due to proliferation of cells or cell death. In one embodiment, the NCs are reseeded at a high density. In further embodiments, NCs are reseeded at a density of about 50000 cells/cm^2 to about 500000 cells/cm^2, at a density of about 75000 cells/cm^2 to about 400000 cells/cm^2, or at a density of about 100000 cells/cm^2 to about 300000 cells/cm^2. In a particular embodiment, the differentiated NCs are reseeded in step b)(ii) at a density of about 200000 cells/cm^2. In one embodiment the cells are dissociated and reseeded after differentiation with BDNF, GDNF, cAMP and ascorbic acid phosphate at a density of about 50000 cells/cm^2 to about 500000 cells/cm^2, about 75000 cells/cm^2 to about 400000 cells/cm^2 or about 100000 cells/cm^2 to about 300000 cells/cm^2. In a particular embodiment the cells are dissociated and reseeded after differentiation with BDNF, GDNF, cAMP and ascorbic acid phosphate at a density of about 200000 cells/cm^2. After dissociation and reseeding (replating), the cells are further differentiated in basal medium supplemented with BDNF, GDNF, cAMP and ascorbic acid for about further 1 day, for about further 2 days, for about further 3 days, for about further 4 days, for about further 5 days, for about further 6 days, for about further 7 days, for about further 8 days, for about further 9 days, for about further 10 days, for about further 11 days, for about further 12 days, for about further 13 days, for about further 14 days or for about further 15 days.

The dissociated and reseeded differentiated NC cultures can be used according to the invention for testing the efficacy of drug candidates. In a particular embodiment the cells are further differentiated in basal medium supplemented with 20 ng/ml BDNF (Peprotech), 10 ng/ml GDNF (Peprotech), 0.5 mM cAMP (BIOLOG Life Science), and 100 µM ascorbic acid phosphate (Sigma) for about 7 days before applying a drug candidate to the cells. Accordingly, the NC cells are ready for screening drug candidates after a total differentiation period of about 10 days to about 50 days, of about 15 days to about 45 days, of about 30 to about 40 days, of about 35 days or about 37 days. In a particular embodiment, the NCs are ready for screening oligonucleotide candidates after a total differentiation period of about 30 to about 40 days.

In further embodiments, the differentiated NC cultures are ready for treatment with a drug candidate after a total differentiation period of about 28 days, of about 29 days, of about 30 days, of about 31 days, of about 32 days, of about 33 days, of about 34 days, of about 35 days, of about 36 days, of about 37 days, of about 38 days, of about 39 days, of about 40 days, of about 41 days, of about 42 days, of about 43 days, of about 44 days, of about 45 days, of about 46 days, of about 47 days, of about 48 days, of about 49 days, of about 50 days, of about 51 days, of about 52 days, of about 53 days, of about 54 days or of about 55 days. In a further

embodiment, treatment with a drug candidate is performed at any step of the cell culture protocols as described herein.

In a particular embodiment, the primate NPCs are differentiated to NCs in a serum free differentiation medium comprising 20 ng/ml BDNF, 10 ng/ml GDNF, 0.5 mM cAMP and 100 µM ascorbic acid phosphate for about 21 to about 23 days prior to dissociating and reseeding. In a further embodiment, the differentiated NCs are incubated with differentiation medium as described herein for further about 1 day, for further about 2 days, for further about 3 days, for further about 4 days, for further about 5 days, for further about 6 days, for further about 7 days, for further about 8 days, for further about 9 days, for further about 10 days, for further about 11 days, for further about 12 days, for further about 13 days, for further about 14 days, or for further about 15 days after reseeding as described herein. In a particular embodiment, the differentiated NCs are incubated in a serum free differentiation medium comprising 20 ng/ml BDNF, 10 ng/ml GDNF, 0.5 mM cAMP and 100 µM ascorbic acid phosphate for about 7 days after reseeding. Thereafter, the differentiated NCs are ready for treatment with a drug candidate.

In one aspect of the invention differentiation of the dissociated and reseeded NCs is continued prior to adding a drug candidate to the cell culture medium. Accordingly, in further embodiments, the reseeded NCs are incubated in a differentiation medium as described herein for about 1 day, for about 2 days, for about 3 days, for about 4 days, for about 5 days, for about 6 days, for about 7 days, for about 8 days, for about 9 days, for about 10 days, for about 11 days, for about 12 days, for about 13 days, for about 14 days or for about 15 days prior to adding a drug candidate. In further embodiments step b)(ii) comprises differentiating the NCs after reseeding for further about 1 to about 20 days, for further about 5 to about 10 days, for about 7 days with basal medium comprising 1 to 50 ng/ml BDNF, 1 to 50 ng/ml GDNF and 0.1-10 mM cAMP and 20 to 200 µM ascorbic acid phosphate prior to adding a drug candidate. In a particular embodiment step b)(ii) comprises differentiating the NCs after reseeding for further about 7 days with basal medium comprising 20 ng/ml BDNF, 10 ng/ml GDNF and 0.5 mM cAMP and 100 µM ascorbic acid phosphate prior to adding a drug candidate.

In one embodiment, the drug candidate is added to the cell culture medium after dissociating and reseeding the differentiated NCs. In further embodiments, the drug candidate is added to the cell culture medium about 1 day to about 15 days, about 5 days to about 10 days or about 7 days after reseeding the differentiated NCs. In a particular embodiment, the drug candidate is added to the cell culture medium about 7 days after reseeding the differentiated NCs.

In one embodiment the primate NPCs are differentiated to NCs according to the methods as described herein. In one embodiment, the neuronal identity is assessed with expression

markers associated with cellular and/or metabolic functions of neurons. Typical neuronal markers include but are not limited to MAP2, HuC/D, Nestin, β-III-Tubulin, DCX/Doublecortin, SYN 1/Synapsin 1 and GPHN/Gephyrin. The expression markers associated with neuronal identity can be expressed at a lower level in NCs derived from NPCs compared to the expression level in primary neurons or neural tissue. The normalized expression level of neuronal expression markers in NSC-derived NCs can be 10000x lower, or 1000x lower, or 100x lower, or 10x lower, or 2x lower compared to the expression level of the respective markers in primary neurons or neural tissue. The fold change of expression level of neuronal expression markers between NPC-derived NCs and primary neurons can be different for different expression markers. Normalization can be achieved by relating the absolute expression level of a given marker to a suitable house-keeping gene, *e.g.*, GAPDH or TBP.

In one embodiment, the innovative method of the present invention is used to generate robust differentiated NC cultures with homogenous NC distribution for different primate species including but not limited to human (Homo sapiens), Cynomolgus monkey (Macaca fascicularis) and Rhesus monkey (Macaca mulatta). Essentially the same cell culture conditions can be applied to all primate species.

Without being bound theory, the present invention distinguishes two different stages of cells along the axis from pluripotent stem cells to fully differentiated NCs which are NPCs and differentiated NCs. Pluripotent NPCs can be obtained as disclosed herein and can be expanded to any suitable cell number, e.g., for a cell culture assay of a desirable format. It is possible to freeze and thaw healthy individuals and patients specific NPC aliquots. Accordingly, said NPCs can be expanded to a suitable cell number, frozen for storing or directly differentiated to produce robust differentiated NC culture assays according to the invention. Unexpectedly, the inventors found that using the specific conditions as disclosed in the present application differentiated primate NCs can be harvested and used as a source for cells with fixed neuronal identity. The differentiated primate NCs can be detached from the cell culture matrix at a late stage of differentiation without substantial loss of viability. The harvested differentiated primate NCs can be reseeded on a defined assay format, wherein the cells attach to the cell culture support and differentiation can be continued before or in parallel to applying a drug candidate to be tested. The method of the present invention solves the problem of non-uniformity of NC cultures from different primate species. Hence the primate NC cultures obtained with the method described herein are valuable models to screen effective and safe drugs and to elaborate new therapeutics for various diseases of the nervous system.

Accordingly, in a further aspect of the invention, the NC cultures produced according to the invention are used for testing the efficacy of at least one drug candidate. The drug candidate

can be added to the cell culture medium at any stage of the method of the present invention. In one embodiment, the drug candidate is added to the differentiated NCs. In one embodiment, the drug candidate is added to the dissociated and reseeded differentiated NC to determine the efficacy profile of the drug candidate. In further embodiments, the drug candidate is added to the cell culture medium at about day 1, at about day 2, at about day 3, at about day 4, at about day 5, at about day 6, at about day 7, at about day 8, at about day 9, at about day 10, at about day 11, at about day 12, at about day 13, at about day 14, at about day 15, at about day 16, at about day 17, at about day 18, at about day 19, at about day 20, at about day 21, at about day 22, at about day 23, at about day 24, at about day 25, at about day 26, at about day 27, at about day 28, at about day 29, at about day 30, at about day 31, at about day 32, at about day 33, at about day 34, at about day 35, at about day 36, at about day 37, at about day 38, at about day 39 or at about day 40 of differentiation.

In a particular embodiment the step b)(i) comprises in this sequence dissociating the differentiated NCs from its support after about day 28 to about day 30 and step b)(ii) comprises reseeding the cells in a suitable cell culture format, continuing differentiation of the NCs for about 7 days, addition of a drug candidate to the cell culture medium, continuing differentiation of the NCs for about further 5 days and assessment of the efficacy profile of the drug candidate.

The primate NC cultures according to the present invention are characterised by uniform cell distribution and, therefore, testing the efficacy of novel drug candidates is straight-forward and well-standardized. The efficacy of a drug candidate can be determined by methods known to the art including but not limited to measuring a phenotypic marker, e.g., the expression of a marker, correlated to the efficacy of the drug candidate. In one embodiment the efficacy of a drug candidate is tested by determining the expression of a disease relevant marker. In one embodiment the efficacy of a drug candidate is tested by determining the expression of a disease relevant protein. In one embodiment the efficacy of a drug candidate is tested by determining the expression of a relevant marker by quantitative real time PCR. The determination of efficacy is performed at a defined time point after addition of a drug candidate. In further embodiments the determination of efficacy is performed at about day 1, at about day 2, at about day 3, at about day 4, at about day 5, at about day 6, at about day 7, at about day 8, at about day 9, at about day 10, at about day 11, at about day 12, at about day 13 or at about day 14 after addition of a drug candidate. In a particular embodiment the step b)(ii) comprises adding a drug candidate to the cell culture medium at about day 7 after dissociating and reseeding, and determining the efficacy of a drug candidate at about day 5 after addition of the drug candidate to the cell culture medium. Robust and uniform differentiated NC cultures according to the invention can be produced for different primate species and used to test the

efficacy profile of drug candidates. In one embodiment, the method is suitable for inter-species comparison of efficacy between primate species, in particular between NHP species and human.

A further aspect of the invention is the use of the uniformly distributed differentiated primate NCs obtained by the methods as described herein. In a preferred embodiment the differentiated primate NCs obtained by the method of the present invention are used as *in vitro* model to study the pathophysiology of CNS diseases. For example, the differentiated primate NCs obtained by the method of the invention can be used for screening for compounds that reverse, inhibit or prevent neurological diseases. In one embodiment, the uniformly distributed differentiated primate NCs are used for screening for compounds that reverse, inhibit or prevent neural side effects of medicaments, for example diabetes medicaments.

In one embodiment, uniformly distributed differentiated primate NCs according to steps a) to b) are used for high-throughput screening of compounds and/or drug candidates selected from the group consisting of small molecules, proteins, peptides and nucleic acids. In a further embodiment, the differentiated NCs according to the invention are used for high-throughput screening of nucleic acid molecules such as a RNAi agent or an antisense oligonucleotide.

In a particular embodiment provided is an *in vitro* method for selecting at least one drug candidate for further development, comprising producing cell cultures of uniformly distributed differentiated neurons individually from human (Homo sapiens) and Cynomolgus monkey (Macaca fascicularis) comprising the steps of

a) providing neuronal precursor cells (NPCs) individually for both human and Cynomolgus monkey at a density of about 30000 cells/cm^2 wherein the NPCs are derived from IPSCs;
b) differentiating the NPCs to neural cells (NCs) comprising the steps of
   (i) incubating the NPCs with basal medium supplemented with Shh, FGF8 and ascorbic acid phosphate for about 7 days, replating the cells at a density of about 45000 cells/cm^2, incubating the replated cells with basal medium supplemented with BDNF, GDNF, cAMP and ascorbic acid phosphate for about 21 days to about 23 days followed by dissociating the differentiated NCs from its support; and
   (ii) individually reseeding the cells in a suitable cell culture format at a density of about 200000 cells/cm^2 and continuing differentiation of the NCs for about 7 days by incubating the cells with basal medium supplemented with BDNF, GDNF, cAMP and ascorbic acid followed by incubating the cells with a drug candidate for about 5 days wherein the drug candidate is added to basal medium supplemented with BDNF, GDNF, cAMP and ascorbic acid; and

establishing the efficacy profiles of the drug candidate on both human and Cynomolgus monkey, and selecting drug candidates for further development if the efficacy profiles are favourable. In one embodiment, establishing the efficacy profile includes assessing target engagement. In one embodiment the further development comprises *in vivo* testing of the drug candidate in NHP species and/or *in vivo* testing in humans.

In one embodiment, a population of differentiated primate NCs produced by any of the foregoing methods is provided. In one embodiment, the differentiated primate NCs are dissociated and reseeded, and further differentiated to obtain uniform and standardized cultures of differentiated NCs. In one embodiment the primate NCs are derived from a healthy individual. In another embodiment, patient-derived primate NCs are used to generate a disease relevant *in vitro* model to study the pathophysiology of CNS diseases. Conversion of patient specific somatic cells to differentiated NCs represents an easy accessible and reproducible technology to generate a source of patient specific NCs for high-throughput cellular assays for disease modelling or compound screening.

In one embodiment, somatic cells from an Angelman syndrome patient are used to generate NPCs. The NPCs derived from one or several patients suffering from Angelman syndrome can be used to generate a disease model of Angelman syndrome. A human monogenic disease model can be recapitulated in NHP species by introduction of the etiologic gene mutation into the respective NHP genome by methods known to the art, e.g., by introducing the respective mutation into NHP NPCs.

In a further embodiment, data generated using the cell assays of the present invention is intended for research purposes with the aim of addressing neural diseases like neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, Huntington's disease, Amyotrophic lateral sclerosis (ALS/Lou Gehrig's Disease) stroke, and spinal cord injury or for therapy of said neurological diseases. Importantly, the present invention leads to consistent and reproducible cell culture assays. Indeed, a major drawback of former cell culture assays deriving from primate stem cells is non-homogenous distribution of differentiating cells among the surface of the cell culture area. The present invention solves this issue by introducing a dissociation and reseeding step which is, surprisingly, tolerated by the differentiating primate NCs, said cells remaining viable and suitable to seed cell culture assays at a defined and uniform cell density. A homogenous cell distribution among the surface of a given cell culture containment leads to improved and more reproducible cell culture assays. Accordingly, a method is provided to generate standardized primate NC culture assays, wherein the obtained differentiated NC cultures according to steps a) to b) are characterized by a homogenous cell distribution, evenly distributed cells in high-throughput plate wells, reduced

formation of clusters and/or clumps and more equal cell distribution. Consequently, the resulting assays display increased robustness, increased homogeneity and decreased variation between assay replicates. In one embodiment, the cells are distributed uniformly over the cell culture area, in particular as assessed by cell nucleus staining.

One embodiment is the use of the standardized NC cultures obtained by the methods according to the invention to determine the efficacy of a drug candidate. In a further aspect of the invention the standardized primate NC culture are used for *in vitro* testing of toxicity of a drug candidate. In a further aspect of the invention the standardized primate NC cultures are used for *in vitro* testing of the efficacy of a drug candidate. The cultures can be derived from healthy individuals and/or from diseased individuals and results from the efficacy and/or toxicity integrated to predict disease and/or therapy relevant physiological effects of a drug candidate. In one embodiment, the *in vitro* efficacy profile of a drug candidate is assessed and drug candidates with favourable efficacy profile are selected for further development. Further development may comprise *in vivo* testing of the drug candidate in NHP species and/or *in vivo* testing in humans.

In a particular embodiment provided is a method for determining the in vitro efficacy profile of a drug candidate using standardized cell cultures of uniformly distributed differentiated neural cells (NCs) from at least two primate species, wherein the differentiated NC cultures are qualified for high throughput screening, the method comprising the steps of:

a) dissociating the differentiated NCs from its support after about 20 days to about 45 days of differentiation and reseeding the differentiated NCs in a high-throughput cell culture format;
b) incubating the reseeded NCs in a differentiation medium;
c) contacting the reseeded NCs with a drug candidate; and
d) determining the in vitro efficacy profile of the drug candidate.

Assessing efficacy data *in vitro* and/or *in vivo* on NHP species prior to *in vivo* testing in humans is conclusive since the genetic distance between NHP species and human is small. This is in contrast to, e.g., rodent species which are genetically more distant to humans compared to NHP species. The small genetic distance between NHP species and humans is especially important when assessing drug candidates targeting a human polynucleotide sequence or if the drug candidate itself comprises a polynucleotide with or close to a sequence derived from a human genome. In one embodiment provided, is a method as described herein, wherein the determined in vitro efficacy profile of the drug candidate is used for inter-species comparison of the efficacy profile of a drug candidate, wherein the cell cultures are produced individually from cells of at least two primate species, wherein essentially the same conditions are applied to the cultures for all primate species and wherein the efficacy profile is determined and compared for

all primate species. In one embodiment, provided is a method for selecting a drug candidate for further development comprising the steps of: (i) determining the *in vitro* efficacy profile of the drug candidate for a first and a second species according to the method as described herein; and (ii) selecting the drug candidate for further development if the efficacy profile of the drug candidate is favourable. In one embodiment the genetic similarity of the protein-coding regions between the first and the second species is high. In one embodiment the genetic similarity of the protein-coding regions between the first and the second species is higher than between human (Homo sapiens) and mouse (Mus musculus). In further embodiments the genetic similarity of the protein-coding regions between the first and the second species is higher than 85%, higher than 90% or higher than 95%. In one embodiment the genetic similarity of the protein-coding regions between the first and the second species is higher than 90%. In one particular embodiment the first species is Cynomolgus monkey (Macaca fascicularis) and the second species is human (Homo sapiens). The differentiated NC cultures according to the invention may be produced sequentially for different species.

Accordingly, in one embodiment the standardized cell cultures of differentiated NCs from one or more primate species according to the present invention are used for *in vitro* efficacy testing of a drug candidate wherein the drug candidate comprises a polynucleotide or targets a specific sequence of a polynucleotide wherein the polynucleotide sequence derives from a human genome. In a further embodiment the drug candidate comprises nucleic acid molecules such as a RNAi agent or an antisense oligonucleotide.

In a further embodiment of the invention, the drug candidate assessed in the *in vitro* efficacy and/or toxicity tests comprises one or more antisense oligonucleotide. In further embodiments, the antisense oligonucleotide comprise or consist of 10 to 30 nucleotides in length with at least 90% identity, preferably 100% identity to a sequence derived from a human genome. It is understood that the antisense oligonucleotide sequences (motif sequence) can be modified to for example increase nuclease resistance and/or binding affinity to the target nucleic acid. In one aspect the antisense oligonucleotides comprise sugar-modified nucleosides and may also comprise DNA or RNA nucleosides. In some embodiments, the oligonucleotide comprise sugar-modified nucleosides and DNA nucleosides. In another aspect incorporation of modified nucleosides into the oligonucleotide enhance the affinity of the oligonucleotide for the target nucleic acid. In that case, the modified nucleosides can be referred to as affinity enhancing modified nucleotides.

In one embodiment, the antisense oligonucleotide comprises at least 1 modified nucleoside, such as at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15 or at least 16 modified

nucleosides. In an embodiment the oligonucleotide comprises from 1 to 10 modified nucleosides, such as from 2 to 9 modified nucleosides, such as from 3 to 8 modified nucleosides, such as from 4 to 7 modified nucleosides, such as 6 or 7 modified nucleosides. In some embodiments, at least 1 of the modified nucleosides is a locked nucleic acid (LNA), such as at least 2, such as at least 3, at least 4, at least 5, at least 6, at least 7, or at least 8 of the modified nucleosides are LNA. In a still further embodiment all the modified nucleosides are LNA.

In one embodiment, the antisense oligonucleotide comprises modifications, which are independently selected from these three types of modifications (modified sugar, modified nucleobase and modified internucleoside linkage) or a combination thereof. Preferably the antisense oligonucleotide comprises one or more sugar modified nucleosides, such as 2' sugar modified nucleosides. Preferably the antisense oligonucleotide comprises the one or more 2' sugar modified nucleoside independently selected from the group consisting of 2'-O-alkyl-RNA, 2'-O-methyl-RNA, 2'-alkoxy-RNA, 2'-O-methoxyethyl-RNA, 2'-amino-DNA, 2'-fluoro-DNA, arabino nucleic acid (ANA), 2'-fluoro-ANA and LNA nucleosides. Even more preferably the one or more modified nucleoside is LNA.

In a further embodiment the antisense oligonucleotide comprises at least one modified internucleoside linkage. In a preferred embodiment the internucleoside linkages within the contiguous nucleotide sequence are phosphorothioate or boranophosphate internucleoside linkages.

In some embodiments, the antisense oligonucleotide comprise at least one modified nucleoside which is a 2'-MOE-RNA, such as 2, 3, 4, 5, 6, 7, 8, 9 or 10 2'-MOE-RNA nucleoside units. In some embodiments, at least one of said modified nucleoside is 2'-fluoro DNA, such as 2, 3, 4, 5, 6, 7, 8, 9 or 10 2'-fluoro-DNA nucleoside units.

In some embodiments, the oligonucleotide of the invention comprises at least one LNA unit, such as 1, 2, 3, 4, 5, 6, 7, or 8 LNA units, such as from 2 to 6 LNA units, such as from 3 to 7 LNA units, 4 to 8 LNA units or 3, 4, 5, 6 or 7 LNA units. In some embodiments, all the modified nucleosides are LNA nucleosides. In a further embodiment, the oligonucleotide may comprise both beta-D-oxy-LNA, and one or more of the following LNA units: thio-LNA, amino-LNA, oxy-LNA, and/or ENA in either the beta-D or alpha-L configurations or combinations thereof. In a further embodiment, all LNA cytosine units are 5-methyl-cytosine. In a preferred embodiment the oligonucleotide or contiguous nucleotide sequence has at least 1 LNA unit at the 5' end and at least 2 LNA units at the 3' end of the nucleotide sequence.

In some embodiments, the antisense oligonucleotide comprise at least one LNA unit and at least one 2' substituted modified nucleoside.

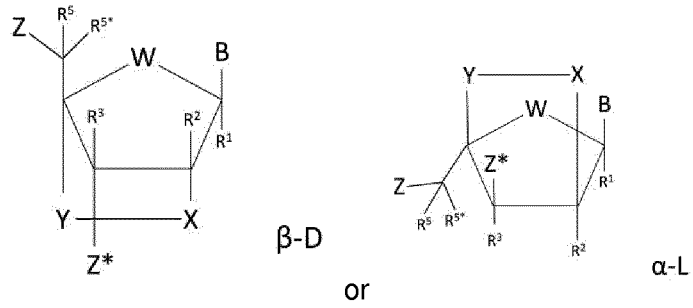
In some embodiments of the invention, the antisense oligonucleotide comprise both 2' sugar modified nucleosides and DNA units. Preferably the antisense oligonucleotide comprise both LNA and DNA units. Preferably, the combined total of LNA and DNA units is 8-30, such as 10 – 25, preferably 12-22, such as 12 – 18, even more preferably 11-16. In some embodiments of the invention, the nucleotide sequence of the antisense oligonucleotide, such as the contiguous nucleotide sequence consists of at least one or two LNA units and the remaining nucleotide units are DNA units. In some embodiments the antisense oligonucleotide comprises only LNA nucleosides and naturally occurring nucleosides (such as RNA or DNA, most preferably DNA nucleosides), optionally with modified internucleoside linkages such as phosphorothioate.

In an embodiment of the invention the antisense oligonucleotide is capable of recruiting RNase H.

In a preferred embodiment the antisense oligonucleotide has a gapmer design or structure also referred herein merely as "Gapmer". In a gapmer structure the antisense oligonucleotide comprises at least three distinct structural regions a 5'-flank, a gap and a 3'-flank, F-G-F' in '5 -> 3' orientation. In this design, flanking regions F and F' (also termed wing regions) comprise a contiguous stretch of modified nucleosides, which are complementary to the target nucleic acid, while the gap region, G, comprises a contiguous stretch of nucleotides which are capable of recruiting a nuclease, preferably an endonuclease such as RNase, for example RNase H, when the antisense oligonucleotide is in duplex with the target nucleic acid. Nucleosides which are capable of recruiting a nuclease, in particular RNase H, can be selected from the group consisting of DNA, alpha-L-oxy-LNA, 2'-Flouro-ANA and UNA. Regions F and F', flanking the 5' and 3' ends of region G, preferably comprise non-nuclease recruiting nucleosides (nucleosides with a 3' endo structure), more preferably one or more affinity enhancing modified nucleosides. In some embodiments, the 3' flank comprises at least one LNA nucleoside, preferably at least 2 LNA nucleosides. In some embodiments, the 5' flank comprises at least one LNA nucleoside. In some embodiments both the 5' and 3' flanking regions comprise a LNA nucleoside. In some embodiments all the nucleosides in the flanking regions are LNA nucleosides. In other embodiments, the flanking regions may comprise both LNA nucleosides and other nucleosides (mixed flanks), such as DNA nucleosides and/or non-LNA modified nucleosides, such as 2' substituted nucleosides. In this case the gap is defined as a contiguous sequence of at least 5 RNase H recruiting nucleosides (nucleosides with a 2' endo structure, preferably DNA) flanked at the 5' and 3' end by an affinity enhancing modified nucleoside, preferably LNA, such as beta-D-oxy-LNA. Consequently, the nucleosides of the 5' flanking region and the 3' flanking region

which are adjacent to the gap region are modified nucleosides, preferably non-nuclease recruiting nucleosides.

In some embodiments, the modified nucleoside or the LNA nucleosides of the oligomer of the invention has a general structure of the formula I or II:



Formula I Formula II

wherein W is selected from -O-, -S-, -N(R^a)-, -C(R^aR^b)-, such as, in some embodiments –O-;

B designates a nucleobase or modified nucleobase moiety;

Z designates an internucleoside linkage to an adjacent nucleoside, or a 5'-terminal group;

Z* designates an internucleoside linkage to an adjacent nucleoside, or a 3'-terminal group;

X designates a group selected from the list consisting of -C(R^aR^b)-, -C(R^a)=C(R^b)-, -(R^a)=N-, -O-, -Si(R^a)$_2$-, -S-, -SO_2-, -N(R^a)-, and >C=Z.

In some embodiments, X is selected from the group consisting of: –O-, -S-, NH-, NR^aR^b, -CH_2-, CR^aR^b, -C(=CH_2)-, and -C(=CR^aR^b)-.

In some embodiments, X is -O- and Y designates a group selected from the group consisting of -C(R^aR^b)-, -C(R^a)=C(R^b)-, -C(R^a)=N-, -O-, -Si(R^a)$_2$-, -S-, -SO_2-, -N(R^a)-, and >C=Z.

In some embodiments, Y is selected from the group consisting of: –CH_2-, -C(R^aR^b)-, –CH_2CH_2-, -C(R^aR^b)-C(R^aR^b)-, –$CH_2CH_2CH_2$-, -C(R^aR^b)C(R^aR^b)C(R^aR^b)-, -C(R^a)=C(R^b)-, and -C(R^a)=N-.

In some embodiments, Y is selected from the group consisting of: -CH_2-, -CHR^a-, -$CHCH_3$-, CR^aR^b- or -X-Y- together designate a bivalent linker group (also referred to as a radicle) together designate a bivalent linker group consisting of 1, 2, 3 or 4 groups/atoms selected from the group consisting of -C(R^aR^b)-, -C(R^a)=C(R^b)-, -C(R^a)=N-, -O-, -Si(R^a)$_2$-, -S-, -SO_2-, -N(R^a)-, and >C=Z.

In some embodiments, -X-Y- designates a biradicle selected from the groups consisting of: -X-CH_2-, -X-CR^aR^b-, -X-CHR^{a-}, -X-$C(HCH_3)^-$, -O-Y-, -O-CH_2-, -S-CH_2-, -NH-CH_2-, -O-$CHCH_3$-, -CH_2-O-CH_2, -O-$CH(CH_3CH_3)$-, -O-CH_2-CH_2-, OCH_2-CH_2-CH_2-,-O-CH_2OCH_2-, -O-NCH_2-, -C(=CH_2)-CH_2-, -NR^a-CH_2-, N-O-CH_2, -S-CR^aR^b- and -S-CHR^a-.

In some embodiments –X-Y- designates –O-CH_2- or –O-$CH(CH_3)$- wherein Z is selected from -O-, -S-, and -N(R^a)-, and R^a and, when present R^b, each is independently selected from hydrogen, optionally substituted C_{1-6}-alkyl, optionally substituted C_{2-6}-alkenyl, optionally substituted C_{2-6}-alkynyl, hydroxyl, optionally substituted C_{1-6}-alkoxy, C_{2-6}-alkoxyalkyl, C_{2-6}-alkenyloxy, carboxy, C_{1-6}-alkoxycarbonyl, C_{1-6}-alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylcarbonyl, heteroaryl, heteroaryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di(C_{1-6}-alkyl)amino, carbamoyl, mono- and di(C_{1-6}-alkyl)-amino-carbonyl, amino-C_{1-6}-alkyl-aminocarbonyl, mono- and di(C_{1-6}-alkyl)amino-C_{1-6}-alkyl-aminocarbonyl, C_{1-6}-alkyl-carbonylamino, carbamido, C_{1-6}-alkanoyloxy, sulphono, C_{1-6}-alkylsulphonyloxy, nitro, azido, sulphanyl, C_{1-6}-alkylthio, halogen, where aryl and heteroaryl may be optionally substituted and where two geminal substituents R^a and R^b together may designate optionally substituted methylene (=CH_2), wherein for all chiral centers, asymmetric groups may be found in either *R* or *S* orientation, wherein R^1, R^2, R^3, R^5 and R^{5*} are independently selected from the group consisting of: hydrogen, optionally substituted C_{1-6}-alkyl, optionally substituted C_{2-6}-alkenyl, optionally substituted C_{2-6}-alkynyl, hydroxy, C_{1-6}-alkoxy, C_{2-6}-alkoxyalkyl, C_{2-6}-alkenyloxy, carboxy, C_{1-6}-alkoxycarbonyl, C_{1-6}-alkylcarbonyl, formyl, aryl, aryloxy-carbonyl, aryloxy, arylcarbonyl, heteroaryl, heteroaryloxy-carbonyl, heteroaryloxy, heteroarylcarbonyl, amino, mono- and di(C_{1-6}-alkyl)amino, carbamoyl, mono- and di(C_{1-6}-alkyl)-amino-carbonyl, amino-C_{1-6}-alkyl-aminocarbonyl, mono- and di(C_{1-6}-alkyl)amino-C_{1-6}-alkyl-aminocarbonyl, C_{1-6}-alkyl-carbonylamino, carbamido, C_{1-6}-alkanoyloxy, sulphono, C_{1-6}-alkylsulphonyloxy, nitro, azido, sulphanyl, C_{1-6}-alkylthio, halogen, where aryl and heteroaryl may be optionally substituted, and where two geminal substituents together may designate oxo, thioxo, imino, or optionally substituted methylene.

In some embodiments R^1, R^2, R^3, R^5 and R^{5*} are independently selected from C_{1-6} alkyl, such as methyl, and hydrogen.

In some embodiments R^1, R^2, R^3, R^5 and R^{5*} are all hydrogen.

In some embodiments R^1, R^2, R^3, are all hydrogen, and either R^5 and R^{5*} is also hydrogen and the other of R^5 and $R^{5*}$is other than hydrogen, such as C_{1-6} alkyl such as methyl.

In some embodiments, R^a is either hydrogen or methyl. In some embodiments, when present, R^b is either hydrogen or methyl.

In some embodiments, one or both of R^a and R^b is hydrogen.

In some embodiments, one of R^a and R^b is hydrogen and the other is other than hydrogen.

In some embodiments, one of R^a and R^b is methyl and the other is hydrogen.

In some embodiments, both of R^a and R^b are methyl.

In some embodiments, the biradicle –X-Y- is –O-CH_2-, W is O, and all of R^1, R^2, R^3, R^5 and R^{5*} are all hydrogen. Such LNA nucleosides are disclosed in WO99/014226, WO00/66604, WO98/039352 and WO2004/046160 which are all hereby incorporated by reference, and include what are commonly known as beta-D-oxy LNA and alpha-L-oxy LNA nucleosides.

In some embodiments, the biradicle –X-Y- is –S-CH_2-, W is O, and all of R^1, R^2, R^3, R^5 and R^{5*} are all hydrogen. Such thio LNA nucleosides are disclosed in WO99/014226 and WO2004/046160 which are hereby incorporated by reference.

In some embodiments, the biradicle –X-Y- is –NH-CH_2-, W is O, and all of R^1, R^2, R^3, R^5 and R^{5*} are all hydrogen. Such amino LNA nucleosides are disclosed in WO99/014226 and WO2004/046160 which are hereby incorporated by reference.

In some embodiments, the biradicle –X-Y- is –O-CH_2-CH_2- or –O-CH_2-CH_2- CH_2-, W is O, and all of R^1, R^2, R^3, R^5 and R^{5*} are all hydrogen. Such LNA nucleosides are disclosed in WO00/047599 and Morita et al, Bioorganic & Med.Chem. Lett. 12 73-76, which are hereby incorporated by reference, and include what are commonly known as 2'-O-4'C-ethylene bridged nucleic acids (ENA).

In some embodiments, the biradicle –X-Y- is –O-CH_2-, W is O, and all of R^1, R^2, R^3, and one of R^5 and R^{5*} are hydrogen, and the other of R^5 and R^{5*} is other than hydrogen such as C_{1-6} alkyl, such as methyl. Such 5' substituted LNA nucleosides are disclosed in WO2007/134181 which is hereby incorporated by reference.

In some embodiments, the biradicle –X-Y- is –O-CR^aR^b-, wherein one or both of R^a and R^b are other than hydrogen, such as methyl, W is O, and all of R^1, R^2, R^3, and one of R^5 and R^{5*} are hydrogen, and the other of R^5 and R^{5*} is other than hydrogen such as C_{1-6} alkyl, such as methyl. Such bis modified LNA nucleosides are disclosed in WO2010/077578 which is hereby incorporated by reference.

In some embodiments, the biradicle –X-Y- designate the bivalent linker group –O-CH(CH_2OCH_3)- (2' O-methoxyethyl bicyclic nucleic acid - Seth at al., 2010, J. Org. Chem. Vol 75(5) pp. 1569-81). In some embodiments, the biradicle –X-Y- designate the bivalent linker group –O-CH(CH_2CH_3)- (2'O-ethyl bicyclic nucleic acid - Seth at al., 2010, J. Org. Chem. Vol 75(5) pp. 1569-81). In some embodiments, the biradicle –X-Y- is –O-CHR^a-, W is O, and all of R^1, R^2, R^3, R^5 and R^{5*} are all hydrogen. Such 6' substituted LNA nucleosides are disclosed in WO10036698 and WO07090071 which are both hereby incorporated by reference.

In some embodiments, the biradicle –X-Y- is –O-CH(CH_2OCH_3)-, W is O, and all of R^1, R^2, R^3, R^5 and R^{5*} are all hydrogen. Such LNA nucleosides are also known as cyclic MOEs in the art (cMOE) and are disclosed in WO07090071.

In some embodiments, the biradicle –X-Y- designate the bivalent linker group –O-CH(CH_3)- in either the R- or S- configuration. In some embodiments, the biradicle –X-Y- together designate the bivalent linker group –O-CH_2-O-CH_2- (Seth at al., 2010, J. Org. Chem). In some embodiments, the biradicle –X-Y- is –O-CH(CH_3)-, W is O, and all of R^1, R^2, R^3, R^5 and R^{5*} are all hydrogen. Such 6' methyl LNA nucleosides are also known as cET nucleosides in the art, and may be either (S)cET or (R)cET stereoisomers, as disclosed in WO07090071 (beta-D) and WO2010/036698 (alpha-L) which are both hereby incorporated by reference.

In some embodiments, the biradicle –X-Y- is –O-CR^aR^b-, wherein in neither R^a or R^b is hydrogen, W is O, and all of R^1, R^2, R^3, R^5 and R^{5*} are all hydrogen. In some embodiments, R^a and R^b are both methyl. Such 6' di-substituted LNA nucleosides are disclosed in WO 2009006478 which is hereby incorporated by reference.

In some embodiments, the biradicle –X-Y- is –S-CHR^a-, W is O, and all of R^1, R^2, R^3, R^5 and R^{5*} are all hydrogen. Such 6' substituted thio LNA nucleosides are disclosed in WO11156202 which is hereby incorporated by reference. In some 6' substituted thio LNA embodiments R^a is methyl.

In some embodiments, the biradicle –X-Y- is –C(=CH2)-C(R^aR^b)-, such as –C(=CH_2)-CH_2- , or –C(=CH_2)-CH(CH_3)-W is O, and all of R^1, R^2, R^3, R^5 and R^{5*} are all hydrogen. Such vinyl carbo LNA nucleosides are disclosed in WO08154401 and WO09067647 which are both hereby incorporated by reference.

In some embodiments the biradicle –X-Y- is –N(-OR^a)-, W is O, and all of R^1, R^2, R^3, R^5 and R^{5*} are all hydrogen. In some embodiments R^a is C_{1-6} alkyl such as methyl. Such LNA nucleosides are also known as N substituted LNAs and are disclosed in WO2008/150729 which is hereby incorporated by reference. In some embodiments, the biradicle –X-Y- together designate the bivalent linker group –O-NR^a-CH_3- (Seth at al., 2010, J. Org. Chem). In some embodiments the

biradicle –X-Y- is –N(R^a)-, W is O, and all of R^1, R^2, R^3, R^5 and R^{5*} are all hydrogen. In some embodiments R^a is C_{1-6} alkyl such as methyl.

In some embodiments, one or both of R^5 and R^{5*} is hydrogen and, when substituted the other of R^5 and R^{5*} is C_{1-6} alkyl such as methyl. In such an embodiment, R^1, R^2, R^3, may all be hydrogen, and the biradicle –X-Y- may be selected from –O-CH2- or –O-C(HCR^a)-, such as –O-C(HCH3)-.

In some embodiments, the biradicle is –C$R^a$$R^b$-O-C$R^a$$R^b$-, such as CH_2-O-CH_2-, W is O and all of R^1, R^2, R^3, R^5 and R^{5*} are all hydrogen. In some embodiments R^a is C_{1-6} alkyl such as methyl. Such LNA nucleosides are also known as conformationally restricted nucleotides (CRNs) and are disclosed in WO2013036868 which is hereby incorporated by reference.

In some embodiments, the biradicle is –O-C$R^a$$R^b$-O-C$R^a$$R^b$-, such as O-$CH_2$-O-$CH_2$-, W is O and all of R^1, R^2, R^3, R^5 and R^{5*} are all hydrogen. In some embodiments R^a is C_{1-6} alkyl such as methyl. Such LNA nucleosides are also known as COC nucleotides and are disclosed in Mitsuoka et al., Nucleic Acids Research 2009 37(4), 1225-1238, which is hereby incorporated by reference.

It will be recognized than, unless specified, the LNA nucleosides may be in the beta-D or alpha-L stereoisoform.

Further gapmer designs are disclosed in WO2004/046160, WO2007/146511 and incorporated by reference.

An aspect of the invention is to modulate the level of pig, primate or human UBE3A protein expression, in particular to increase the expression of paternal UBE3A expression in neuronal cells, in particular in human neuronal cells. The human UBE3A protein exists in several isoforms which are listed under Uniprot nr. Q05086. Several mutations in the maternal UBE3A gene can results in Angelman syndrome.

The target nucleic acid for the oligonucleotides of this aspect of the invention is RNA, in particular a long non-coding RNA. The long non-coding RNA which is targeted by the oligonucleotides of the present invention is human SNHG14 (also known as UBE3A-ATS with Ensembl entry number ENSG00000224078, version GRCh38.p2). In particular the target nucleic acid is the region downstream of SNORD109B corresponding to position 25278410 to 25419462 on chromosome 15 (SEQ ID NO: 1). In Rhesus monkey (Macaca mulatta) the UBE3A supressor is defined as region downstream of SNORD109A corresponding to position 4222848 to 4373084 (forward strand) on chromosome 7 using the Ensembl assembly MMUL 1.0 (SEQ ID NO: 2).

In some embodiments, the target nucleic acid is SEQ ID NO: 1, or naturally occurring variants thereof. In certain embodiments target nucleic acid correspond to regions which are conserved between human (SEQ ID NO: 1) and Rhesus monkey (SEQ ID NO: 2). In certain embodiments target nucleic acid correspond to regions which are conserved between human (SEQ ID NO:1), Rhesus monkey (SEQ ID NO: 2) and mouse (SEQ ID NO: 3).

In certain embodiments target nucleic acid is the region that is antisense to the UBE3A pre-mRNA, this region corresponds to position 55319 to 141053 of SEQ ID NO: 1.

In some embodiments, the target nucleic acid is present in a cell, such as a mammalian cell in particular a human cell *in vitro* or *in vivo* (the target cell). In certain embodiments the target cell is a neuron, preferably a human neuronal cell.

The target sequence may be a sub-sequence of the target nucleic acid. In some embodiments the oligonucleotide targets sub-sequence selected from the group consisting of the antisense region of exon 9, exon10, exon13, exon14, intron 14, exon 15, intron15 and exon 16 of UBE3A. In some embodiments the oligonucleotide or contiguous nucleotide sequence hybridize or is complementary to a single stranded nucleic acid molecule selected from the group consisting of positions: 55319-76274, 77483-77573, 92157-93403 and 97056-97354 of SEQ ID NO: 1. In some embodiments the oligonucleotide or contiguous nucleotide sequence hybridize or is complementary to a single stranded nucleic acid molecule selected from the group consisting of positions: 60821-60849, 77567-77583, 92323-92339 and 97156-97172 of SEQ ID NO: 1.

Particular Embodiments

1. An *in vitro* method for producing standardized cell cultures of uniformly distributed differentiated neural cells (NCs) from different primate species, the method comprising the steps of
   a) providing neuronal precursor cells (NPCs);
   b) differentiating the NPCs to neural cells (NCs) comprising the steps of
      (i) dissociating the differentiated NCs from its support after about 20 days to about 45 days of differentiation; and
      (ii) reseeding the cells in a suitable cell culture format and continuing differentiation of the NCs for about 4 days to about 15 days.

2. The method according to embodiment 1, wherein the primate species are selected from the group consisting of human (Homo sapiens), Cynomolgus monkey (Macaca fascicularis) and Rhesus monkey (Macaca mulatta).

3. The method according to embodiment 1, wherein the primate species are selected from the group consisting of human (Homo sapiens) and Cynomolgus monkey (Macaca fascicularis).

4. The method according to embodiment 1, wherein standardized cell cultures of differentiated NCs are individually produced for 2 primate species, wherein the first primate species is human (Homo sapiens) and the second primate species is Cynomolgus monkey (Macaca fascicularis).

5. The method according to any one of embodiments 1 to 4, wherein the NPCs are derived from induced pluripotent stem cells (IPSCs).

6. The method according to any one of embodiments 1 to 5, wherein step b) comprises cultivating the NPCs with one or more differentiating agents selected from the group consisting of Shh, FGF8, BDNF, GDNF, cAMP and ascorbic acid phosphate.

7. The method according to any one of embodiments 1 to 6, wherein step b)(i) comprises cultivating the cells in basal medium comprising Shh, FGF8 and ascorbic acid phosphate for a period of about 5 to about 10 days followed by cultivating the cells in basal medium comprising BDNF, GDNF, cAMP and ascorbic acid phosphate for a further period of about 15 days to about 35 days prior to dissociating the differentiated NCs from its support.

8. The method according to embodiment 6 or 7, wherein the concentration of the one or more differentiating agent in basal medium is 50-500 ng/ml for Shh, 25-250 ng/ml for FGF8, 1-50 ng/ml for BDNF, 1-50 ng/ml for GDNF, 0.1-10 mM for cAMP and 20-200 µM for ascorbic acid phosphate.

9. The method according to any one of embodiments 6 to 8 wherein the concentration of the one or more differentiating agent in basal medium is 200 ng/ml for Shh, 100 ng/ml for FGF8, 20 ng/ml for BDNF, 10 ng/ml for GDNF, 0.5 mM for cAMP and 100 µM for ascorbic acid phosphate.

10. The method according to any one of embodiments 6 to 9, wherein the cultivation period with Shh, FGF8 and ascorbic acid phosphate is about 7 days and the cultivation period with BDNF, GDNF, cAMP and ascorbic acid phosphate is about 20 to about 40 days.

11. The method according to any one of embodiments 1 to 10, wherein the NPCs are provided on a Laminin521 support.

12. The method according to any one of embodiments 1 to 11, wherein step b)(i) comprises dissociating the cells with a cell detachment solution.

13. The method according to embodiment 12, wherein the cell detachment solution is Accutase.

14. The method according to any one of embodiments 1 to 13, wherein step b)(ii) comprises reseeding the cells at 200000 cells/cm^2 on a Laminin521 support.

15. The method according to any one of embodiments 1 to 14, wherein the cells are reseeded in 96 well or 384 well plates.

16. The method according to any one of embodiments 1 to 15, wherein step b)(ii) comprises reseeding the cells in the presence of a ROCK inhibitor.

17. The method according to embodiment 16, wherein the ROCK inhibitor is Y-27632.

18. The method according to any one of embodiments 1 to 17, wherein step b)(ii) comprises continuing differentiation by cultivating the cells in basal medium supplemented with BDNF, GDNF, cAMP and ascorbic acid after reseeding the cells.

19. The method according to any one of embodiments 1 to 18, wherein the differentiated NCs express MAP2.

20. The method according to any one of embodiments 1 to 19, wherein the differentiated NCs comprise MAP2 positive neurites.

21. The method according to any one of embodiments 1 to 20, wherein the NPCs are derived from an individual with a neuronal disorder.

22. The method according to any one of embodiments 1 to 21, wherein the NPCs are derived from a healthy individual.

23. The method according to any one of embodiment 1 to 22, wherein the cell cultures are produced sequentially for different species.

24. The method according to any one of embodiment 1 to 23, wherein the cells are essentially uniformly distributed over the cell culture area as assessed by cell nucleus staining.

25. The method according to any one of embodiment 1 to 24, wherein the distribution of the cells is assessed by DNA staining, in particular by Hoechst staining.

26. A cell culture system obtained by a method according to any one of embodiments 1 to 25.

27. Use of the cell cultures obtained according to any one of embodiments 1 to 26 for *in vitro* testing of the toxicity of a drug candidate.

28. Use of the cell cultures obtained according to any one of embodiments 1 to 27 for *in vitro* testing of the efficacy of a drug candidate.

29. The use according to embodiment 28, wherein the efficacy is tested in a cell culture derived from the method of embodiment 21 and a cell culture derived from the method of embodiment 22.

30. Use of the cell cultures obtained according to any one of embodiments 1 to 25 for selecting a drug candidate, in particular for selecting a drug candidate for further development.

31. The cell cultures for use according to any one of embodiments 27 to 30 wherein the drug candidate comprises a polynucleotide or targets a specific sequence of a polynucleotide.

32. The cell cultures for use according to any one of embodiments 27 to 31 wherein the drug candidate comprises at least one nucleic acid molecule such as a RNAi agent or an antisense oligonucleotide.

33. The cell cultures for use according to embodiment 32, wherein the nucleic acid molecule comprise one or more 2' sugar modified nucleosides, independently selected from the group consisting of 2'-O-alkyl-RNA, 2'-O-methyl-RNA, 2'-alkoxy-RNA, 2'-O-methoxyethyl-RNA, 2'-amino-DNA, 2'-fluoro-DNA, arabino nucleic acid (ANA), 2'-fluoro-ANA and LNA nucleosides.

34. The cell cultures for use according to embodiment 33, wherein the one or more 2' sugar modified nucleoside is a LNA nucleoside.

35. The cell cultures for use according to embodiment 34, wherein the LNA nucleoside is selected from beta-D-oxy-LNA, alpha-L-oxy-LNA, beta-D-amino-LNA, alpha-L-amino-LNA, beta-D-thio-LNA, alpha-L-thio-LNA, (S)cET, (R)cET, beta-D-ENA or alpha-L-ENA.

36. The cell cultures for use according to any one of embodiments 32 to 35, wherein the nucleic acid molecule comprises at least one modified internucleoside linkage.

37. The cell cultures for use according to embodiment 36, wherein the internucleoside linkages within the contiguous nucleotide sequence are phosphorothioate internucleoside linkages.

38. The cell cultures for use according to any one of embodiments 32 to 37, wherein the antisense oligonucleotide is capable of recruiting RNase H.

39. The cell cultures for use according to any one of embodiments 32 to 38, wherein the antisense oligonucleotide is a gapmer.

40. The cell cultures for use according embodiment 38 or 39, wherein the oligonucleotide is a gapmer of formula 5'-F-G-F'-3', where region F and F' independently comprise 1 to 7 modified nucleosides and G is a region between 6 and 16 nucleosides which are capable of recruiting RNaseH.

41. The methods and uses essentially as described herein.

Any of the embodiments as described herein may be used singly or in combination. The examples below explain the invention in more detail. The following preparations and examples are given to enable those skilled in the art to more clearly understand and to practice the present invention. The present invention, however, is not limited in scope by the exemplified embodiments, which are intended as illustrations of single aspects of the invention only, and methods which are functionally equivalent are within the scope of the invention. Indeed, various modifications of the invention in addition to those disclosed herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

EXAMPLES

Materials and methods

Table 3: List of oligonucleotides or contiguous nucleobase sequences complementary to SEQ ID NO: 1 (motif sequences indicated by SEQ ID NO), oligonucleotide designs made from these, as well as specific oligonucleotide compounds (indicated by CMP ID NO) designed based on the motif sequence.

Seq ID NO	Motif	Design	Compound	CMP ID NO	ΔG°	Start SEQ ID NO 1
4	AACTTCATCAATATTTCCC	3-13-3	AACttcatcaatatttCCC	4_1	-23,36	1677
4	AACTTCATCAATATTTCCC	2-15-2	AActtcatcaatatttcCC	4_2	-19,60	1677
5	ACTTCATCAATATTTCCC	3-12-3	ACTtcatcaatatttCCC	5_1	-23,80	1677
5	ACTTCATCAATATTTCCC	2-14-2	ACttcatcaatatttcCC	5_2	-20,24	1677
6	CAACTTCATCAATATTTCCC	2-14-4	CAacttcatcaatattTCCC	6_1	-25,64	1677
6	CAACTTCATCAATATTTCCC	2-16-2	CAacttcatcaatatttcCC	6_2	-22,28	1677
7	CAACTTCATCAATATTTCC	4-13-2	CAACttcatcaatatttCC	7_1	-21,47	1678
7	CAACTTCATCAATATTTCC	2-15-2	CAacttcatcaatatttCC	7_2	-19,46	1678
8	CCAACTTCATCAATATTTCC	3-14-3	CCAacttcatcaatattTCC	8_1	-25,64	1678
9	CCCAACTTCATCAATATTTC	4-14-2	CCCAacttcatcaatattTC	9_1	-25,64	1679
10	ACCCAACTTCATCAATATTT	2-16-2	ACccaacttcatcaatatTT	10_1	-20,05	1680
11	CCCAACTTCATCAATATTT	4-13-2	CCCAacttcatcaatatTT	11_1	-23,96	1680
11	CCCAACTTCATCAATATTT	2-15-2	CCcaacttcatcaatatTT	11_2	-20,28	1680
12	ACCCAACTTCATCAATATT	4-13-2	ACCCaacttcatcaataTT	12_1	-23,64	1681
12	ACCCAACTTCATCAATATT	2-15-2	ACccaacttcatcaataTT	12_2	-19,18	1681
13	CCCAACTTCATCAATATT	4-12-2	CCCAacttcatcaataTT	13_1	-23,09	1681
13	CCCAACTTCATCAATATT	2-14-2	CCcaacttcatcaataTT	13_2	-19,41	1681
14	TACCCAACTTCATCAATAT	2-15-2	TAcccaacttcatcaatAT	14_1	-19,31	1682
15	TACCCAACTTCATCAATA	2-14-2	TAcccaacttcatcaaTA	15_1	-19,14	1683
16	TTACCCAACTTCATCAATA	2-15-2	TTacccaacttcatcaaTA	16_1	-19,74	1683
17	TTTACCCAACTTCATCAAT	4-13-2	TTTAcccaacttcatcaAT	17_1	-21,68	1684
17	TTTACCCAACTTCATCAAT	2-15-2	TTtacccaacttcatcaAT	17_2	-19,22	1684
18	ATACTTTACCCAACTTCAT	3-13-3	ATActttacccaacttCAT	18_1	-23,44	1688
18	ATACTTTACCCAACTTCAT	2-15-2	ATactttacccaacttcAT	18_2	-20,13	1688
19	TACTTTACCCAACTTCAT	3-12-3	TACtttacccaacttCAT	19_1	-22,78	1688
19	TACTTTACCCAACTTCAT	2-14-2	TActttacccaacttcAT	19_2	-19,30	1688
20	TTATACTTTACCCAACTTCA	2-16-2	TTatactttacccaacttCA	20_1	-21,40	1689
21	TCACTGTTCTGACTTT	3-10-3	TCActgttctgacTTT	21_1	-19,11	1712
22	TTCAATCTCTATCTCATCAT	2-16-2	TTcaatctctatctcatcAT	22_1	-19,42	4169
23	CTTCAATCTCTATCTCATCA	4-14-2	CTTCaatctctatctcatCA	23_1	-24,21	4170
23	CTTCAATCTCTATCTCATCA	2-16-2	CTtcaatctctatctcatCA	23_2	-22,04	4170
24	TTCAATCTCTATCTCATCA	2-15-2	TTcaatctctatctcatCA	24_1	-19,44	4170

Seq ID NO	Motif	Design	Compound	CMP ID NO	ΔG°	Start SEQ ID NO 1
25	CTTCAATCTCTATCTCATC	2-15-2	CTtcaatctctatctcaTC	25_1	-19,87	4171
26	ACTTCAATCTCTATCTCAT	3-13-3	ACTtcaatctctatctCAT	26_1	-22,36	4172
26	ACTTCAATCTCTATCTCAT	2-15-2	ACttcaatctctatctcAT	26_2	-19,08	4172
27	CACTTCAATCTCTATCTCAT	2-16-2	CActtcaatctctatctcAT	27_1	-20,98	4172
28	ACTTCAATCTCTATCTCA	2-12-4	ACttcaatctctatCTCA	28_1	-21,96	4173
28	ACTTCAATCTCTATCTCA	2-14-2	ACttcaatctctatctCA	28_2	-19,10	4173
29	CACTTCAATCTCTATCTCA	2-13-4	CActtcaatctctatCTCA	29_1	-23,86	4173
29	CACTTCAATCTCTATCTCA	2-15-2	CActtcaatctctatctCA	29_2	-21,00	4173
30	ACACTTCAATCTCTATCTC	2-15-2	ACacttcaatctctatcTC	30_1	-19,38	4174
31	TACACTTCAATCTCTATCTC	2-14-4	TAcacttcaatctctaTCTC	31_1	-23,31	4174
31	TACACTTCAATCTCTATCTC	2-16-2	TAcacttcaatctctatcTC	31_2	-20,53	4174
32	TACACTTCAATCTCTATCT	4-13-2	TACActtcaatctctatCT	32_1	-22,34	4175
33	CTTTGTCTCTCTTTACT	2-13-2	CTttgtctctctttaCT	33_1	-19,36	4374
34	TATACCTTTCTTTAACCC	3-12-3	TATacctttctttaaCCC	34_1	-24,89	8118
34	TATACCTTTCTTTAACCC	2-14-2	TAtacctttctttaacCC	34_2	-20,83	8118
35	TGTTTATACCCTTTCC	2-12-2	TGtttatacccttTCC	35_1	-20,33	9212
36	TCTCCTTTATGACTCC	2-10-4	TCtcctttatgaCTCC	36_1	-23,29	10839
37	CTTCTCCTTTATGACTC	2-13-2	CTtctcctttatgacTC	37_1	-19,26	10840
38	CCATTTATTTCCATTTATT	4-13-2	CCATttatttccatttaTT	38_1	-22,32	15567
38	CCATTTATTTCCATTTATT	2-15-2	CCatttatttccatttaTT	38_2	-19,61	15567
39	CTTTCCATTTATTTCCATTT	2-14-4	CTttccatttatttccATTT	39_1	-23,14	15570
40	TCTTTCCATTTATTTCCATT	2-14-4	TCtttccatttatttcCATT	40_1	-24,62	15571
41	ATTACCCATCCGTTCT	2-12-2	ATtacccatccgttCT	41_1	-21,15	21965
42	GCATTAGGCACATTACAT	3-12-3	GCAttaggcacattaCAT	42_1	-23,96	22211
43	ATTATTATTTAACCTTCCTA	2-16-2	ATtattatttaaccttccTA	43_1	-19,28	30451
44	ACATTATTATTTAACCTTCC	4-14-2	ACATtattatttaaccttCC	44_1	-22,84	30453
44	ACATTATTATTTAACCTTCC	2-16-2	ACattattatttaaccttCC	44_2	-20,13	30453
45	CATTATTATTTAACCTTCC	4-13-2	CATTattatttaaccttCC	45_1	-22,04	30453
45	CATTATTATTTAACCTTCC	2-15-2	CAttattatttaaccttCC	45_2	-19,55	30453
46	CCTCTGCTTATAACTTT	2-13-2	CCtctgcttataactTT	46_1	-19,15	30699
47	CTACTATACTTTCCTCT	2-11-4	CTactatactttcCTCT	47_1	-22,32	30711
48	GTTCTACTATACTTTCC	4-11-2	GTTCtactatactttCC	48_1	-21,69	30714
48	GTTCTACTATACTTTCC	2-13-2	GTtctactatactttCC	48_2	-19,21	30714
49	CACCTGATAACAGACCCT	3-12-3	CACctgataacagacCCT	49_1	-26,38	36068
50	CACCTGATAACAGACC	3-10-3	CACctgataacagACC	50_1	-21,10	36070
51	CCCACCAAAGGATATATT	3-12-3	CCCaccaaaggatatATT	51_1	-23,47	37208
52	ACCAGCTACAGGAACCTC	3-12-3	ACCagctacaggaacCTC	52_1	-26,57	46132
53	CTATATCTCACTCCTATTT	4-13-2	CTATatctcactcctatTT	53_1	-23,07	48143
53	CTATATCTCACTCCTATTT	2-13-4	CTatatctcactcctATTT	53_2	-22,12	48143
54	CTATATCTCACTCCTATT	2-14-2	CTatatctcactcctaTT	54_1	-19,40	48144

Seq ID NO	Motif	Design	Compound	CMP ID NO	ΔG°	Start SEQ ID NO 1
54	CTATATCTCACTCCTATT	2-12-4	CTatatctcactccTATT	54_2	-22,28	48144
54	CTATATCTCACTCCTATT	3-12-3	CTAtatctcactcctATT	54_3	-21,44	48144
55	CTACTATATCTCACTCCTAT	2-16-2	CTactatatctcactcctAT	55_1	-22,00	48145
55	CTACTATATCTCACTCCTAT	2-14-4	CTactatatctcactcCTAT	55_2	-25,54	48145
56	TACTATATCTCACTCCTAT	2-13-4	TActatatctcactcCTAT	56_1	-23,29	48145
57	CTACTATATCTCACTCCTA	2-15-2	CTactatatctcactccTA	57_1	-21,91	48146
58	TACTATATCTCACTCCTA	2-14-2	TActatatctcactccTA	58_1	-19,66	48146
58	TACTATATCTCACTCCTA	2-12-4	TActatatctcactCCTA	58_2	-23,59	48146
58	TACTATATCTCACTCCTA	3-12-3	TACtatatctcactcCTA	58_3	-22,62	48146
59	CTACTATATCTCACTCCT	2-14-2	CTactatatctcactcCT	59_1	-21,25	48147
59	CTACTATATCTCACTCCT	4-12-2	CTACtatatctcactcCT	59_2	-23,87	48147
60	CTACTATATCTCACTCC	2-13-2	CTactatatctcactCC	60_1	-20,13	48148
60	CTACTATATCTCACTCC	2-11-4	CTactatatctcaCTCC	60_2	-23,00	48148
60	CTACTATATCTCACTCC	3-11-3	CTActatatctcacTCC	60_3	-22,56	48148
61	CCTACTATATCTCACTC	2-11-4	CCtactatatctcACTC	61_1	-21,93	48149
62	CTCCTACTATATCTCACTC	4-13-2	CTCCtactatatctcacTC	62_1	-25,69	48149
63	TCCTACTATATCTCACTC	3-12-3	TCCtactatatctcaCTC	63_1	-23,88	48149
64	CTCCTACTATATCTCACT	4-12-2	CTCCtactatatctcaCT	64_1	-24,87	48150
64	CTCCTACTATATCTCACT	3-12-3	CTCctactatatctcACT	64_2	-22,93	48150
65	TTTCCTCTCCTACTATATC	2-15-2	TTtcctctcctactataTC	65_1	-21,23	48155
66	ATCCATATCCTTTCCT	3-10-3	ATCcatatcctttCCT	66_1	-24,02	48168
67	CATCCATATCCTTTCCT	4-11-2	CATCcatatcctttcCT	67_1	-24,94	48168
68	ATCATCCATATCCTTTCC	4-12-2	ATCAtccatatcctttCC	68_1	-25,69	48169
69	CATCATCCATATCCTTTC	4-12-2	CATCatccatatccttTC	69_1	-23,32	48170
69	CATCATCCATATCCTTTC	2-14-2	CAtcatccatatccttTC	69_2	-20,72	48170
69	CATCATCCATATCCTTTC	2-12-4	CAtcatccatatccTTTC	69_3	-22,56	48170
70	TACATCATCCATATCCTTTC	2-16-2	TAcatcatccatatccttTC	70_1	-22,45	48170
70	TACATCATCCATATCCTTTC	4-14-2	TACAtcatccatatccttTC	70_2	-25,00	48170
70	TACATCATCCATATCCTTTC	2-14-4	TAcatcatccatatccTTTC	70_3	-24,29	48170
71	ACATCATCCATATCCTTT	3-12-3	ACAtcatccatatccTTT	71_1	-22,11	48171
72	CATCATCCATATCCTTT	2-13-2	CAtcatccatatcctTT	72_1	-19,04	48171
72	CATCATCCATATCCTTT	4-11-2	CATCatccatatcctTT	72_2	-21,64	48171
73	TACATCATCCATATCCTTT	2-15-2	TAcatcatccatatcctTT	73_1	-20,76	48171
73	TACATCATCCATATCCTTT	2-13-4	TAcatcatccatatcCTTT	73_2	-23,36	48171
73	TACATCATCCATATCCTTT	3-13-3	TACatcatccatatccTTT	73_3	-22,88	48171
74	ATACATCATCCATATCCTT	2-15-2	ATacatcatccatatccTT	74_1	-20,80	48172
74	ATACATCATCCATATCCTT	4-13-2	ATACatcatccatatccTT	74_2	-23,12	48172
75	TACATCATCCATATCCTT	2-14-2	TAcatcatccatatccTT	75_1	-19,97	48172
75	TACATCATCCATATCCTT	4-12-2	TACAtcatccatatccTT	75_2	-22,52	48172
76	TATACATCATCCATATCCTT	2-16-2	TAtacatcatccatatccTT	76_1	-21,36	48172

Seq ID NO	Motif	Design	Compound	CMP ID NO	ΔG°	Start SEQ ID NO 1
77	ATACATCATCCATATCCT	3-12-3	ATAcatcatccatatCCT	77_1	-24,15	48173
77	ATACATCATCCATATCCT	2-14-2	ATacatcatccatatcCT	77_2	-20,55	48173
77	ATACATCATCCATATCCT	2-13-3	ATacatcatccatatCCT	77_3	-22,92	48173
78	ATATACATCATCCATATCCT	2-16-2	ATatacatcatccatatcCT	78_1	-22,04	48173
79	TACATCATCCATATCCT	2-11-4	TAcatcatccataTCCT	79_1	-23,21	48173
79	TACATCATCCATATCCT	2-13-2	TAcatcatccatatcCT	79_2	-19,71	48173
79	TACATCATCCATATCCT	4-11-2	TACAtcatccatatcCT	79_3	-22,27	48173
80	TATACATCATCCATATCCT	2-15-2	TAtacatcatccatatcCT	80_1	-21,11	48173
80	TATACATCATCCATATCCT	3-13-3	TATacatcatccatatCCT	80_2	-25,15	48173
80	TATACATCATCCATATCCT	4-13-2	TATAcatcatccatatcCT	80_3	-24,01	48173
81	ATACATCATCCATATCC	3-11-3	ATAcatcatccataTCC	81_1	-21,79	48174
82	ATATACATCATCCATATCC	4-13-2	ATATacatcatccatatCC	82_1	-23,73	48174
82	ATATACATCATCCATATCC	2-15-2	ATatacatcatccatatCC	82_2	-20,93	48174
83	TATACATCATCCATATCC	2-14-2	TAtacatcatccatatCC	83_1	-20,00	48174
83	TATACATCATCCATATCC	4-12-2	TATAcatcatccatatCC	83_2	-22,90	48174
84	TATATACATCATCCATATCC	2-16-2	TAtatacatcatccatatCC	84_1	-21,49	48174
84	TATATACATCATCCATATCC	4-14-2	TATAtacatcatccatatCC	84_2	-24,29	48174
85	GCTTCATATTTCTCCA	2-12-2	GCttcatatttctcCA	85_1	-20,44	49345
86	CATCTTGTTCTTTACCT	2-13-2	CAtcttgttctttacCT	86_1	-19,67	49581
87	TATATTCACCATTGCC	2-10-4	TAtattcaccatTGCC	87_1	-22,70	49724
88	CCTTATATTCACCATTG	2-13-2	CCttatattcaccatTG	88_1	-19,44	49726
88	CCTTATATTCACCATTG	2-11-4	CCttatattcaccATTG	88_2	-21,25	49726
89	CCTCCTTATATTCACC	4-10-2	CCTCcttatattcaCC	89_1	-24,64	49730
90	CCCTTCCTTTATTCAA	3-10-3	CCCttcctttattCAA	90_1	-23,86	50189
91	CCTTACTGTTAAATCCT	2-13-2	CCttactgttaaatcCT	91_1	-19,81	50475
92	CAGGCAGATAACCTCCAA	3-12-3	CAGgcagataacctcCAA	92_1	-25,31	52419
93	CAGCAGGCAGATAACCTC	3-12-3	CAGcaggcagataacCTC	93_1	-25,88	52422
94	CGAATCTTGACATACAGG	3-12-3	CGAatcttgacatacAGG	94_1	-21,47	53955
95	CTCATACTTGCTTTAAT	4-11-2	CTCAtacttgctttaAT	95_1	-19,10	60821
95	CTCATACTTGCTTTAAT	2-13-2	CTcatacttgctttaAT	95_2	-16,35	60821
96	ACATCTCATACTTGCTT	2-11-4	ACatctcatacttGCTT	96_1	-21,31	60825
96	ACATCTCATACTTGCTT	2-13-2	ACatctcatacttgcTT	96_2	-17,66	60825
96	ACATCTCATACTTGCTT	2-12-3	ACatctcatacttgCTT	96_3	-19,52	60825
97	ACATCTCATACTTGCT	2-10-4	ACatctcatactTGCT	97_1	-21,18	60826
97	ACATCTCATACTTGCT	2-12-2	ACatctcatacttgCT	97_2	-17,70	60826
97	ACATCTCATACTTGCT	2-11-3	ACatctcatacttGCT	97_3	-19,49	60826
97	ACATCTCATACTTGCT	4-10-2	ACATctcatacttgCT	97_4	-20,48	60826
98	TACATCTCATACTTGCT	2-11-4	TAcatctcatactTGCT	98_1	-22,33	60826
98	TACATCTCATACTTGCT	2-13-2	TAcatctcatacttgCT	98_2	-18,85	60826
98	TACATCTCATACTTGCT	4-11-2	TACAtctcatacttgCT	98_3	-21,40	60826

Seq ID NO	Motif	Design	Compound	CMP ID NO	ΔG°	Start SEQ ID NO 1
99	CCTACATCTCATACTTGC	3-12-3	CCTacatctcatactTGC	99_1	-26,29	60827
99	CCTACATCTCATACTTGC	2-14-2	CCtacatctcatacttGC	99_2	-22,98	60827
99	CCTACATCTCATACTTGC	2-13-3	CCtacatctcatactTGC	99_3	-24,67	60827
99	CCTACATCTCATACTTGC	2-12-4	CCtacatctcatacTTGC	99_4	-25,70	60827
100	CTACATCTCATACTTGC	3-11-3	CTAcatctcatactTGC	100_1	-22,33	60827
100	CTACATCTCATACTTGC	2-13-2	CTacatctcatacttGC	100_2	-19,41	60827
100	CTACATCTCATACTTGC	2-12-3	CTacatctcatactTGC	100_3	-21,10	60827
101	TACATCTCATACTTGC	3-10-3	TACatctcatactTGC	101_1	-19,94	60827
101	TACATCTCATACTTGC	2-12-2	TAcatctcatacttGC	101_2	-17,15	60827
101	TACATCTCATACTTGC	2-11-3	TAcatctcatactTGC	101_3	-18,85	60827
101	TACATCTCATACTTGC	4-10-2	TACAtctcatacttGC	101_4	-19,71	60827
102	CCTACATCTCATACTTG	4-11-2	CCTAcatctcatactTG	102_1	-22,52	60828
102	CCTACATCTCATACTTG	2-13-2	CCtacatctcatactTG	102_2	-19,67	60828
102	CCTACATCTCATACTTG	3-12-2	CCTacatctcatactTG	102_3	-21,29	60828
102	CCTACATCTCATACTTG	3-11-3	CCTacatctcatacTTG	102_4	-22,31	60828
103	ACCTACATCTCATACTT	3-11-3	ACCtacatctcataCTT	103_1	-21,93	60829
103	ACCTACATCTCATACTT	2-13-2	ACctacatctcatacTT	103_2	-17,76	60829
103	ACCTACATCTCATACTT	2-11-4	ACctacatctcatACTT	103_3	-20,03	60829
103	ACCTACATCTCATACTT	3-12-2	ACCtacatctcatacTT	103_4	-20,26	60829
104	CCTACATCTCATACTT	3-10-3	CCTacatctcataCTT	104_1	-21,50	60829
104	CCTACATCTCATACTT	2-12-2	CCtacatctcatacTT	104_2	-18,21	60829
104	CCTACATCTCATACTT	2-10-4	CCtacatctcatACTT	104_3	-20,48	60829
105	TACCTACATCTCATACTT	4-12-2	TACCtacatctcatacTT	105_1	-22,49	60829
105	TACCTACATCTCATACTT	2-14-2	TAcctacatctcatacTT	105_2	-18,81	60829
105	TACCTACATCTCATACTT	2-13-3	TAcctacatctcataCTT	105_3	-20,48	60829
105	TACCTACATCTCATACTT	2-12-4	TAcctacatctcatACTT	105_4	-21,08	60829
106	TTACCTACATCTCATACTT	3-13-3	TTAcctacatctcataCTT	106_1	-22,30	60829
106	TTACCTACATCTCATACTT	2-15-2	TTacctacatctcatacTT	106_2	-19,40	60829
106	TTACCTACATCTCATACTT	2-14-3	TTacctacatctcataCTT	106_3	-21,08	60829
106	TTACCTACATCTCATACTT	2-13-4	TTacctacatctcatACTT	106_4	-21,67	60829
107	ACCTACATCTCATACT	4-10-2	ACCTacatctcataCT	107_1	-21,72	60830
107	ACCTACATCTCATACT	2-12-2	ACctacatctcataCT	107_2	-17,61	60830
107	ACCTACATCTCATACT	3-11-2	ACCtacatctcataCT	107_3	-20,10	60830
107	ACCTACATCTCATACT	2-10-4	ACctacatctcaTACT	107_4	-20,11	60830
108	TACCTACATCTCATACT	4-11-2	TACCtacatctcataCT	108_1	-22,34	60830
108	TACCTACATCTCATACT	2-13-2	TAcctacatctcataCT	108_2	-18,66	60830
108	TACCTACATCTCATACT	3-12-2	TACctacatctcataCT	108_3	-19,85	60830
108	TACCTACATCTCATACT	3-11-3	TACctacatctcatACT	108_4	-20,44	60830
109	TTACCTACATCTCATACT	2-12-4	TTacctacatctcaTACT	109_1	-21,75	60830
109	TTACCTACATCTCATACT	2-14-2	TTacctacatctcataCT	109_2	-19,25	60830

Seq ID NO	Motif	Design	Compound	CMP ID NO	ΔG°	Start SEQ ID NO 1
109	TTACCTACATCTCATACT	3-13-2	TTAcctacatctcataCT	109_3	-20,48	60830
109	TTACCTACATCTCATACT	3-12-3	TTAcctacatctcatACT	109_4	-21,08	60830
110	TTACCTACATCTCATAC	3-11-3	TTAcctacatctcaTAC	110_1	-19,50	60831
110	TTACCTACATCTCATAC	2-13-2	TTacctacatctcatAC	110_2	-16,37	60831
111	GTTACCTACATCTCATA	2-11-4	GTtacctacatctCATA	111_1	-21,69	60832
111	GTTACCTACATCTCATA	2-13-2	GTtacctacatctcaTA	111_2	-18,74	60832
111	GTTACCTACATCTCATA	3-12-2	GTTacctacatctcaTA	111_3	-19,98	60832
112	GTTACCTACATCTCAT	3-10-3	GTTacctacatctCAT	112_1	-20,69	60833
112	GTTACCTACATCTCAT	2-12-2	GTtacctacatctcAT	112_2	-17,37	60833
113	ATATACCCAAAGGCACCT	3-12-3	ATAtacccaaaggcaCCT	113_1	-25,99	62200
114	TCTACTCATCCTTTAACTCA	2-14-4	TCtactcatcctttaaCTCA	114_1	-25,63	62251
115	CCTTAATCTGTATCACT	2-13-2	CCttaatctgtatcaCT	115_1	-19,58	62286
116	CCATACACAGCACATA	2-12-2	CCatacacagcacaTA	116_1	-19,04	62424
117	CTCCATACACAGCACAT	2-13-2	CTccatacacagcacAT	117_1	-20,08	62425
118	CAGAATAATTCTCCTCC	2-13-2	CAgaataattctcctCC	118_1	-19,86	62441
119	GTCCTACATATATACC	4-10-2	GTCCtacatatataCC	119_1	-22,09	66380
120	TGCTTCCTTACTAACC	4-10-2	TGCTtccttactaaCC	120_1	-23,93	66701
120	TGCTTCCTTACTAACC	2-12-2	TGcttccttactaaCC	120_2	-20,10	66701
121	CCCTTTGTAATCATCT	4-10-2	CCCTttgtaatcatCT	121_1	-23,44	66838
122	TCCCTTTGTAATCATCT	2-13-2	TCcctttgtaatcatCT	122_1	-19,97	66838
123	CTGCCATCAATACCAT	2-12-2	CTgccatcaataccAT	123_1	-19,14	68918
124	TCACTGCCATCAATACC	2-13-2	TCactgccatcaataCC	124_1	-21,35	68920
125	ATTCTTACTTTATTCCTCA	2-15-2	ATtcttactttattcctCA	125_1	-20,16	70033
126	TCACTTTCCAGATATCA	4-11-2	TCACtttccagatatCA	126_1	-21,61	77567
126	TCACTTTCCAGATATCA	2-13-2	TCactttccagatatCA	126_2	-18,65	77567
127	TCCTTCAAATTCCACATAC	3-13-3	TCCttcaaattccacaTAC	127_1	-24,09	82053
128	ACATGTCCCTTTATATT	4-11-2	ACATgtcccttataTT	128_1	-20,87	92323
128	ACATGTCCCTTTATATT	2-13-2	ACatgtcccttataTT	128_2	-17,66	92323
128	ACATGTCCCTTTATATT	3-12-2	ACAtgtcccttataTT	128_3	-19,13	92323
128	ACATGTCCCTTTATATT	3-11-3	ACAtgtcccttatATT	128_4	-20,03	92323
129	ACATGTCCCTTTATAT	3-10-3	ACAtgtcccttaTAT	129_1	-20,11	92324
129	ACATGTCCCTTTATAT	2-12-2	ACatgtcccttatAT	129_2	-16,74	92324
130	CCAAGAAAGGAGCAAGCT	3-12-3	CCAagaaaggagcaaGCT	130_1	-25,26	97146
131	TCCAAGAAAGGAGCAAGC	3-12-3	TCCaagaaaggagcaAGC	131_1	-24,12	97147
132	CTCATCCCTCCAAGAAA	4-11-2	CTCAtccctccaagaAA	132_1	-22,58	97156
132	CTCATCCCTCCAAGAAA	2-13-2	CTcatccctccaagaAA	132_2	-19,83	97156
132	CTCATCCCTCCAAGAAA	3-12-2	CTCatccctccaagaAA	132_3	-21,11	97156
133	TCATCCCTCCAAGAAA	4-10-2	TCATccctccaagaAA	133_1	-20,41	97156
133	TCATCCCTCCAAGAAA	2-12-2	TCatccctccaagaAA	133_2	-17,63	97156
133	TCATCCCTCCAAGAAA	3-11-2	TCAtccctccaagaAA	133_3	-19,09	97156

Seq ID NO	Motif	Design	Compound	CMP ID NO	ΔG°	Start SEQ ID NO 1
133	TCATCCCTCCAAGAAA	3-10-3	TCAtccctccaagAAA	133_4	-19,81	97156
134	CACCTCCCTATTACATAAA	4-13-2	CACCtccctattacataAA	134_1	-24,18	100018
134	CACCTCCCTATTACATAAA	2-15-2	CAcctccctattacataAA	134_2	-20,51	100018
135	CACCTCCCTATTACATAA	4-12-2	CACCtccctattacatAA	135_1	-23,75	100019
135	CACCTCCCTATTACATAA	2-14-2	CAcctccctattacatAA	135_2	-20,07	100019
136	CCTCCCTATTACATAA	2-12-2	CCtccctattacatAA	136_1	-18,40	100019
137	CTAAATCTTCCAATTCATA	2-15-2	CTaaatcttccaattcaTA	137_1	-18,12	106139
138	TATCCCTTGATTATCCT	2-13-2	TAtcccttgattatcCT	138_1	-20,68	109406
139	CCTCTTTGTCAAATACT	2-13-2	CCtctttgtcaaataCT	139_1	-19,30	110768
140	CAGCTTATTTACCTCTT	2-13-2	CAgcttatttacctcTT	140_1	-19,30	114828
141	ACTCTTTACCTCTAACACT	4-13-2	ACTCtttacctctaacaCT	141_1	-24,26	117468
142	TTACTCTTTACCTCTAACAC	3-14-3	TTActctttacctctaaCAC	142_1	-23,23	117469
143	CCAACCTAATACCTTAATA	2-15-2	CCaacctaataccttaaTA	143_1	-20,27	118639
144	TACCAACCTAATACCTTAA	2-15-2	TAccaacctaataccttAA	144_1	-18,32	118641
145	CCAATACCCACAAACC	3-10-3	CCAatacccacaaACC	145_1	-23,17	124162
145	CCAATACCCACAAACC	2-12-2	CCaatacccacaaaCC	145_2	-20,85	124162
146	CCATTATTCTACTTTGT	3-11-3	CCAttattctacttTGT	146_1	-21,79	125501
146	CCATTATTCTACTTTGT	2-13-2	CCattattctactttGT	146_2	-18,63	125501
147	CATTTCCTTATCTTCACA	2-14-2	CAtttccttatcttcaCA	147_1	-20,39	125529
148	TCATTTCCTTATCTTCACA	4-13-2	TCATttccttatcttcaCA	148_1	-24,13	125529
149	AATAATTCCTCATTTCCT	2-14-2	AAtaattcctcatttcCT	149_1	-18,01	125539
150	ACAATAATTCCTCATTTCC	3-13-3	ACAataattcctcattTCC	150_1	-22,71	125540
150	ACAATAATTCCTCATTTCC	2-15-2	ACaataattcctcatttCC	150_2	-20,23	125540

Designs refer to the gapmer design, F-G-F', where each number represents the number of consecutive modified nucleosides, e.g2' modified nucleosides (first number=5' flank), followed by the number of DNA nucleosides (second number= gap region), followed by the number of modified nucleosides, e.g. 2' modified nucleosides (third number=3' flank), optionally preceded by or followed by further repeated regions of DNA and LNA, which are not necessarily part of the contiguous sequence that is complementary to the target nucleic acid.

For the oligonucleotide compounds capital letters represent beta-D-oxy LNA nucleosides, lowercase letters represent DNA nucleosides, all LNA C are 5-methyl cytosine, and 5-methyl DNA cytosines are presented by "e", all internucleoside linkages are phosphorothioate internucleoside linkages

Oligonucleotide synthesis

Oligonucleotide synthesis is generally known in the art. Below is a protocol which may be applied. The oligonucleotides of the present invention may have been produced by slightly varying methods in terms of apparatus, support and concentrations used.

Oligonucleotides are synthesized on uridine universal supports using the phosphoramidite approach on a MerMade12 or an Oligomaker DNA/RNA synthesizer at 1-4 µmol scale. At the end of the synthesis, the oligonucleotides are cleaved from the solid support using aqueous ammonia for 5-16 hours at 60˚C. The oligonucleotides are purified by reverse phase HPLC (RP-HPLC) or by solid phase extractions and characterized by UPLC, and the molecular mass is further confirmed by ESI-MS.

Elongation of the oligonucleotide:

The coupling of β-cyanoethyl- phosphoramidites (DNA-A(Bz), DNA- G(ibu), DNA- C(Bz), DNA-T, LNA-5-methyl-C(Bz), LNA-A(Bz), LNA- G(dmf), LNA-T or amino-C6 linker) is performed by using a solution of 0.1 M of the 5'-O-DMT-protected amidite in acetonitrile and DCI (4,5–dicyanoimidazole) in acetonitrile (0.25 M) as activator. For the final cycle a phosphoramidite with desired modifications can be used, e.g. a C6 linker for attaching a conjugate group or a conjugate group as such. Thiolation for introduction of phosphorthioate linkages is carried out by using xanthane hydride (0.01 M in acetonitrile/pyridine 9:1). Phosphordiester linkages can be introduced using 0.02 M iodine in THF/Pyridine/water 7:2:1. The rest of the reagents are the ones typically used for oligonucleotide synthesis.

Purification by RP-HPLC:

The crude compounds are purified by preparative RP-HPLC on a Phenomenex Jupiter C18 10µ 150x10 mm column. 0.1 M ammonium acetate pH 8 and acetonitrile is used as buffers at a flow rate of 5 mL/min. The collected fractions are lyophilized to give the purified compound typically as a white solid.

Abbreviations:

DCI: 4,5-Dicyanoimidazole

DCM: Dichloromethane

DMF: Dimethylformamide

DMT: 4,4'-Dimethoxytrityl

THF: Tetrahydrofurane

Bz: Benzoyl

Ibu: Isobutyryl

RP-HPLC: Reverse phase high performance liquid chromatography

T_m Assay

Oligonucleotide and RNA target duplexes are diluted to 3 mM in 500 ml RNase-free water and mixed with 500 ml 2x T_m-buffer (200mM NaCl, 0.2mM EDTA, 20mM Naphosphate, pH 7.0). The solution is heated to 95ºC for 3 min and then allowed to anneal in room temperature for 30 min. The duplex melting temperatures (T_m) is measured on a Lambda 40 UV/VIS Spectrophotometer equipped with a Peltier temperature programmer PTP6 using PE Templab software (Perkin Elmer). The temperature is ramped up from 20ºC to 95ºC and then down to 25ºC, recording absorption at 260 nm. First derivative and the local maximums of both the melting and annealing are used to assess the duplex T_m.

Preparation of mouse primary cortical neuron cell cultures

Primary cortical neuron cultures were prepared from mouse embryo brains of 15 days of age according to standard procedure. In brief, culture plates were coated with Poly-L-Lysine (50 µg/ml Poly-L-Lysine, 10 mM Na-tetraborate, pH 8 buffer) for 2-3 hrs at room temperature. The plates were washed with 1xPBS before use. Harvested mouse embryo brains were dissected and homogenized by a razor blade and submerged into 38 ml dissection medium (HBSS, 0.01 M Hepes, Penicillin/Streptomycin). Then, 2 ml trypsin was added and cells were incubated for 30 min at 37 °C and centrifuged down. The cells were dissolved in 20 ml DMEM (+ 10% FBS) and passed through a syringe for further homogenization. This was followed by centrifugation at 500 rpm for 15 mins. The cells were dissolved in DMEM (+10% FBS) and seeded in 96 well plates (0.1 x 10^6 cells/well in 100 µl). The neuronal cell cultures were ready for use directly after seeding.

Screening oligonucleotides in mouse primary cortical neuron cell cultures

Cells were cultured in growth medium (Gibco Neurobasal medium, B27 supplement, Glutamax, Pencillin-streptomycin) in 96-well plates and incubated with oligonucleotides for 3 days at the desired concentrations. Total RNA was isolated from the cells and the knock-down efficacy was measured by qPCR analysis using the qScript™ XLT One-Step RT-qPCR ToughMix®, Low ROX™ kit from Quanta Bioscience (95134-500). A commercial taqman assays from Thermo Fisher Scientific was used to measure Ube3a_ATS including GAPDH for normalization.

Generation of human primary neuronal cell cultures

Any cell lines at any described time point was incubated at 37°C, 5% CO2 concentration and 95% relative humidity.

Human induced pluripotent stem cells (hIPSC) culture

Whole human blood samples were obtained from patients diagnosed with Angelman syndrome. The subsequent cultures of primary Peripheral Blood Mononuclear Cells (PBMCs) were

enriched for erythroblasts. Patient-specific IPSC lines were generated by reprogramming erythroblast with CytoTune-iPS Sendai Reprogramming Kit (Thermo Fisher Scientific). Derived IPSC lines were maintained in feeder-free conditions using hESC-qualified Matrigel (Corning) in mTESR1 (STEMCELL Technologies) with daily medium replacement. Upon reaching confluence, colonies were dissociated into cell cluster of 50 - 200 µm in size using Gentle Cell Dissociation Reagent (STEMCELL Technologies) and subcultured at a ratio of 1:10 – 1:20 in the presence of 10 µM Y-27632 (Calbiochem).

Differentiation into Neural progenitor cells (NPC)

Upon induction of neural differentiation IPSC-derived cells were maintained in basal medium composed of equal volumes of DMEM:F12 Glutamax medium and Neurobasal medium (Gibco, Invitrogen), supplemented with 1x B27 (Gibco, Invitrogen), 1x N2 (Gibco, Invitrogen), 0.1 mM beta-mercaptoethanol (Gibco, Invitrogen) and indicated supplements.

Neural progenitor cells (NPCs) were derived from hIPSCs by dual SMAD inhibition and according to published procedures with slight modifications (Chambers et al. 2009 Nat Biotechnol. Vol. 3 pp.275-80, Boissart et al., 2013 Transl Psychiatry. 3:e294). HIPSCs were dissociated with Accutase (Innovative Cell Technologies Inc.) into a single cell suspension and resuspended in MT medium further supplemented with 10 µM Y-27632 (Calbiochem), 5 ng/ml FGF (Peprotech), 10 µM SB-431542 (Calbiochem) and 100 nM LDN (Calbiochem). Single cell suspension was transferred to AggreWell800 plates (STEMCELL Technologies) enabling the formation of aggregates consisting of 8000 cells. After 5 days neural aggregates were transferred onto plates coated with poly-L-ornithine (Sigma) and laminin (Roche) and allowed to form neural rosettes under continued dual SMAD inhibition (SB-431542 and LDN) in basal medium supplemented with FGF. Neural rosettes were selectively isolated using STEMdiff™ Neural Rosette Selection Reagent (STEMCELL Technologies), replated onto dishes coated with poly-L-ornithine and Laminin521 (BioLamina) and expanded in basal medium supplemented with 10 ng/ml FGF (Peprotech), 10 ng/ml EGF (RnD), and 20 ng/ml BDNF (Peprotech). When reaching confluency, cells were enzymatically dissociated with 0.05% Trypsin/EDTA (Gibco, Invitrogen) and sub-cultured. Continued passaging in basal medium supplemented with FGF, EGF and BDNF leads to a stable neural progenitor cell line (NPC line) within 10 to 20 passages. A stable neural progenitor cell line is defined by its capacity to self-renew and by the expression of the developmental stage-specific markers Sox2 and Nestin. Upon specific stimuli, NPCs differentiate into neuronal (MAP2+, Tau+, HuC/D+) and astroglial (GFAP+) progenies (Dunkley et al., 2015 Proteomics Clin Appl. Vol. 7-8 pp.684-94).

NPC culture

Conditions for NPC culture have been described previously and were used with slight modifications (Boissart et al., 2013 Transl Psychiatry. 3:e294). In brief, cells were maintained in dishes coated with Laminin521 (BioLamina) and cultured in basal medium [composed of equal volumes of DMEM:F12 Glutamax medium and Neurobasal medium (Gibco, Invitrogen), supplemented with 1x B27 (Gibco, Invitrogen), 1x N2 (Gibco, Invitrogen), 0.1 mM beta-mercaptoethanol (Gibco, Invitrogen)] and supplemented with 10 ng/ml FGF (Peprotech), 10 ng/ml EGF (RnD), and 20 ng/ml BDNF (Peprotech).

Differentiation into neuronal cell culture

To induce neuronal differentiation of NPC, cells were dissociated with 0.05% Trypsin/EDTA (Gibco, Invitrogen) into single cell suspension and seeded onto Laminin521 (BioLamina) coated dishes at a density of 12.000 cells/cm2 and maintained in basal medium supplemented with 200 ng/ml Shh (Peprotech), 100 ng/ml FGF8 (Peprotech), and 100 µM ascorbic acid phosphate (Sigma) for a period of 7 days. Subsequently, cells were replated in basal medium supplemented with 20 ng/ml BDNF (Peprotech), 10 ng/ml GDNF (Peprotech), 0.5 mM cAMP (BIOLOG Life Science), and 100 µM ascorbic acid phosphate (Sigma) at a density of 45000 cells/cm2 and differentiated for a period of 21 days. At day 21 of differentiation, differentiated neuronal cultures were replated onto the screening-compatible plate format. Replating was performed by dissociating the cultures with Accutase (Innovative Cell Technologies Inc.) into a single cell suspension. Cells were seeded at a density of 200.000 cells/cm2 in presence of 10 µM Y-27632 (a cell-permeable, reversible, inhibitor of Rho kinases from Calbiochem) into the 384 well microtiter plates for final oligonucleotides screening assay. Neuronal cultures were further differentiated for additional 7 days in basal medium supplemented with 20 ng/ml BDNF (Peprotech), 10 ng/ml GDNF (Peprotech), 0.5 mM cAMP (BIOLOG Life Science), and 100 µM ascorbic acid phosphate (Sigma). Differentiation medium was exchanged twice per week. After a total differentiation period of 35 days neuronal cell cultures were ready for oligonucleotide treatment.

Screening oligonucleotides in human neuronal cell cultures

For screening, oligonucleotide stocks were pre-diluted to the indicated concentrations with water into 384 well microtiter plates (compound plate). The plate layout served as a treatment template. Two microliter oligonucleotide dilution from each well was transferred from the compound plate to a respective culture plate. All liquid handling was done under sterile conditions in a laminar flow using a semi-automated laboratory robotic system (Beckmancoulter). Neuronal cell cultures were incubated with oligonucleotides for 5 days without media change. Subsequently, neuronal cultures were lysed and processed for qPCR assay with RealTime

ready Cell lysis and RNA Virus Master kit (Roche). Liquid handling was performed using a semi-automated laboratory robotic system (Beckmancoulter). Samples were analyzed by a Lightcycler480 real-time PCR system (Roche).

Activity of the oligonucleotides was assessed by qPCR monitoring transcript abundance of UBE3A using the following primers and probes

UBE3a-Sense: Forward primer: ATATGTGGAAGCCGGAATCT,

Reverse primer: TCCCAGAACTCCCTAATCAGAA,

Internal probe labeled with dye FAM: ATGACGGTGGCTATACCAGG

The RT-qPCR was multiplexed with PPIA (peptidylprolyl isomerase A) as housekeeping gene for normalization. PPIA primers and probe labeled with the dye VIC were purchased from Thermo Fisher Scientific (assay ID Hs99999904_m1). Each plate includes a non-targeting oligonucleotide (mock) as negative control (TTGaataagtggaTGT) and a reference oligonucleotide CMP ID NO: 41_1, resulting in up-regulation of UBE3A mRNA.

Selectivity of oligonucleotides was verified by counter screening for SNORD 115 transcript, which is located upstream of SNORD109B on chromosome 15. Expression of SNORD115 was monitored by qPCR using the following primers and probe

Forward primer: GGGTCAATGATGAGAACCTTAT,

Reverse primer: GGGCCTCAGCGTAATCCTATT,

Internal probe labeled with the dye FAM: TTCTGAAGAGAGGTGATGACTTAAAA

The RT-qPCR was multiplexed with PPIA (Thermo Fisher Scientific) upon oligonucleotide treatment.

The reduction of the SNHG14 transcript downstream of SNORD109B (also termed the UBE3A suppressor) was measured by RT-qPCR using the following primers and probe

Forward primer: ATCCGAGGCATGAATCTCAC,

Reverse primer: CAGGCCAAAACCCTTGATAA,

Internal probe labeled with dye FAM: TTGCTGAGCATTTTTGCATC)

The RT-qPCR was multiplexed with PPIA (Thermo Fisher Scientific).

Data are presented as average % expression relative to mock across all plates and normalized to the reference oligonucleotide to account for plate to plate variation

Screening oligonucleotides in human neuronal cell cultures – 96 well system

For screening, oligonucleotide stocks were pre-diluted to the indicated concentrations with water into 96 well microtiter plates (compound plate). The plate layout served as a treatment template. Two microliter oligonucleotide dilution from each well was transferred from the compound plate to a respective culture plate. All liquid handling was done under sterile conditions in a laminar flow using a semi-automated laboratory robotic system (Beckman

Coulter). Neuronal cell cultures were incubated with oligonucleotides for 5 days without media change. Subsequently, neuronal cultures were lysed and RNA purified using RNA purification kit Pure Link Pro96 (12173011A) LifeTechnologies. Liquid handling was performed using a semi-automated laboratory robotic system (Beckmancoulter). qPCR analysis of Ube3a and Ube3a-ATS was carried out on a ViiA™ 7 Real-Time PCR System Thermo Fisher Scientific using the qScriptTM XLT 1-Step RT-qPCR ToughMix Low ROX, from Quanta (95134-50).

The following primers and probes were used:

qPCR UBE3a-Sense:

Forward primer: ATATGTGGAAGCCGGAATCT,

Reverse primer: TCCCAGAACTCCCTAATCAGAA,

Internal probe labeled with dye FAM: ATGACGGTGGCTATACCAGG

qPCR SNHG14 transcript downstream of SNORD109B (also termed the UBE3A suppressor):

Commercially available primer and probe set from Thermo Fisher: Hs01372957_m1. These primers amplifies a 87 bp exon-exon spanning sequence in the Genbank transcript AF400500.1

QPCR GAPDH transcript:

Commercially available primer and probe set from Thermo Fisher: Gene Symbol: with following assay details: RefSeq: NM_002046.3, Probe Exon Location:3, Amplicon Size: 122 bp. Corresponding TaqMan Assay ID: Hs99999905_m1.

The RT-qPCR for both Ube3a and Ube3a-ATS was multiplexed with GAPDH as housekeeping gene for normalization. Each plate includes a non-targeting oligonucleotide (mock) as negative control (TTGaataagtggaTGT) and a reference oligonucleotide CMP ID NO: 21_1, resulting in up-regulation of UBE3A mRNA. Moreover panel of control oligonucleotides not targeting Ub3a or SNHG14 transcript downstream of SNORD109B (also termed the UBE3A suppressor) were included to monitor the assay noise and risk of detecting false positives. These were randomly distributed over the plates.

Control oligonucleotides:

SEQUENCE

CGAaccactgaaCAA

CGAaccactgaacAAA

CGAagtgcacaCG

GCGtaaagagaGGT

GAGAaggcacagaCGG

GCGaagtgcacaCGG

GAGaaggcacagaCGG

CGAaccactgAACA

GAAccactgaacAAA

caGCGtaaagagaGG
GCgtaaagagAGG
CGAaccactgaAC
CGAAccactgaaCAAA
AGCgaagtgcacaCGG
AGGtgaagcgaAGTG
TAGTaaactgagCCA
AGAaggcacagaCGG
CCGcagtatggaTCG

Generation of cynomolgus primary neuronal cell cultures

Any cell lines at any described time point was incubated at 37°C, 5% CO2 concentration and 95% relative humidity.

Cynomolgus induced pluripotent stem cells (cIPSC) culture

All animal procedures were performed in accordance with the guidelines of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee (IACUC) affiliated with Roche 340 Kingsland Street Nutley NJ 07110, USA (closed 2014). Cynomolgus IPSCs were established from kidney fibroblasts from a female, 14-year-old Mauritian Cynomolgus monkey using Sendai virus particles (CytoTune-iPS Sendai Reprogramming Kit, Thermo Fisher Scientific) harboring the Yamanaka factors (Oct4, Sox2, Klf4, and C-Myc) (Takahashi, K. & Yamanaka, S., 2006). Five days post-transfection, cells were passed onto mitomycin C inactivated feeders at varying densities and cultured in hESC media (knock-out DMEM:F12 supplemented with 20% knock-out serum replacement, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 8 ng/ml bFGF, all from Life Technologies). Twenty days post-transfection clones with ES-like morphologies were selected for further passaging. The cells were routinely passaged every 3–4 days by manual dissociation of colonies. After at least 10 passages, cIPSCs were adapted to feeder-free culture conditions (MT medium supplemented with 15ng/ml FGF2 (Peprotech) and 10ng/ml ActivinA (Peprotech)) based on Ono et al.; 2014 with the modification that cells were cultured on Matrigel (BD Bioscience)-coated plates and that Y-27632 (Calbiochem) was used instead of Thiazovivin when the cells were in single cell suspension. MT medium is a defined medium that contains Dulbecco's Modified Eagle Medium with Ham's F12 Nutrient Mixture (DMEM/F12) with 2.5 mM GlutaMAX™, 7 µg/ml insulin, 450 µM monothioglycerole, 1x Lipid concentrate, 5 mg/ml BSA, 14 ng/ml sodium selenite, 1x non-essential amino acids, 2 mg/ml heparin, 15 µg/ml transferrin, and 220 µM ascorbic acid-2-phosphate. The cells were passaged every 2–4 days using Gentle Cell

Dissociation Reagent (Stem Cell Technologies). One clone was chosen for further experiments and used for all the following assays.

Differentiation into Neural progenitor cells (NPC)

Upon induction of neural differentiation cIPSC-derived cells (see Figure 4: day 1) were maintained in basal medium composed of equal volumes of DMEM:F12 Glutamax medium and Neurobasal medium (Gibco, Invitrogen), supplemented with 1x B27 (Gibco, Invitrogen), 1x N2 (Gibco, Invitrogen), 0.1 mM beta-mercaptoethanol (Gibco, Invitrogen) and indicated supplements.

Neural progenitor cells (NPCs) were derived from cIPSCs by dual SMAD inhibition and according to published procedures with slight modifications (Chambers et al. 2009 Nat Biotechnol. Vol. 3 pp.275-80, Boissart et al., 2013 Transl Psychiatry. 3:e294). CynoIPSCs were dissociated with Accutase (Innovative Cell Technologies Inc.) into a single cell suspension and resuspended in MT medium supplemented with 10 µM Y-27632 (Calbiochem). Single cell suspension was transferred to AggreWell800 plates (STEMCELL Technologies) enabling the formation of aggregates consisting of 12000 cells (see Figure 4: EB formation). For the next five consecutive days 1.5 ml of medium per AggreWell was replaced by basal medium supplemented with 5 ng/ml FGF (Peprotech), 10 µM SB-431542 (Calbiochem) and 100 nM LDN-193189 (Calbiochem). After 5 days neural aggregates were transferred onto plates coated with poly-L-ornithine (Sigma) and laminin (Roche) and allowed to form neural rosettes under continued dual SMAD inhibition (SB-431542 and LDN-193189) in basal medium supplemented with FGF (see Figure 4: Harvesting). Neural rosettes were selectively isolated by manual dissection, thereafter replated onto dishes coated with poly-L-ornithine and Laminin521 from BioLamina (see Figure 4: neural rosette isolation) and expanded in basal medium supplemented with 10 ng/ml FGF (Peprotech), 10 ng/ml EGF (RnD), and 20 ng/ml BDNF (Peprotech). When reaching confluency, cells were enzymatically dissociated with 0.05% Trypsin/EDTA (Gibco, Invitrogen) and sub-cultured. Continued passaging in basal medium supplemented with FGF, EGF and BDNF leads to a stable neural progenitor cell line (NPC line) within 5 to 10 passages (see Figure 4, day 24-45). A stable neural progenitor cell line is defined by its capacity to self-renew and by the expression of the developmental stage-specific markers Sox2 and Nestin. Upon specific stimuli, cyno NPCs differentiate into neuronal MAP2+ cells.

NPC culture

Conditions for NPC culture have been described previously and were used with slight modifications (Boissart et al., 2013 Transl Psychiatry. 3:e294). In brief, cells were maintained in dishes coated with Laminin521 (BioLamina) and cultured in basal medium [composed of equal

volumes of DMEM:F12 Glutamax medium and Neurobasal medium (Gibco, Invitrogen), supplemented with 1x B27 (Gibco, Invitrogen), 1x N2 (Gibco, Invitrogen), 0.1 mM beta-mercaptoethanol (Gibco, Invitrogen)] supplemented with 10 ng/ml FGF (Peprotech), 10 ng/ml EGF (RnD), and 20 ng/ml BDNF (Peprotech).

Differentiation into neuronal cell culture

To induce neuronal differentiation of NPC, cells were dissociated with 0.05% Trypsin/EDTA (Gibco, Invitrogen) into single cell suspension and seeded onto Laminin521 (BioLamina) coated dishes at a density of 30.000 cells/cm2 and maintained in basal medium supplemented with 200 ng/ml Shh (Peprotech), 100 ng/ml FGF8 (Peprotech), and 100 µM ascorbic acid phosphate (Sigma) for a period of 7 days. Subsequently, cells were replated in basal medium supplemented with 20 ng/ml BDNF (Peprotech), 10 ng/ml GDNF (Peprotech), 0.5 mM cAMP (BIOLOG Life Science), and 100 µM ascorbic acid phosphate (Sigma) at a density of 45000 cells/cm2 and differentiated for a period of 21 days. At day 21 of differentiation, differentiated NCs were dissociated and reseeded onto the screening-compatible plate format. Replating was performed by dissociating the cultures with Accutase (Innovative Cell Technologies Inc.) into a single cell suspension. Cells were seeded at a density of 200000 cells/cm2 in presence of 10 µM Y-27632 (a cell-permeable, reversible, inhibitor of Rho kinases from Calbiochem) into the 384 well microtiter plates for final oligonucleotides screening assay. Neuronal cultures were further differentiated for additional 7 days in basal medium supplemented with 20 ng/ml BDNF (Peprotech), 10 ng/ml GDNF (Peprotech), 0.5 mM cAMP (BIOLOG Life Science) and 100 µM ascorbic acid phosphate (Sigma). Differentiation medium was exchanged twice per week. After a total differentiation period of 35 days neuronal cell cultures were ready for oligonucleotide treatment.

Screening oligonucleotides in cyno neuronal cell cultures – 96 well system

For screening, oligonucleotide stocks were pre-diluted to the indicated concentrations with water into 96 well microtiter plates (compound plate). The plate layout served as a treatment template. Two microliter oligonucleotide dilution from each well was transferred from the compound plate to a respective culture plate. All liquid handling was done under sterile conditions in a laminar flow using a semi-automated laboratory robotic system (Beckmancoulter). Neuronal cell cultures were incubated with oligonucleotides for 5 days without media change. Subsequently, neuronal cultures were lysed and purified using PureLink® Pro 96 total RNA Purification Kit (Thermo Fisher) and thereafter processed for qPCR assay with qScript XLT One-Step RT-qPCR Tough Mix, Low ROX (Quanta Biosiences). Liquid handling was performed

using a semi-automated laboratory robotic system (Integra Assist and Integra Viaflo). Samples were analyzed by a Lightcycler480 real-time PCR system (Roche).

Activity of the oligonucleotides was assessed by qPCR monitoring transcript abundance of UBE3A-Sense and UBE3A-Antisense using the following primers and probes:

UBE3a-Sense: HS00166580_VIC Taqman assay (Thermo Fisher)

UBE3A-Antisense: HS01372957_VIC Taqman assay (Thermo Fisher)

The RT-qPCR was multiplexed with TBP (TATA-Box Binding Protein) as housekeeping gene for normalization. TBP primers and FAM labeled probe were purchased from Roche (REF.:05532957001, Assay Id. 101145). Each plate includes a non-targeting oligonucleotide (mock) as negative control (TTGaataagtggaTGT).

Data are presented as average percentage expression relative to mock treated conditions.

Immunocytofluorescence staining

Cells were fixed with 4% PFA and permeabilized with 0.1% TritonX (Sigma) in PBS (with Ca2+ and Mg2+). Blocking was performed using SuperBlock solution (Thermo Fisher Scientific) supplemented with 0.1% TritonX. Cells were stained with the following primary antibodies: anti-SOX2 (Milipore AB5603), anti-GFAP (Dako Z033401), anti-HuC/D (Invitrogen A21271), anti-Nestin (Millipore mab5326) and anti-Map2 (Neuromics CH22103). Subsequently, the cells were washed and stained with secondary antibodies conjugated either to Alexa488, Alexa555 or Alexa647 (all Molecular Probes). Nuclei were stained with Hoechst 1:1000 (Molecular Probes). The cells were imaged using an Axiovert microscope (Zeiss) or an Operetta high content imaging system (Perkin Elmer). Images were analyzed using ImageJ software.

qPCR analysis

RNA from cIPSC, cyno differentiated NCs, hESCs and human differentiated NCs was isolated using the miRNeasy Mini kit (QIAGEN) and qPCR analysis was performed (for human cells using the Ag-Path-ID One-Step RT-PCR kit, Ambion; for cyno cells reverse transcription was performed using the Transcriptor First Strand cDNA synthesis kit, Roche and qPCR using LightCycler 480 Probes Master, Roche). The following primers and probes were used: for human cells: NES Hs04187831_g1; MAP2 Hs00258900_m1; ASCL1 Hs00269932_m1; SOX2 Hs01053049_s1; ELAVL3 Hs00154959_m1; GAPDH 4352665, all from Thermo Fisher/Applied Biosystems; UBE3A-ATS Fw primer: CAA ATG CCT CAC CCA CTC TT, RV primer: CCA GCT GTC AAC ATG TGC TT, internal oligo: AAG TGC GCT CCT GTG AAA AG; UBE3A Fw primer: TCT GGG AAA TCG TT CATT CA, RV primer: TGT AGG TAA CCT TTC TGT GTC TGG, internal oligo TAC AAC GGG CAC AGA CAG AG. For cyno cells: NANOG Assay ID 700103,

POU5F1 Assay ID 113034, SOX2 Assay ID 111867, NESTIN Assay ID 138150, PAX6 Assay ID 136139, SOX1 Assay ID 136988, ZIC1 Assay ID 112077, ASCL1 Assay ID 700027, TUBB3 Assay ID 700047, GAPDH Cat N.04694333001, Probe #147; TBP Assay ID 101145, all purchased from Roche. UBE3A-ATS Fw primer: AATGCAAAGGCAGCAGTACA, Rv primer: TTGGGGAGTTGGTTATTGGA, internal oligo TGACACCACCAGAAGAACACA;
UBE3A sense: Fw primer: ATATGTGGAAGCCGGAATCT,
Rv primer: TCCCAGAACTCCCTAATCAGAA,
internal oligo: ATGACGGTGGCTATACCAGG.

RNA sequencing analysis

RNA isolation was performed using the miRNeasy Mini kit (QIAGEN). Library preparation was performed using the TruSeq Stranded Total RNA LT kit (illumina) with Ribo-Zero Gold depletion. Cluster generation was performed using the cBOT instrument and the HiSeq PE Cluster kit v4. Sequencing was performed on the HiSeq 2500 instrument as paired-end Single-indexed Sequencing run (2 x 125 cycles) with the following reagents: HiSeq SBS Kit v4 250 cycles (illumina).

Western Blot analysis

For protein expression studies, the cells were collected by scraping and flash frozen. The pellets were lysed using Cytobuster (Millipore) completed with phosphatase and protease inhibitors and DNAse (all from Roche). The protein concentration in the extracts was measured by BCA assay (Thermo Scientific) and the solution loaded to a polyacrylamide gel (Invitrogen). After electrophoresis, the proteins were blotted on a nitrocellulose membrane using the iBlot system (Invitrogen). The antibodies used to detect the proteins of interest were anti-hTau HT7 (Pierce MN1000), anti-pTauS422 (Roche) anti-pTauS404 (Abcam ab92676); these were detected by an appropriate HRP-conjugated secondary antibody. The complex was detected by luminescence using SuperSignal West Dura Extended Duration Substrate (Thermo Fisher) using an imager (Biorad).

References:

Ono, T. et al. A single-cell and feeder-free culture system for monkey embryonic stem cells. PLoS One 9, e88346, doi: 10.1371/ journal.pone.0088346 (2014).

Takahashi, K. & Yamanaka, S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663–676, doi: 10.1016/j.cell.2006.07.024 (2006).

Example 1 – Oligonucleotide activity in mouse primary neuronal cell cultures

Oligonucleotides targeting the part of SNHG14 long non-coding RNA which is antisense to the UBE3A pre-mRNA (position 55319 to 141053 of SEQ ID NO: 1) were tested for their ability to reduce the SNHG14 long non-coding RNA transcript preventing UBE3A expression (also termed UBE3A suppressor or UBE3A-SUP in the data table) and their ability to induce UBE3A mRNA re-expression in mouse primary cortical neuron cell cultures, obtained as described in the "Materials and methods" section above. The oligonucleotide concentration was 5 microM.

The oligonucleotides were screened according to the protocol for screening in mouse cortical neuron cell cultures described in the section "Materials and methods". The results are shown in table 4.

Table 4: Oligonucleotide activity in primary mouse neuronal cell cultures.

CMP ID NO	oligonucleotide	% of Mock UBE3A_SUP	sd	% of Mock UBE3A	sd
95_1	CTCAtacttgctttaAT	3,6	0,1	154,1	15,1
95_2	CTcatacttgctttaAT	15,9	2,6	119,8	12,4
96_1	ACatctcatacttGCTT	4,0	0,5	149,9	11,5
96_2	ACatctcatacttgcTT	9,3	3,9	139,9	36,4
96_3	ACatctcatacttgCTT	3,1	0,2	143,2	3,9
97_1	ACatctcatactTGCT	4,0	1,5	154,5	10,0
97_2	ACatctcatacttgCT	6,1	1,7	141,1	14,1
97_3	ACatctcatacttGCT	3,7	0,6	162,7	15,0
97_4	ACATctcatacttgCT	5,2	0,4	156,7	24,4
98_1	TAcatctcatactTGCT	5,0	0,9	159,0	15,6
98_2	TAcatctcatacttgCT	15,5	5,3	130,4	3,4
98_3	TACAtctcatacttgCT	4,7	0,4	140,3	38,2
101_1	TACatctcatactTGC	2,6	0,5	152,6	10,2
101_2	TAcatctcatacttGC	19,2	6,0	112,0	15,0
101_3	TAcatctcatactTGC	3,5	0,4	117,2	13,7
101_4	TACAtctcatacttGC	3,0	0,7	140,5	12,4
100_1	CTAcatctcatactTGC	5,4	0,8	160,4	4,1
100_2	CTacatctcatacttGC	9,6	3,7	159,2	14,5
100_3	CTacatctcatactTGC	3,0	0,1	133,2	5,9
99_2	CCtacatctcatacttGC	7,8	1,4	150,7	11,0
99_3	CCtacatctcatactTGC	3,2	0,6	134,7	12,5
99_4	CCtacatctcatacTTGC	2,7	0,2	145,2	4,7
102_1	CCTAcatctcatactTG	5,8	1,7	127,0	24,5
102_2	CCtacatctcatactTG	20,2	6,6	129,7	9,2
102_4	CCTacatctcatacTTG	4,0	0,6	140,2	7,2
102_3	CCTacatctcatactTG	3,9	1,0	133,3	10,0
104_1	CCTacatctcataCTT	6,6	1,5	136,5	8,7
104_3	CCtacatctcatACTT	3,5	0,4	131,4	6,0
103_1	ACCtacatctcataCTT	5,8	1,4	130,8	0,7
103_2	ACctacatctcatacTT	11,4	2,2	123,6	12,4
103_3	ACctacatctcatACTT	5,8	0,8	132,2	4,5
105_1	TACCtacatctcatacTT	5,2	0,8	152,3	7,2
106_1	TTAcctacatctcataCTT	13,3	3,0	140,1	17,5
106_2	TTacctacatctcatacTT	21,0	1,4	116,9	15,0

CMP ID NO	oligonucleotide	% of Mock UBE3A_SUP	sd	% of Mock UBE3A	sd
107_1	ACCTacatctcataCT	6,2	0,9	119,2	3,4
107_2	ACctacatctcataCT	14,3	7,4	142,9	13,7
108_1	TACCtacatctcataCT	5,6	1,0	127,0	10,7
108_2	TAcctacatctcataCT	21,4	12,5	117,1	8,5
109_1	TTacctacatctcaTACT	4,4	0,4	138,9	1,2
109_2	TTacctacatctcataCT	22,9	3,3	117,1	13,0
110_1	TTAcctacatctcaTAC	8,7	2,1	133,2	5,1
110_2	TTacctacatctcatAC	21,0	5,1	111,4	11,1
111_1	GTtacctacatctCATA	8,0	2,4	143,8	14,8
111_2	GTtacctacatctcaTA	19,0	2,3	115,4	4,1
112_1	GTTacctacatctCAT	6,6	1,4	145,5	16,8
112_2	GTtacctacatctcAT	15,8	4,5	120,3	8,1
126_1	TCACtttccagatatCA	8,0	1,9	133,8	5,4
126_3	TCactttccagatatCA	53,4	75,9	112,0	11,4
128_1	ACATgtccctttataTT	16,3	2,5	114,7	11,1
128_2	ACatgtccctttataTT	14,8	1,1	136,9	6,2
129_1	ACAtgtccctttaTAT	11,8	1,9	135,0	14,3
132_1	CTCAtccctccaagaAA	9,1	1,6	131,7	8,4
132_2	CTcatccctccaagaAA	11,2	3,9	159,3	17,7

Example 2 – Oligonucleotide activity in human neuronal cell cultures

Oligonucleotides targeting human SNHG14 in the region downstream of SNORD109B corresponding to position 25278410 to 25419462 on chromosome 15 (SEQ ID NO: 1) were tested in patient derived human neuronal cell cultures (see protocol in “Materials and methods” section). The oligonucleotides ability to reduce the SNHG14 transcript in the region downstream of SNORD109B (also termed UBE3A suppressor or UBE3A-SUP in the data table), without affecting expression of SNORD115 was analyzed. Furthermore, the ability to induce UBE3A mRNA re-expression was analyzed.

The oligonucleotides were screened according to the protocol for screening oligonucleotides in human neuronal cell cultures described in the section “Materials and methods” above.

The results are shown in table 5. The expression of UBE3A mRNA has been measured for all compounds, whereas the knock-down of the UBE3A suppressor and the maintenance of SNORD1115 levels have not been analyzed for all compounds.

Table 5: Oligonucleotide activity in patient derived human neuronal cell cultures.

Start SEQ ID NO 1	CMP ID NO	Target	% of Mock Oligo conc 0.2 µM	sd	% of Mock Oligo conc 1.0 µM	sd	% of Mock Oligo conc 5.0 µM	sd
1678	10_1	UBE3A	107	14	88	10	151	8
1679	12_2	UBE3A	100	9	87	14	158	16
1687	20_1	UBE3A	87	7	102	22	213	44
1712	21_1	UBE3A	127	23	166	6	178	13

Start SEQ ID NO 1	CMP ID NO	Target	% of Mock Oligo conc 0.2 µM	sd	% of Mock Oligo conc 1.0 µM	sd	% of Mock Oligo conc 5.0 µM	sd
1712	21_1	UBE3A-SUP	81	3	82	8	72	12
1712	21_1	SNORD115	115	6	142	24	169	26
4167	22_1	UBE3A	87	5	90	8	146	20
4170	27_1	UBE3A	94	16	106	11	170	10
4171	29_2	UBE3A	86	13	100	12	194	35
4172	30_1	UBE3A	96	6	121	12	209	27
9210	35_1	UBE3A	88	5	112	23	195	27
10838	37_1	UBE3A	77	7	85	9	169	24
15565	38_2	UBE3A	93	11	108	6	167	34
22209	42_1	UBE3A	125	16	143	14	180	17
22209	42_1	UBE3A-SUP	108	14	98	15	85	18
22209	42_1	SNORD115	101	14	93	25	127	21
30449	43_1	UBE3A	99	5	95	13	115	8
30451	44_1	UBE3A	99	15	80	20	141	17
30451	44_2	UBE3A	98	31	104	16	119	7
30697	46_1	UBE3A	91	8	87	5	167	20
36066	49_1	UBE3A	95	6	111	10	155	29
36066	49_1	UBE3A-SUP	76	7	84	24	110	31
36066	49_1	SNORD115	99	14	111	20	94	6
36068	50_1	UBE3A	109	15	105	11	92	14
36068	50_1	UBE3A-SUP	122	24	93	28	73	7
36068	50_1	SNORD115	120	15	113	12	99	6
37206	51_1	UBE3A	114	16	101	7	101	3
37206	51_1	UBE3A-SUP	128	21	67	9	84	13
37206	51_1	SNORD115	140	26	110	9	100	11
46130	52_1	UBE3A	139	3	160	1	236	36
46130	52_1	UBE3A-SUP	135	16	133	26	160	32
46130	52_1	SNORD115	104	8	119	14	100	8
48145	59_1	UBE3A	179	3	122	17	115	NA
48170	76_1	UBE3A	85	16	100	8	155	12
48171	80_1	UBE3A	120	7	114	10	172	20
48171	78_1	UBE3A	136	31	103	20	169	11
48172	82_2	UBE3A	96	11	121	4	186	32
48172	84_1	UBE3A	95	14	100	8	158	14
49343	85_1	UBE3A	97	22	121	10	189	17
49722	87_1	UBE3A	111	9	126	11	177	22
52417	92_1	UBE3A	133	7	140	30	140	8
52417	92_1	UBE3A-SUP	88	14	80	14	82	8
52417	92_1	SNORD115	102	8	114	20	91	9

Start SEQ ID NO 1	CMP ID NO	Target	% of Mock Oligo conc 0.2 µM	sd	% of Mock Oligo conc 1.0 µM	sd	% of Mock Oligo conc 5.0 µM	sd
52420	93_1	UBE3A	111	14	120	9	126	16
52420	93_1	UBE3A-SUP	104	23	82	20	79	8
52420	93_1	SNORD115	110	11	114	17	95	7
53953	94_1	UBE3A	117	12	147	15	166	15
53953	94_1	UBE3A-SUP	92	18	81	5	86	22
53953	94_1	SNORD115	124	33	122	17	106	14
60819	95_1	UBE3A	103	11	131	14	175	7
60819	95_1	UBE3A-SUP	93	13	87	3	74	6
60819	95_1	SNORD115	162	19	158	20	201	11
60819	95_2	UBE3A	147	10	129	20	117	2
60819	95_2	UBE3A-SUP	118	24	87	13	83	8
60819	95_2	SNORD115	104	17	118	10	129	6
60823	96_1	UBE3A	115	16	135	19	174	17
60823	96_1	UBE3A-SUP	104	25	93	32	91	11
60823	96_2	UBE3A	108	7	114	9	115	13
60823	96_2	UBE3A-SUP	99	17	92	19	93	10
60824	97_1	UBE3A	111	12	134	23	169	14
60824	97_1	UBE3A-SUP	110	27	105	33	92	10
60824	97_2	UBE3A	124	13	126	12	124	11
60824	97_2	UBE3A-SUP	113	17	107	33	96	20
60824	98_1	UBE3A	111	16	119	11	138	14
60824	98_1	UBE3A-SUP	118	34	98	23	82	19
60824	98_1	SNORD115	109	11	123	18	114	16
60824	98_2	UBE3A	128	10	109	7	136	12
60824	98_2	UBE3A-SUP	91	15	77	11	110	16
60824	98_2	SNORD115	101	3	110	7	124	11
60825	99_1	UBE3A	125	6	115	5	131	10
60825	99_1	UBE3A-SUP	139	18	121	34	127	45
60825	99_1	SNORD115	110	18	112	12	99	19
60825	99_2	UBE3A	120	21	111	11	135	22
60825	99_2	UBE3A-SUP	96	21	79	15	75	11
60825	99_2	SNORD115	104	34	113	22	131	24
60825	100_1	UBE3A	123	34	139	34	145	21
60825	100_1	UBE3A-SUP	104	37	127	46	99	17
60825	100_2	UBE3A	124	46	138	37	145	31
60825	100_2	UBE3A-SUP	111	36	120	47	92	11
60825	101_1	UBE3A	112	18	123	15	150	13
60825	101_1	UBE3A-SUP	96	18	102	14	88	12
60825	101_2	UBE3A	118	15	138	24	139	32

Start SEQ ID NO 1	CMP ID NO	Target	% of Mock Oligo conc 0.2 µM	sd	% of Mock Oligo conc 1.0 µM	sd	% of Mock Oligo conc 5.0 µM	sd
60825	101_2	UBE3A-SUP	100	29	110	39	92	10
60826	102_1	UBE3A	132	17	120	7	125	9
60826	102_1	UBE3A-SUP	113	16	83	5	88	18
60826	102_1	SNORD115	121	36	131	23	100	9
60826	102_2	UBE3A	90	6	116	23	103	7
60826	102_2	UBE3A-SUP	91	7	90	12	64	18
60826	102_2	SNORD115	116	15	146	27	183	28
60827	103_1	UBE3A	106	8	112	10	115	9
60827	103_1	UBE3A-SUP	99	15	110	28	94	8
60827	103_2	UBE3A	107	14	120	13	112	14
60827	103_2	UBE3A-SUP	97	14	118	38	93	20
60827	104_1	UBE3A	128	14	111	9	111	6
60827	104_1	UBE3A-SUP	111	12	97	9	87	19
60827	104_1	SNORD115	114	10	110	12	109	13
60827	104_2	UBE3A	108	10	111	16	109	10
60827	104_2	UBE3A-SUP	103	13	103	33	89	9
60827	105_1	UBE3A	122	13	121	12	121	4
60827	105_1	UBE3A-SUP	119	7	97	15	93	7
60827	105_1	SNORD115	114	21	128	12	118	9
60827	105_2	UBE3A	123	5	110	9	114	8
60827	105_2	UBE3A-SUP	110	11	89	17	94	21
60827	105_2	SNORD115	102	15	108	16	107	18
60827	106_1	UBE3A	114	17	133	23	125	9
60827	106_1	UBE3A-SUP	112	35	103	15	87	12
60827	106_2	UBE3A	110	12	130	22	123	14
60827	106_2	UBE3A-SUP	105	19	107	27	93	10
60828	107_1	UBE3A	83	11	117	13	112	6
60828	107_1	UBE3A-SUP	86	11	114	16	67	7
60828	107_1	SNORD115	108	17	130	21	137	24
60828	107_2	UBE3A	143	42	117	10	122	11
60828	107_2	UBE3A-SUP	116	12	92	4	100	8
60828	107_2	SNORD115	108	4	127	16	108	14
60828	108_1	UBE3A	120	7	127	31	132	31
60828	108_1	UBE3A-SUP	153	33	118	34	89	17
60828	108_1	SNORD115	114	9	114	9	105	15
60828	108_2	UBE3A	122	18	133	26	128	9
60828	108_2	UBE3A-SUP	101	19	100	28	89	17
60828	109_1	UBE3A	108	10	129	14	128	5
60828	109_1	UBE3A-SUP	106	21	107	24	84	8

Start SEQ ID NO 1	CMP ID NO	Target	% of Mock Oligo conc 0.2 µM	sd	% of Mock Oligo conc 1.0 µM	sd	% of Mock Oligo conc 5.0 µM	sd
60828	109_2	UBE3A	109	11	110	8	111	13
60828	109_2	UBE3A-SUP	95	15	86	14	83	9
60829	110_1	UBE3A	104	6	83	3	101	15
60829	110_1	UBE3A-SUP	100	13	95	12	79	4
60829	110_1	SNORD115	126	21	125	6	182	13
60829	110_2	UBE3A	92	7	87	8	96	7
60829	110_2	UBE3A-SUP	99	7	108	9	81	5
60829	110_2	SNORD115	118	15	139	22	198	39
60830	111_1	UBE3A	110	6	122	13	124	10
60830	111_1	UBE3A-SUP	104	14	90	28	79	11
60830	111_2	UBE3A	115	10	120	15	121	10
60830	111_2	UBE3A-SUP	114	20	89	19	87	9
60831	112_1	UBE3A	93	8	94	13	106	10
60831	112_1	UBE3A-SUP	97	1	68	29	82	7
60831	112_1	SNORD115	116	20	110	13	158	20
60831	112_2	UBE3A	83	8	78	7	83	6
60831	112_2	UBE3A-SUP	106	35	80	23	69	9
60831	112_2	SNORD115	107	6	106	8	159	21
62198	113_1	UBE3A	110	3	122	6	134	9
62198	113_1	UBE3A-SUP	113	20	85	19	79	24
62198	113_1	SNORD115	116	18	123	9	91	9
62284	115_1	UBE3A	105	14	98	19	141	36
62422	116_1	UBE3A	130	19	142	29	172	18
62423	117_1	UBE3A	76	8	93	13	171	17
62439	118_1	UBE3A	75	7	88	9	150	19
66378	119_1	UBE3A	96	14	93	5	110	10
77565	126_1	UBE3A	94	6	113	5	125	14
77565	126_1	UBE3A-SUP	83	17	95	33	85	5
77565	126_1	SNORD115	105	11	123	19	152	15
77565	126_2	UBE3A	95	5	126	9	111	2
77565	126_2	UBE3A-SUP	77	27	106	21	83	15
77565	126_2	SNORD115	115	17	157	13	180	15
92321	128_1	UBE3A	102	7	91	5	111	13
92321	128_1	UBE3A-SUP	115	3	104	25	91	13
92321	128_1	SNORD115	135	9	132	12	196	35
92321	128_2	UBE3A	91	5	96	8	104	8
92321	128_2	UBE3A-SUP	112	20	92	20	79	7
92321	128_2	SNORD115	125	7	111	13	169	12
92322	129_1	UBE3A	101	5	103	2	110	7

Start SEQ ID NO 1	CMP ID NO	Target	% of Mock Oligo conc 0.2 µM	sd	% of Mock Oligo conc 1.0 µM	sd	% of Mock Oligo conc 5.0 µM	sd
92322	129_1	UBE3A-SUP	99	39	113	12	94	13
92322	129_1	SNORD115	124	25	114	6	140	13
92322	129_2	UBE3A	93	2	100	4	113	16
92322	129_2	UBE3A-SUP	109	4	102	22	85	7
92322	129_2	SNORD115	103	11	99	9	152	31
97154	132_1	UBE3A	100	10	128	13	142	13
97154	132_1	UBE3A-SUP	103	9	115	8	109	6
97154	132_1	SNORD115	49	7	90	12	143	25
97154	132_2	UBE3A	111	8	128	17	128	17
97154	132_2	UBE3A-SUP	95	7	116	9	105	13
97154	133_2	SNORD115	86	7	106	9	121	9
97154	133_1	UBE3A	101	3	107	11	124	19
97154	133_1	UBE3A-SUP	112	9	117	7	146	25
97154	133_1	SNORD115	60	7	110	15	141	15
97154	133_2	UBE3A	94	13	116	14	138	12
97154	133_2	UBE3A-SUP	116	6	128	13	148	38
97154	132_2	SNORD115	70	5	108	9	160	34
106137	137_1	UBE3A	83	12	74	11	124	20
109404	138_1	UBE3A	80	20	92	7	120	21
110766	139_1	UBE3A	76	5	85	12	121	17
114826	140_1	UBE3A	87	10	88	11	136	9
118637	143_1	UBE3A	83	7	104	30	141	28
118639	144_1	UBE3A	74	17	31	39	106	33
124160	145_2	UBE3A	89	6	95	10	115	25
125499	146_1	UBE3A	83	13	76	7	124	16
125499	146_2	UBE3A	123	30	79	14	102	23
125538	150_2	UBE3A	82	17	82	7	119	24

Of the 187 compounds tested approximately 90 % showed re-expression of UBE3A when compared to the mock oligonucleotide at the 5 micro Molar concentration. The number of oligonucleotides capable of inducing re-expression of UBE3A is higher in the region between position 1 to 55318 of SEQ ID NO: 1 (non-overlapping region) then in the region complementary to UBE3A coding region (overlapping region. Figure 2 plots the distribution of the oligonucleotides according to their position on chromosome 15 versus the UBE3A mRNA expression relative to the mock oligonucleotide.

For the oligonucleotides where SNORD115 has been tested there is no significant down regulation when compared to mock at 1 and 5 microM.

Example 3 – Generation and validation of cyno neuronal differentiation *in vitro* system

Cyno IPSCs were induced to differentiate into NPCs following the protocol as described in the Materials and Methods section and depicted in Figure 4. The protocol allows to derive NPCs which can be maintained and expanded in basal medium supplemented with FGF, EGF and BDNF. To verify the efficient induction of cyno NPCs, expression of the neural stem cell markers SOX2 and NESTIN was evaluated by immunostaining (see Figure 5). Cyno NPCs express SOX2 and NESTIN and the expression pattern is highly comparable to human NPCs.

To derive differentiated NCs, expanding NPCs are dissociated and plated in SFA medium for a week. Afterwards the cells are exposed to differentiation medium (BGAA). To evaluate the neuronal differentiation potential of cyno NPCs, a transcriptional analysis was performed and results were compared to the transcriptional profile of differentiated human NPCs (see Figure 6). After 14 days of differentiation in BGAA medium, cyno cultures show decrease in the expression of pluripotency markers like NANOG and POU5F1 and increased expression of ectodermal and neuronal markers like PAX6, SOX1, ZIC1, ASCL1, TUBB3 if compared to cIPSCs (see Figure 6A). Similarly, transcriptional analysis (RNA sequencing in Figure 6B and qPCR in Figure 6C) of human pluripotent stem cell-derived NCs differentiated for 14 days in BGAA reveals decreased expression of pluripotency markers and increased expression of ectodermal and neuronal markers in day 14 BGAA differentiated NCs if compared to hESCs. Importantly, changes in the expression of UBE3A, a disease gene implicated in Angelman syndrome, and UBE3A-ATS, a non-coding transcript described to be expressed in neurons, are comparable between differentiated cyno and human NCs (see Figure 6D). These data demonstrate that cyno IPCS differentiate into NCs and the expression profile of specific markers is similar to differentiated human NCs.

Example 4 – Dissociation and reseeding of differentiated cynomolgus NCs

Stem cell-derived cells have a great potential as *in vitro* system for drug screening, especially if the corresponding primary cell type is not available or only in limited quantities as it is the case for neurons. However, drug screening requires robust cellular screening systems, which are available in suitable plate formats. For this purpose, we tested different strategies to differentiate NPCs derived from NHP-IPSCs into neuronal cells (NCs). In order to identify a robust cellular system with uniformly distributed NCs, expanding NPCs were dissociated and exposed to differentiation medium in one of two ways: either they were plated directly in the final assay format or, alternatively, the cells were plated in a cell culture flask and, thereafter, dissociated and reseeded into the final assay format after further differentiation (see Figure 7). In method A and B, NPCs were differentiated for 23 days in flasks and then dissociated and reseeded in the assay format. After an additional differentiation period of 7 days cells were

treated with oligonucleotides. Alternatively, in methods C and D, NPCs were dissociated and directly plated onto 96 well plates. The differentiation medium in method A and C was supplemented with a mitotic inhibitors cocktail (Uridine 35 µg/ml and 15 µg/ml 5-Fuoro-2'-deoxyuridine) to inhibit cell proliferation. On day 35 cultures were fixed and stained for the neural progenitor cell and glia cell marker SOX2 and neuronal marker MAP2 (see Figure 8). The method B (with replating and without mitotic inhibitor) resulted in a more uniformly distributed cell culture with MAP2 positive cells and with fewer SOX2 positive cells than in the other methods. For this reason, the neuronal differentiation method B was chosen for subsequent described oligonucleotide treatments.

Example 5 - Dissociation and reseeding of differentiated human NCs

As described in Example 4, we established that replating differentiating cyno neurons was a necessary step to make these cells amenable for screening purposes. We therefore set out to verify whether it was possible to utilize a similar procedure for human IPSC-derived neurons. To this end, previously established neural progenitor cells (NPCs) from two IPSC lines (ED4 and SFC808) were differentiated following the procedure described in the Materials and Methods section. Briefly, expanding NPCs were dissociated and plated in SFA medium for a week. Afterwards the cells were exposed to differentiation medium (BGAA) in one of two ways: either they were plated directly in assay format or in a cell culture flask. In the latter case, the cells were differentiated for 21 days and then dissociated and reseeded in the assay format. Both experimental groups (i.e. directly differentiated or dissociated and reseeded) were differentiated for a total of 6 weeks (6 weeks in assay format or 3 weeks in flask and 3 weeks in assay format, respectively) before being processed.

For a first set of experiments, the neurons were analyzed by immunofluorescence and high content imaging. The markers used were specific for neural progenitors (SOX2), glial cells (GFAP) or neurons (MAP2 and HuC/D). As exemplified in Figure 9, the replating process did not seem to influence the differentiation or morphology of the human IPSC-derived neurons. This was further verified by the quantitation of the staining, which showed no significant difference in the presence of HuC/D positive cells in the direct differentiation and replated groups (Figure 10; for ED4, $p=0.542$, for SFC808, $p=0.579$).

To further confirm that it is possible to dissociate and replate differentiated human IPSC-derived neurons without altering their features, we wanted to confirm that cytoskeleton-associated proteins were not disrupted by the dissociation process. We analyzed the expression of the microtubule associated protein Tau as it is crucial for neuronal function and is directly implicated in neurodegenerative diseases such as Frontotemporal dementia and Alzheimer's disease. The cells were cultured as described above (with and without dissociation and reseeding), and the

protein extracts were analyzed by Western Blot. As shown in Figure 11, the constant expression of Tau and two of its phosphorylated forms proves that replating did, surprisingly, not disrupt the cytoskeletal characteristics of human IPSC-derived neurons, suggesting that physiological features will not be altered by the process.

These results demonstrate that human IPSC-derived neurons can undergo dissociation and reseeding during differentiation and maintain their key features, suggesting that the replating step (dissociation and reseeding) is feasible for the differentiation of primate stem cell derived neurons.

Example 6 - High-throughput screening using primate IPSC-derived dissociated and reseeded differentiated NCs

The primate IPSC-derived neuronal cultures described above are highly flexible and reproducible systems amenable to be used for screening purposes. We developed a workflow to treat the cells with oligonucleotides and verify the expression of target and associated genes by quantitative polymerase chain reaction (qPCR) (see Figure 12). To this end, either human or cynomolgus neural progenitors were treated as described above. For screening, the cells were dissociated after 3 weeks of differentiation and reseeded in 96 well plates at the density of 200'000 cells/cm^2. After one additional week of differentiation, the cells were treated with oligonucleotides for 5 days and then processed for expression analysis as described in the Materials and Methods section. This method allows constant availability of samples to be tested in a high-throughput manner.

For this example, we utilized 5 different oligonucleotides that target the Ube3A antisense (Ube3A_ATS) transcript at 8 concentrations, starting at 20 µM and decreasing by 3 fold at each step, resulting in doses of 20.000, 6.325, 2.000, 0.632, 0.200, 0.063, 0.020 and 0.006 µM. We tested these compounds on neurons differentiated from Angelman Syndrom patient IPSCs (human) or cynomologus IPSCs (cyno).

After the treatment, the cells were lysed, the RNA was extracted and the qPCR reaction was performed by using commercially available TaqMan assays for Ube3A and Ube3A_ATS; GAPDH was used in human cells and TBP in cyno cells as housekeeping gene to normalize the expression data. In all cases, the treatment resulted in a marked decrease of Ube3A_ATS, which was matched by increased expression of the sense transcript. This effect was evident in a dose-response manner; for simplicity of visualization, we report in Figure 13 the expression levels at a high (2µM) and a low (0.02 µM) dose.

At the dose 2 µM, the antisense transcript was expressed at ~50% of the level of vehicle (PBS) treated cells; this was true for both human and cyno cells. The knockdown of Ube3A_ATS resulted in the increased expression of Ube3A, to levels of ~250% and ~130% of vehicle for

human and cyno, respectively. This discrepancy is not surprising, as the patient derived neurons, because of the deletion of one allele of the Ube3A gene, express very low levels of the sense transcript.

These experiments demonstrate target engagement of oligonucleotides directed at Ube3A_ATS in human and cyno neurons. Therefore, the method described is suitable for high-throughput screening of therapeutic oligonucleotides in primate IPSC-derived neurons.

Claims

1. An *in vitro* method for producing standardized cell cultures of uniformly distributed differentiated neural cells (NCs) from different primate species, the method comprising the steps of
   a) providing neuronal precursor cells (NPCs);
   b) differentiating the NPCs to neural cells (NCs) comprising the steps of
      (i) dissociating the differentiated NCs from its support after about 20 days to about 45 days of differentiation; and
      (ii) reseeding the cells in a suitable cell culture format and continuing differentiation of the NCs for about 4 days to about 15 days.

2. The method according to claim 1, wherein the primate species are selected from the group consisting of human (Homo sapiens), Cynomolgus monkey (Macaca fascicularis) and Rhesus monkey (Macaca mulatta).

3. The method according to claim 1, wherein standardized cell cultures of differentiated NCs are individually produced for 2 primate species, wherein the first primate species is human (Homo sapiens) and the second primate species is Cynomolgus monkey (Macaca fascicularis).

4. The method according to any one of claims 1 to 3, wherein the NPCs are derived from induced pluripotent stem cells (IPSCs).

5. The method according to any one of claims 1 to 4, wherein step b)(i) comprises cultivating the cells in basal medium comprising Shh, FGF8 and ascorbic acid phosphate for a period of about 5 to about 10 days followed by cultivating the cells in basal medium comprising BDNF, GDNF, cAMP and ascorbic acid phosphate for a further period of about 15 days to about 35 days prior to dissociating the differentiated NCs from its support.

6. The method according to any one of claims 1 to 5, wherein step b)(i) comprises dissociating the cells with a cell detachment solution.

7. The method according to claim 6, wherein the cell detachment solution is Accutase.

8. The method according to any one of claims 1 to 7, wherein the cells are reseeded in 96 well or 384 well plates.

9. The method according to any one of claims 1 to 8, wherein step b)(ii) comprises continuing differentiation by cultivating the cells in basal medium supplemented with BDNF, GDNF, cAMP and ascorbic acid after reseeding the cells.

10. The method according to any one of claim 1 to 9, wherein the differentiated NCs are essentially uniformly distributed over the cell culture area as assessed by cell nucleus staining.

11. Use of the cell cultures obtained according to any one of claims 1 to 10 for *in vitro* testing of the toxicity of a drug candidate.

12. Use of the cell cultures obtained according to any one of claims 1 to 10 for *in vitro* testing of the efficacy of a drug candidate.

13. Use of the cell cultures obtained according to any one of claims 1 to 10 for selecting a drug candidate, in particular for selecting a drug candidate for further development.

14. The cell cultures for use according to any one of claims 11 to 13 wherein the drug candidate comprises a polynucleotide or targets a specific sequence of a polynucleotide.

15. The cell cultures for use according to any one of claims 11 to 13 wherein the drug candidate comprises at least one nucleic acid molecule such as a RNAi agent or an antisense oligonucleotide.

16. The methods and uses essentially as described herein.

Figure 1

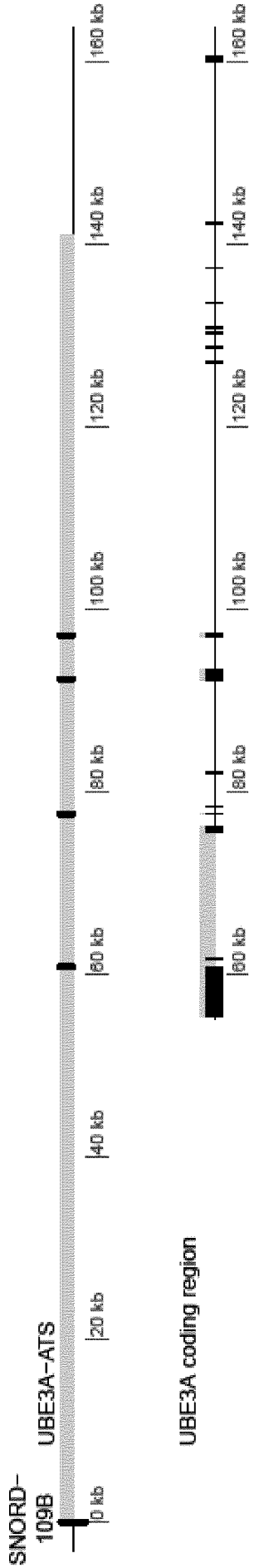
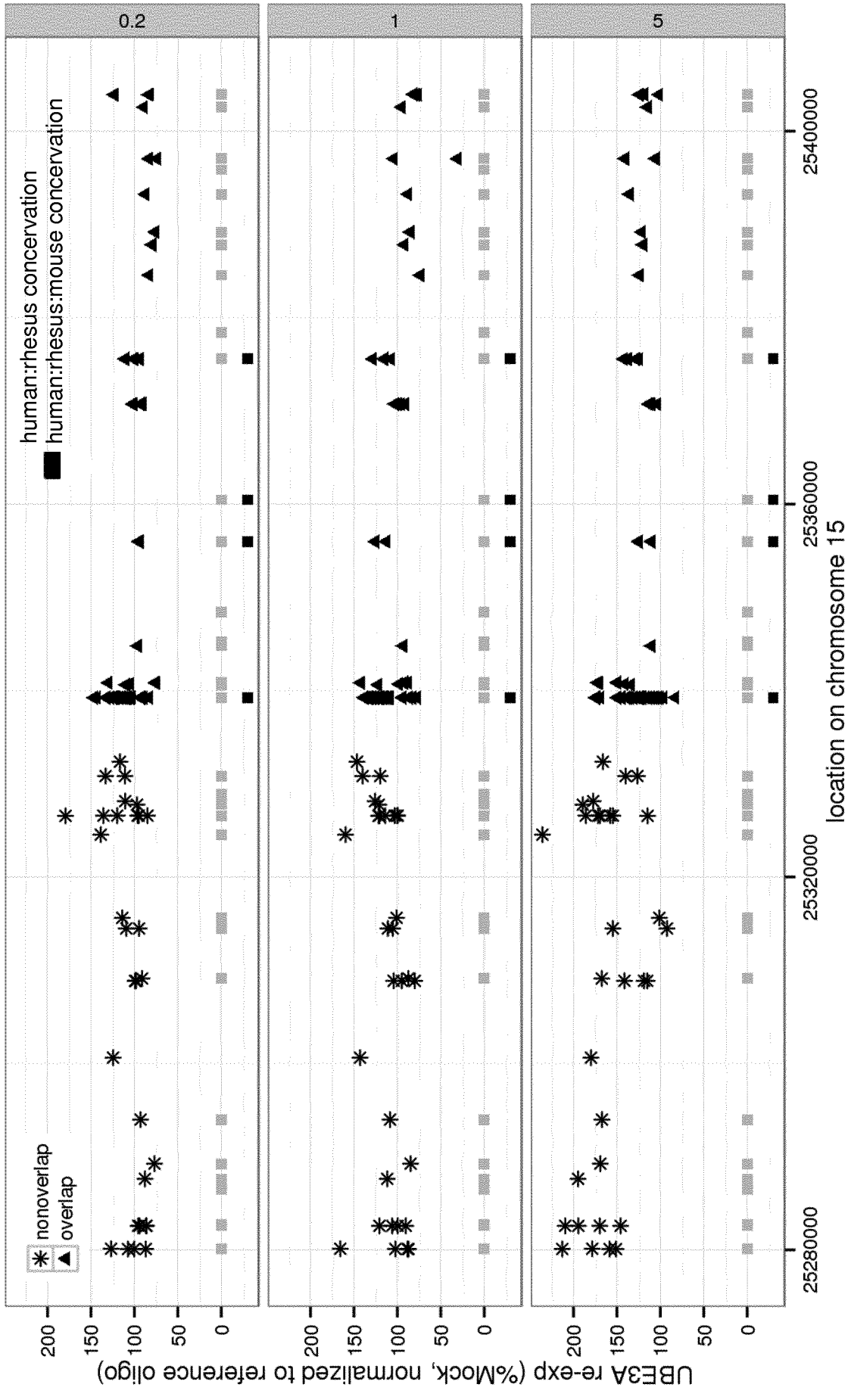

SNORD-109B
UBE3A-ATS
0 kb
20 kb
40 kb
60 kb
80 kb
100 kb
120 kb
140 kb
160 kb
UBE3A coding region
60 kb
80 kb
100 kb
120 kb
140 kb
160 kb

Figure 2


0.2
1
5
human:rhesus concervation
human:rhesus:mouse concervation
nonoverlap
overlap
UBE3A re-exp (%Mock, normalized to reference oligo)
0
50
100
150
200
25280000
25320000
25360000
25400000
location on chromosome 15

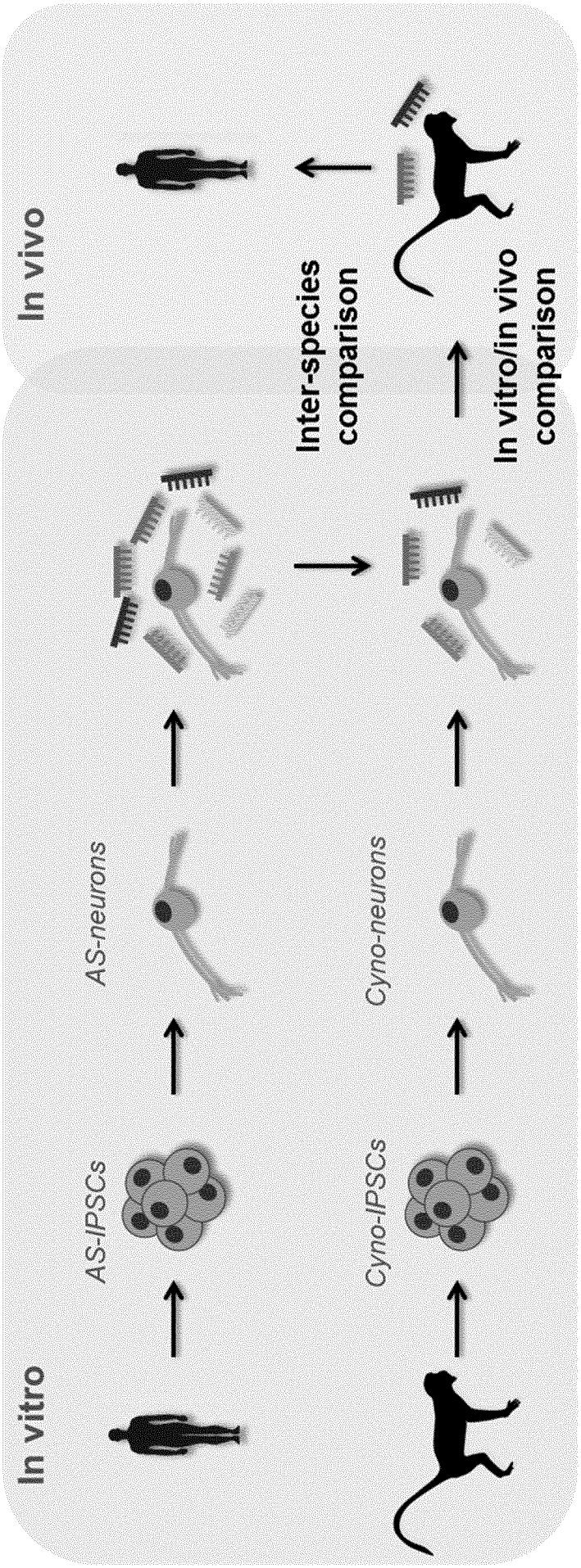

In vivo
Inter-species comparison
In vitro/in vivo comparison
In vitro
AS-neurons
AS-IPSCs
Cyno-neurons
Cyno-IPSCs

Figure 3

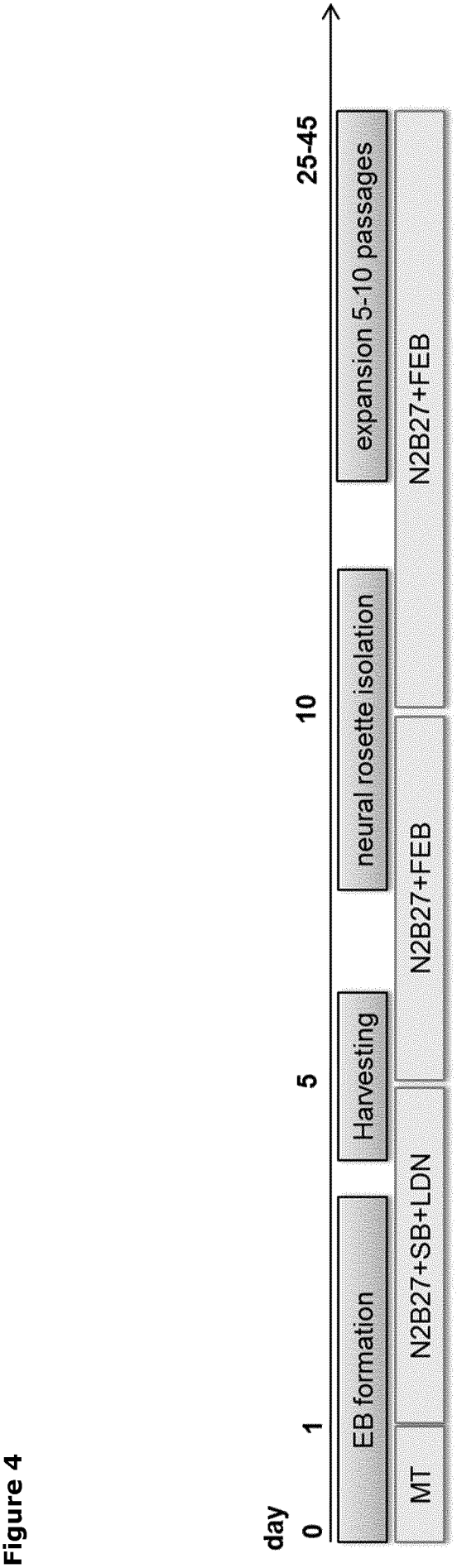

day
0
1
5
10
25-45
EB formation
Harvesting
neural rosette isolation
expansion 5-10 passages
MT
N2B27+SB+LDN
N2B27+FEB
N2B27+FEB

Figure 4

Figure 5B

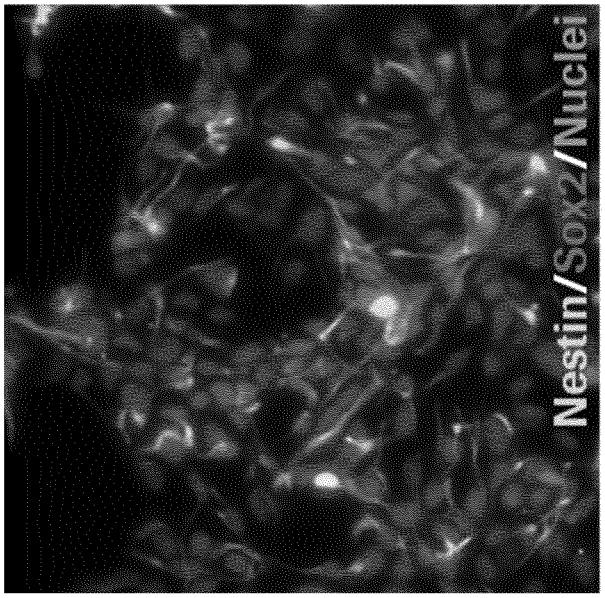


Figure 5A

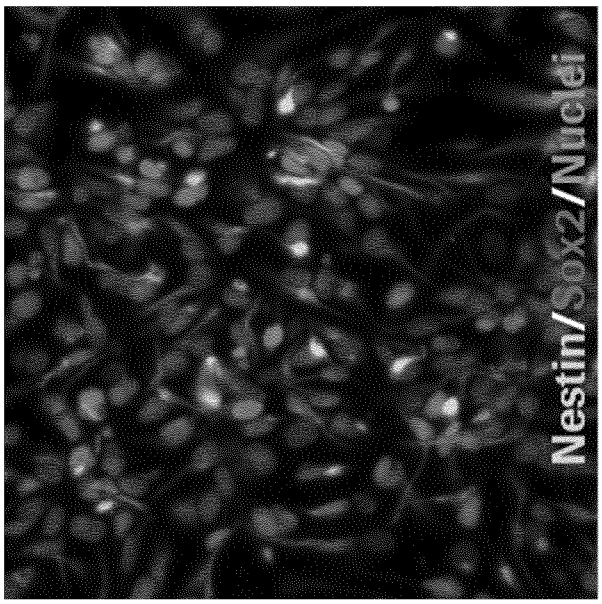


Figure 6A

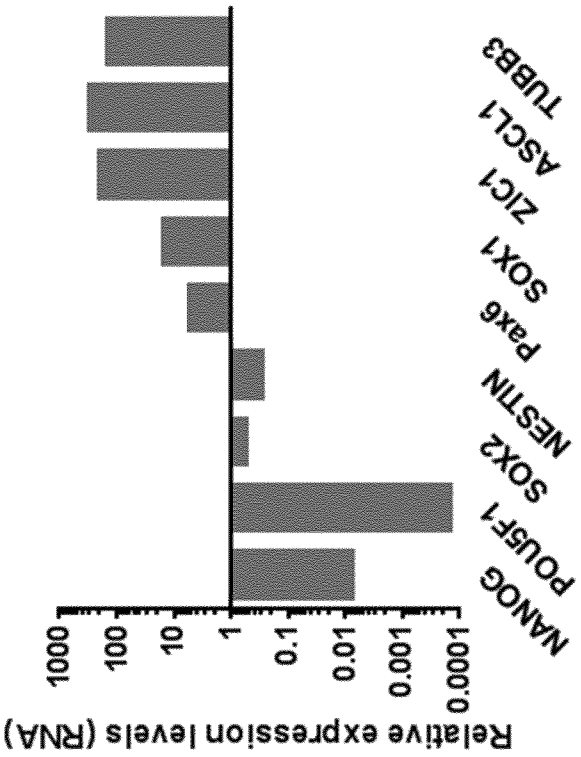


Figure 6B

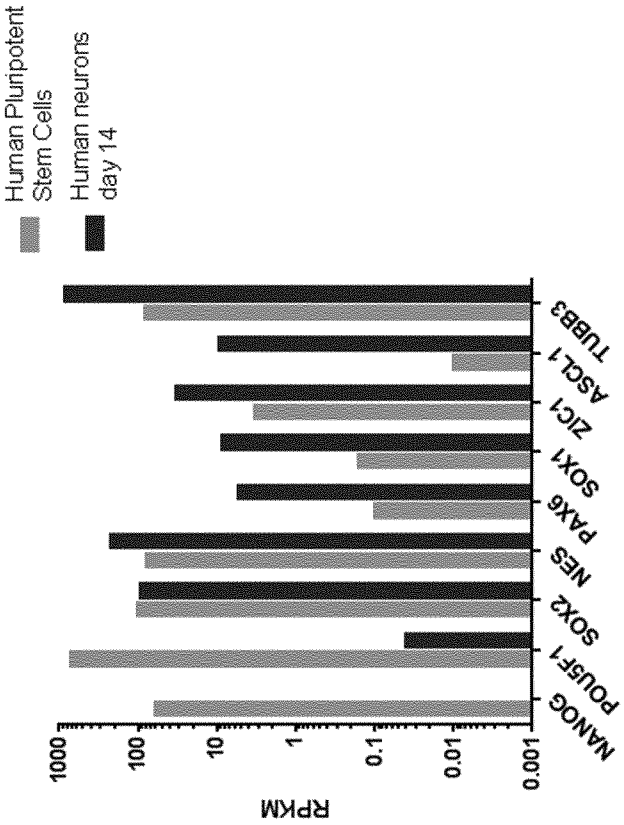


Figure 6C

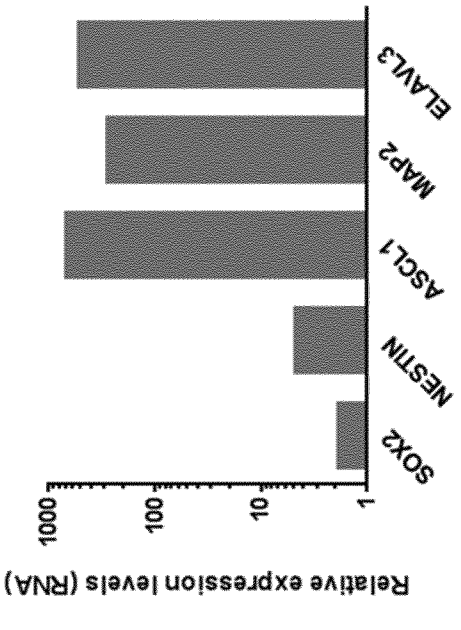


Figure 6D

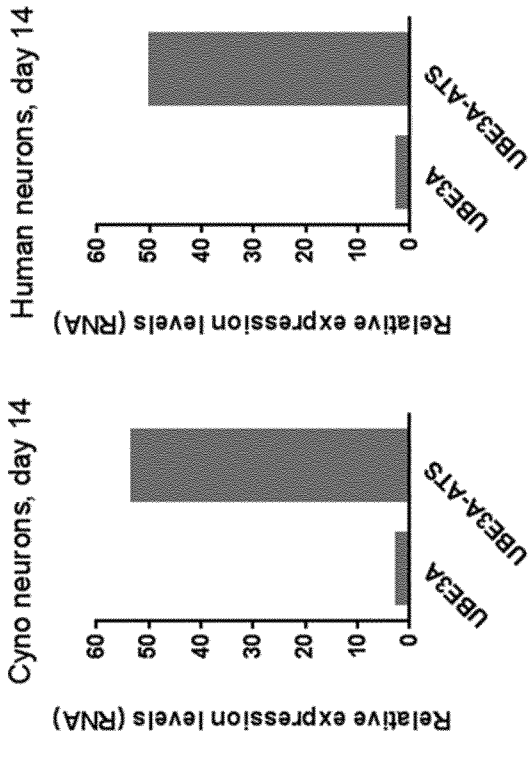
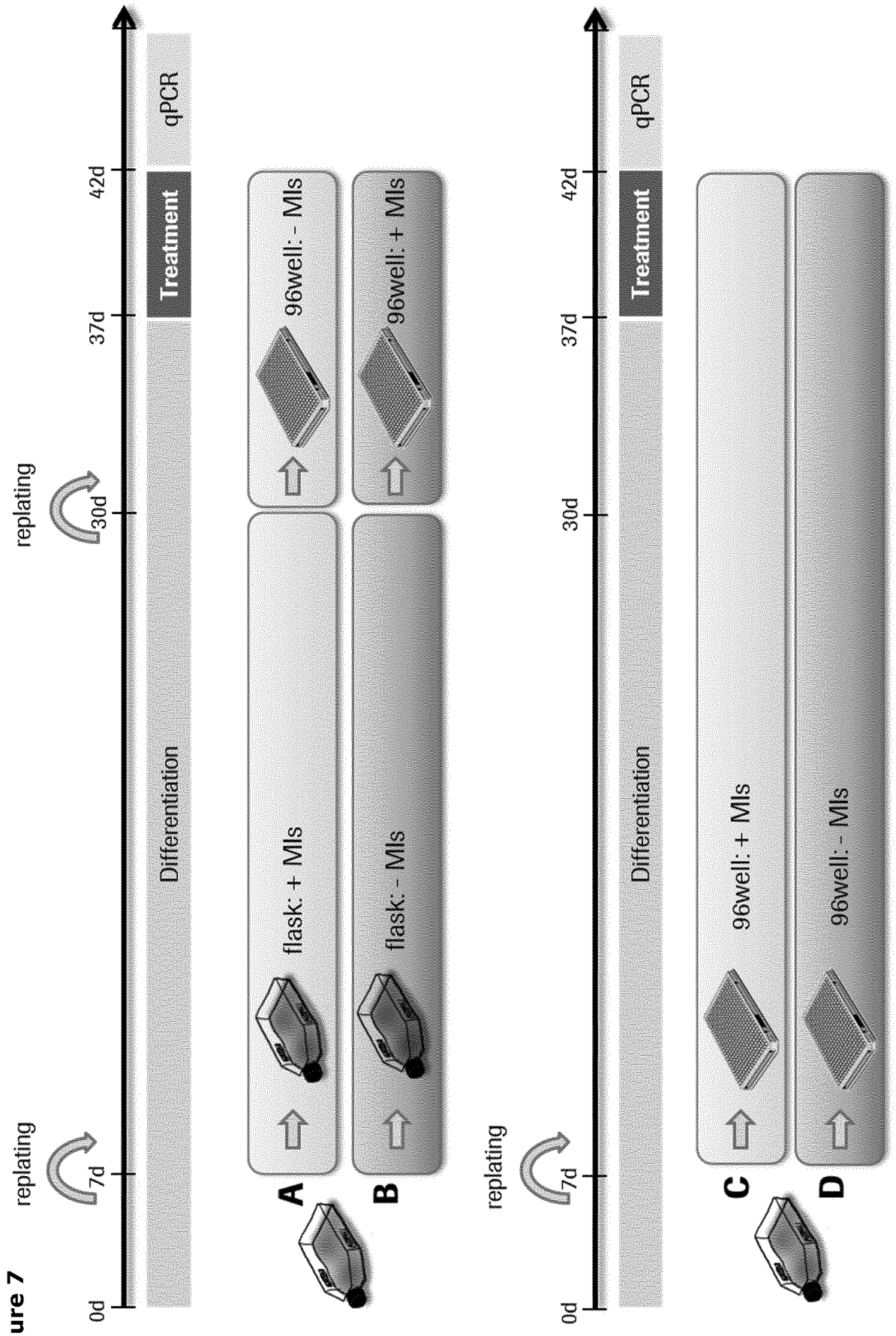


Figure 7


replating
7d
0d
replating
30d
37d
42d
Differentiation
Treatment
qPCR
A
flask: + MIs
96well: - MIs
B
flask: - MIs
96well: + MIs
C
96well: + MIs
D
96well: - MIs

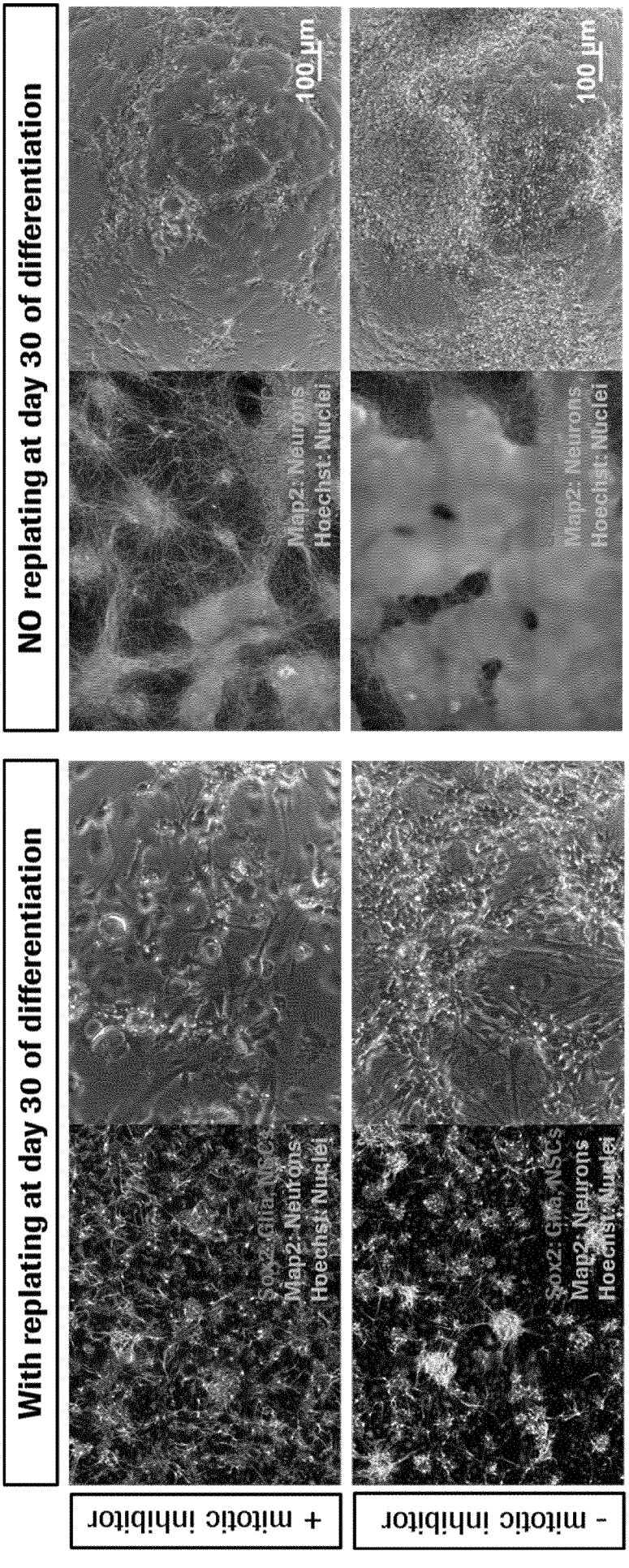

With replating at day 30 of differentiation
NO replating at day 30 of differentiation
+ mitotic inhibitor
- mitotic inhibitor
Sox2: Glia, NSCs
Map2: Neurons
Hoechst: Nuclei
100 µm

Figure 8

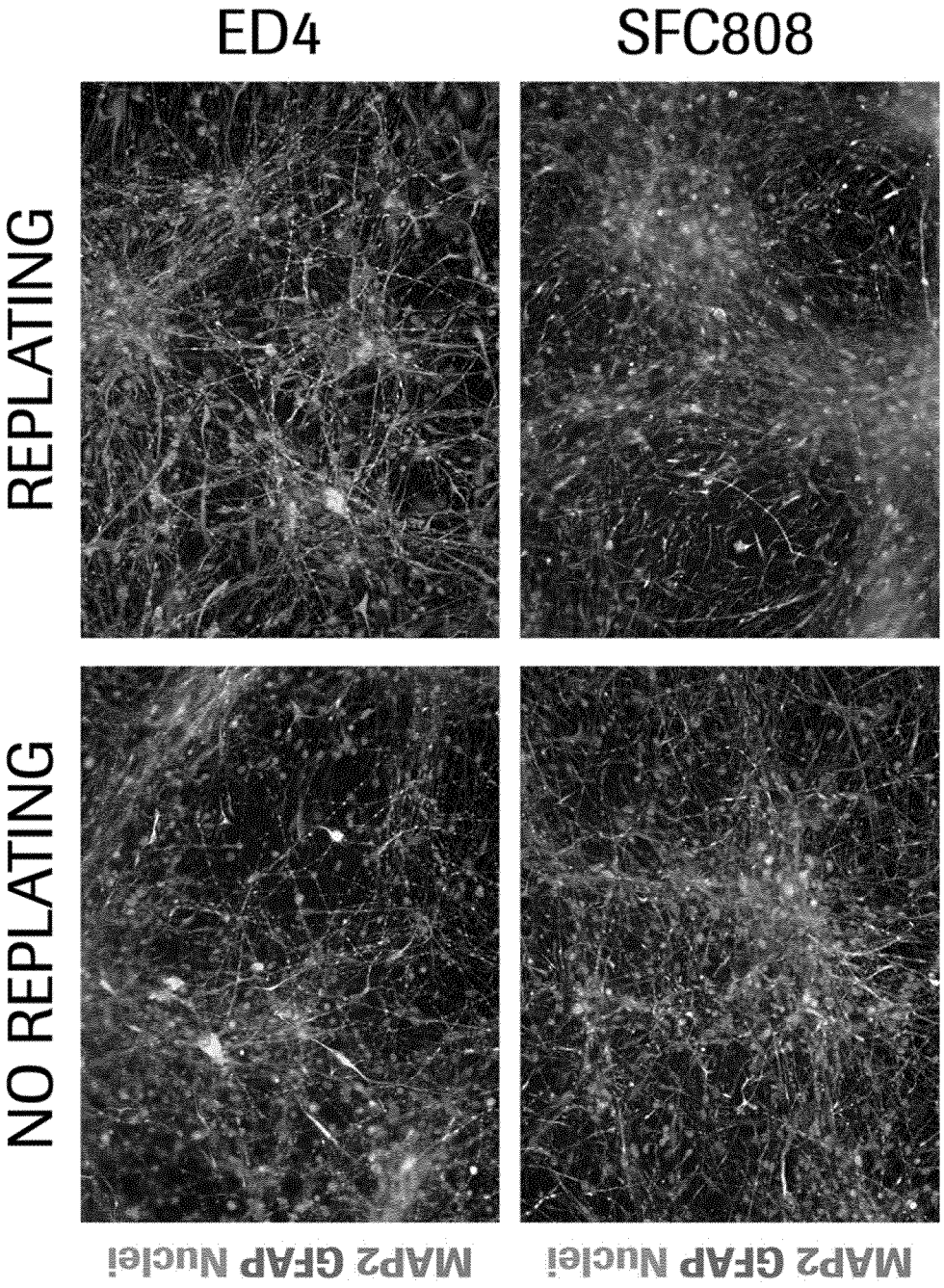

ED4
SFC808
REPLATING
NO REPLATING
MAP2 GFAP Nuclei
MAP2 GFAP Nuclei

Figure 9

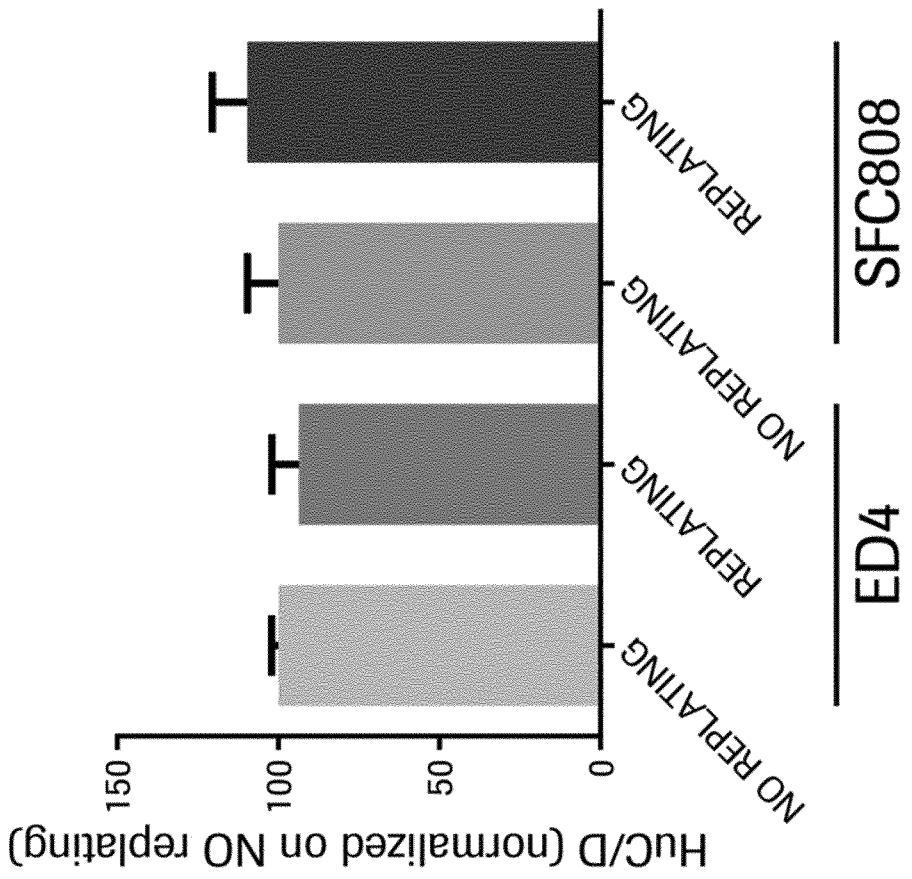

HuC/D (normalized on NO replating)
150
100
50
0
NO REPLATING
REPLATING
NO REPLATING
REPLATING
ED4
SFC808

Figure 10

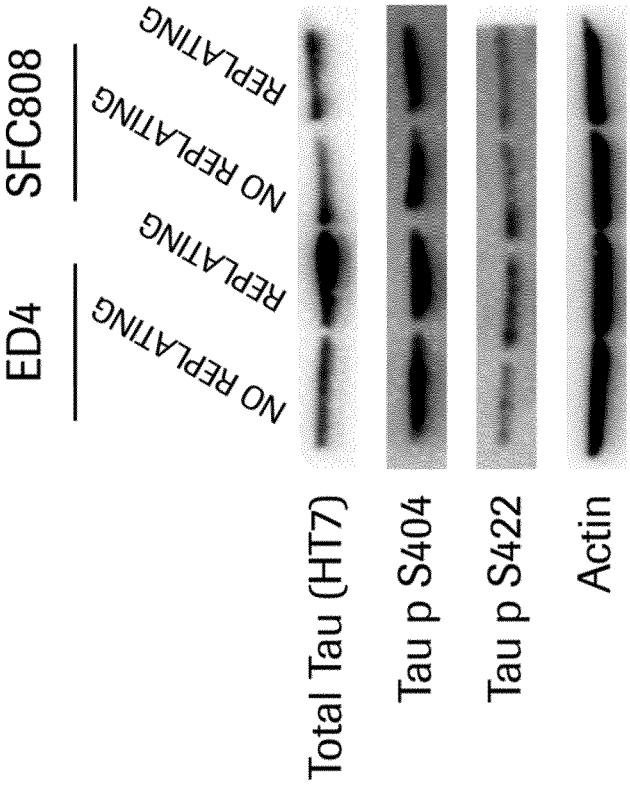
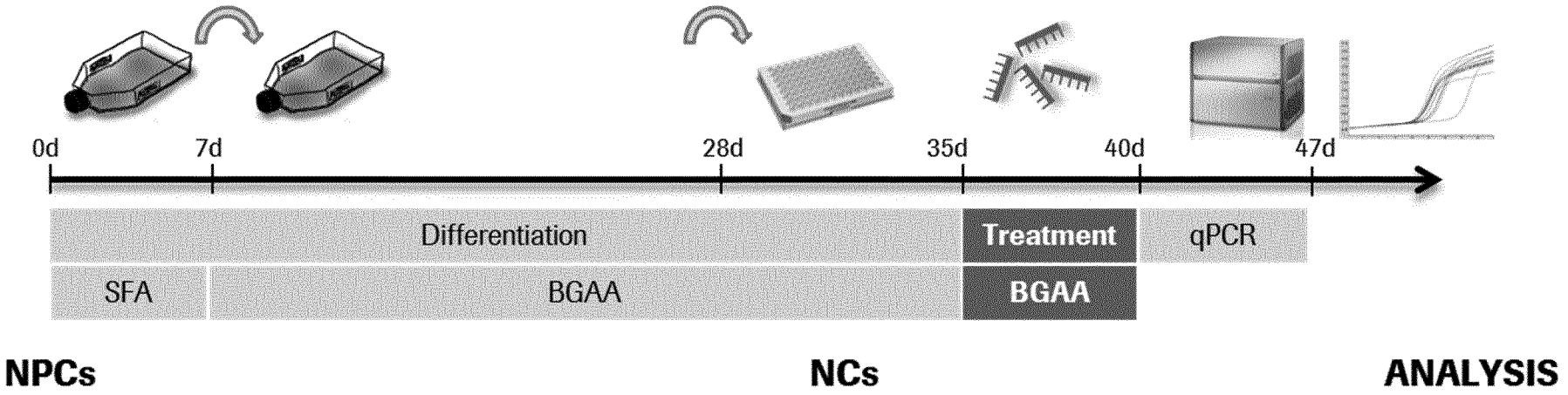

ED4
NO REPLATING
REPLATING
SFC808
NO REPLATING
REPLATING
Total Tau (HT7)
Tau p S404
Tau p S422
Actin

Figure 11

Figure 12


0d
7d
28d
35d
40d
47d
Differentiation
Treatment
qPCR
SFA
BGAA
BGAA
NPCs
NCs
ANALYSIS

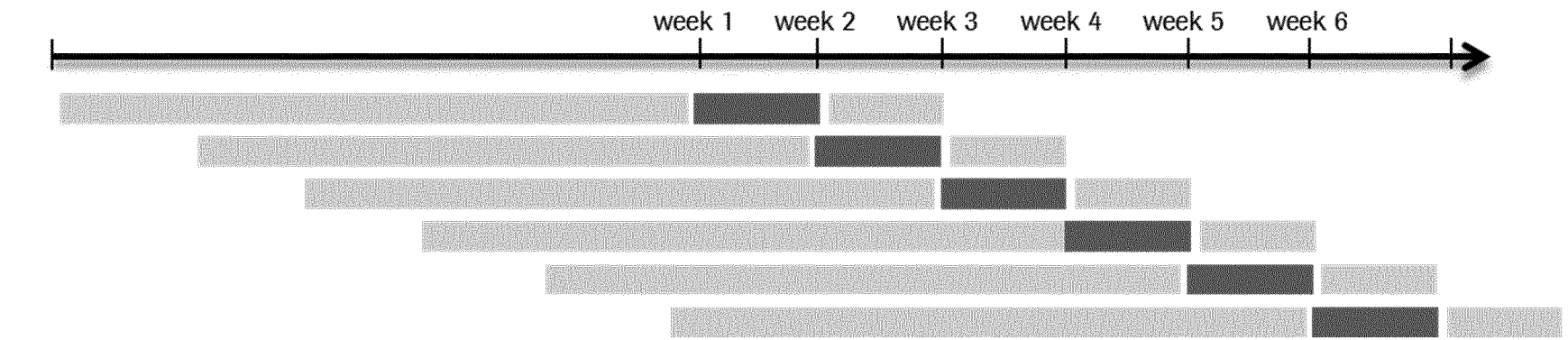

Throughput of the screening platform
week 1
week 2
week 3
week 4
week 5
week 6

Figure 13A

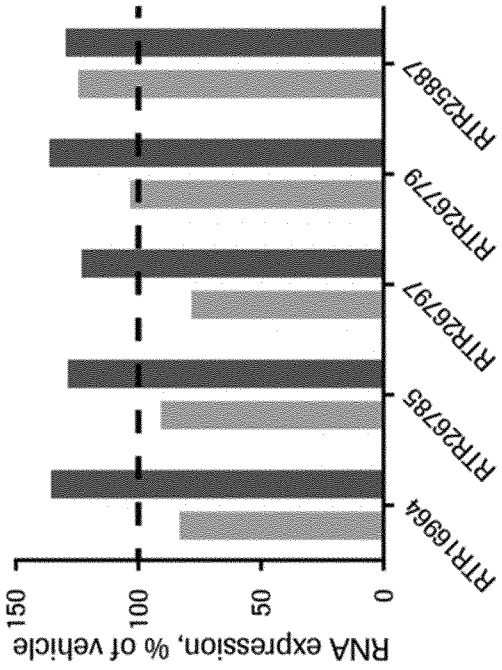


Figure 13B

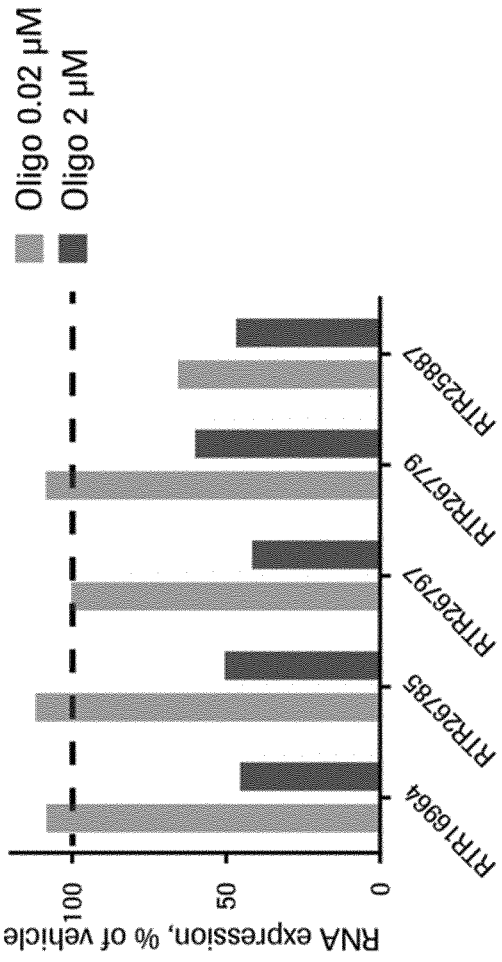


Figure 13C

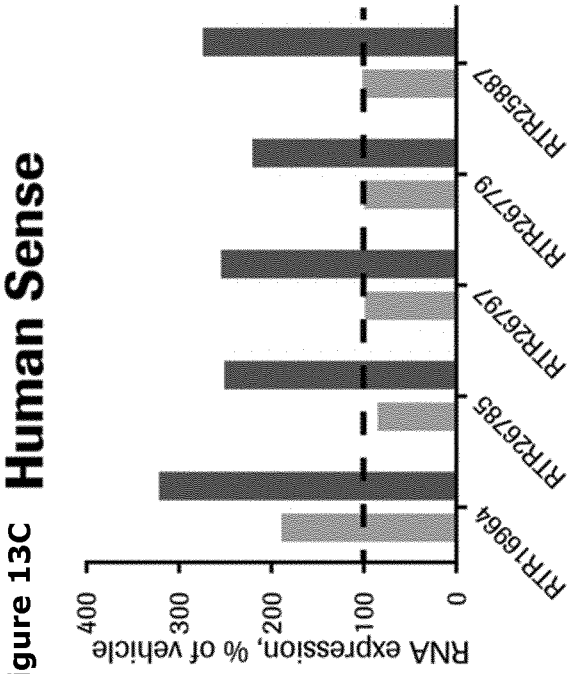
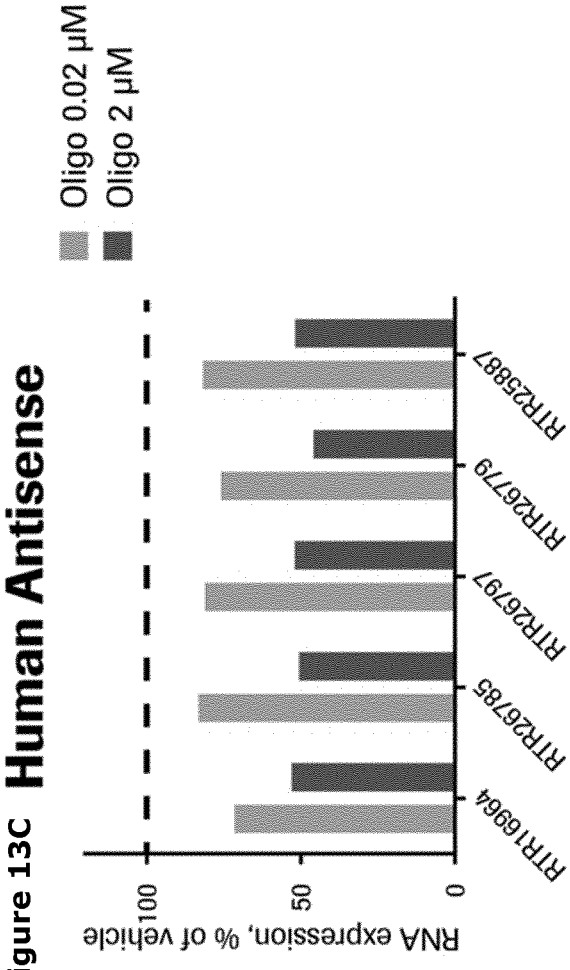


Figure 13C



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/077429

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/0793 C12N5/0797 G01N33/50
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 G01N

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.
X	Efthymiou A: "Functional screening assays with neurons generated from pluripotent stem cell-derived neural stem cells", Journal of Biomolecular Screening Society for Laboratory Automation and Screening, 9 September 2013 (2013-09-09), pages 32-43, XP055340253, Retrieved from the Internet: URL:http://journals.sagepub.com/doi/pdf/10.1177/1087057113501869 [retrieved on 2017-01-30] the whole document ----- -/--	1-4,6,7, 10-16

[X] Further documents are listed in the continuation of Box C. [ ] 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" 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
8 February 2017	16/02/2017

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	Manu, Dominique

1

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.
X	LEONARDO D'AIUTO ET AL: "Large-scale generation of human iPSC-derived neural stem cells/early neural progenitor cells and their neuronal differentiation", ORGANOGENESIS, vol. 10, no. 4, 2 October 2014 (2014-10-02), pages 365-377, XP055242556, US ISSN: 1547-6278, DOI: 10.1080/15476278.2015.1011921 the whole document	11-16
X	TOM DUNKLEY ET AL: "Characterization of a human pluripotent stem cell-derived model of neuronal development using multiplexed targeted proteomics", PROTEOMICS - CLINICAL APPLICATIONS, vol. 9, no. 7-8, 26 May 2015 (2015-05-26), pages 684-694, XP055343143, DE ISSN: 1862-8346, DOI: 10.1002/prca.201400150 cited in the application the whole document	1-7,9,10
X,P	JUN CUI ET AL: "Quantification of dopaminergic neuron differentiation and neurotoxicity via a genetic reporter", SCIENTIFIC REPORTS, vol. 6, 28 April 2016 (2016-04-28), page 25181, XP055343024, DOI: 10.1038/srep25181 the whole document	1-16

1

(19)中华人民共和国国家知识产权局

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代理人 张莉 黄革生

(51)Int.Cl.
C12N 5/0793(2006.01)
C12N 5/0797(2006.01)
G01N 33/50(2006.01)

权利要求书1页 说明书50页
序列表154页 附图15页

(54)发明名称

来自灵长类物种的标准化神经元细胞测定试验

(57)摘要

本申请涉及一种从灵长类物种产生标准化且稳健的神经元细胞培养物测定试验的方法，例如适用于药物候选物的高通量筛选的试验。该方法包括将人和/或非人灵长类神经元前体细胞(NPC)分化成神经元细胞(NC)，基于在分化的NC上进行的解离和再接种步骤产生均一的NC培养物，导致适用于高通量药物筛选测定试验，特别是筛选反义寡核苷酸的稳健培养物。

CN 108350432 A

1.用于自不同灵长类物种产生均匀分布的分化神经细胞(NC)的标准化细胞培养物的体外方法,所述方法包括以下步骤:

a)提供神经元前体细胞(NPC);

b)将NPC分化为神经细胞(NC),包括以下步骤

(i)在分化的约20天至约45天后,将分化的NC从其支持物解离;和

(ii)以合适的细胞培养形式再接种细胞并继续分化NC约4天至约15天。

2.根据权利要求1所述的方法,其中所述灵长类物种选自由人(Homo sapiens),食蟹猴(Macaca fascicularis)和恒河猴(Macaca mulatta)组成的组。

3.根据权利要求1所述的方法,其中分化NC的标准化细胞培养物针对2种灵长类物种分别单独产生,其中所述第一灵长类物种是人(Homo sapiens),并且所述第二灵长类物种是食蟹猴(Macaca fascicularis)。

4.根据权利要求1至3中任一项所述的方法,其中所述NPC源自诱导的多能干细胞(IPSCs)。

5.根据权利要求1至4中任一项所述的方法,其中步骤b)(i)包括在含有Shh,FGF8和抗坏血酸磷酸盐的基础培养基中培养细胞约5至约10天的时间,随后在包含BDNF,GDNF,cAMP和抗坏血酸磷酸盐的基础培养基中再培养细胞约15天至约35天的时间,然后将分化的NC从其支持物解离。

6.根据权利要求1至5中任一项所述的方法,其中步骤b)(i)包括用细胞分离溶液解离所述细胞。

7.根据权利要求6所述的方法,其中所述细胞分离溶液是Accutase。

8.根据权利要求1至7中任一项所述的方法,其中所述细胞在96孔或384孔板中再接种。

9.根据权利要求1至8中任一项所述的方法,其中步骤b)(ii)包括在再接种细胞后通过在补充了BDNF,GDNF,cAMP和抗坏血酸的基础培养基中培养细胞来继续分化。

10.根据权利要求1至9中任一项所述的方法,其中通过细胞核染色评估,所述分化的NC基本上均匀分布在细胞培养区域上。

11.根据权利要求1至10中任一项获得的细胞培养物用于体外测试药物候选物的毒性的用途。

12.根据权利要求1至10中任一项获得的细胞培养物用于体外测试药物候选物功效的用途。

13.根据权利要求1至10中任一项获得的细胞培养物用于选择药物候选物,特别是选择用于进一步开发的药物候选物的用途。

14.根据权利要求11至13中任一项所述的细胞培养物的用途,其中所述药物候选物包含多核苷酸或靶向多核苷酸的特定序列。

15.根据权利要求11至13中任一项所述的细胞培养物的用途,其中所述药物候选物包含至少一个核酸分子,例如RNAi剂或反义寡核苷酸。

16.基本上如本文所述的方法和用途。

来自灵长类物种的标准化神经元细胞测定试验

技术领域

[0001] 本申请涉及一种从灵长类物种产生标准化且稳健的神经元细胞培养物测定试验的方法，例如适用于高通量筛选药物候选物的测定试验。该方法包括，将人和/或非人灵长类动物神经元前体细胞(NPC)分化成神经元细胞(NC)，和基于在分化的NC上进行的解离(dissociate)和再接种(reseed)步骤，产生均一的NC培养物，从而获得适用于高通量药物筛选测定，特别是筛选反义寡核苷酸的稳健培养物。

背景技术

[0002] 基于显示各种疾病(包括中枢神经系统(CNS)疾病)的病理生理学的各种细胞类型或细胞培养物模型的高通量筛选试验，广泛用于早期评估药物候选物的功效和毒性特征。在过去的十年中，通过建立从体细胞产生多能细胞的方案，克服了由有限的细胞类型或细胞的胚胎干细胞来源所造成的局限性。自从Yamanaka及其同事(Takahashi，K。&Yamanaka，S.Cell.2006;126:663-676)证明了体细胞可重编程为诱导的多能干细胞(IPSCs)，已经可以从多种细胞来源产生多能细胞。已经将不同类型的体细胞，包括成纤维细胞，角质形成细胞，脂肪细胞和血细胞，重编程为IPSC多能状态。最近，特定的体细胞类型可以被转分化成完全不同的体细胞类型，例如神经元。Vierbuchen及其同事证明，通过转导三种关键基因Mash1，Brn2和Myt11，小鼠成纤维细胞可以直接转化为功能性神经元(Vierbuchen等，Nature。2010;463:1035-41)。值得注意的是，US2010/0021437公开了从成纤维细胞产生诱导的多能干细胞并诱导这些细胞分化成NPC的方法。最近已经描述了分化的体细胞向NPC的直接转化(WO2012/022725)。这些神经元细胞被认为是模建各种CNS疾病的病理生理学的有价值的工具。

[0003] NPC是多能干细胞并在特定条件下繁殖。它们可以生长为单层贴壁培养物，也可以在非贴壁细胞培养板中生长为漂浮神经球(neurosphere)。两种类型的NPC培养物(神经球，贴壁培养物)似乎是完全可以相互转化的。NPC可以无限生长，并仍然保持真正的多能性。在特定条件下，它们可以分化成组成成年大脑的神经元细胞类型，包括分化的NC。分化的NC培养物是筛选有效和安全药物的有价值的疾病模型。事实上，NC培养物在药物开发中对评估药物候选物的毒性和功效非常重要。

[0004] 在首次给予人类患者之前，需要在体外细胞培养系统中、然后在啮齿动物和非人灵长类(NHP)物种的体内测试中彻底地评估药物候选物。目前，新药药物候选物的毒性和功效评估可以针对不同的物种采用不同的方案以不同试验形式进行。虽然药物候选物的体外测试可以减少为药物测试而处死的实验动物的数量，但这也对结果从体外到体内和人体生理学的转化提出了挑战。事实上，由于物种之间的非密切遗传关系，药物候选物的功效和毒性特征从啮齿动物物种到人类的转化可能是具有挑战性的。值得注意的是，小鼠和人类基因组的蛋白质编码区约85％相同。遗传组成的差异可能造成对药物的不同生理反应、以及对毒素或诱变剂的不同耐受性。目前，对于靶向特定DNA或RNA序列(例如，反义寡核苷酸)或基因或基因产物的遗传变体的新药类型，物种间的可转化性变得甚至更加重要。因此，鉴于

遗传关系密切(>90%),NHP物种代表了最有意义的模型系统,用于产生功效和毒性数据以预测人类的反应率和不良事件发生率。不受理论束缚,认为在NHP物种(例如,食蟹猴)中的体内测试,在所有药物类别的首次人体试验之前均是优选的,而且对于靶向确定的人类遗传组成的药物类别尤其如此。另一方面,在药物开发中使用NHP物种仍然是一个有争议的问题。NHP物种在遗传上与人类接近的这一事实引发伦理问题。因此,NHP实验动物例如食蟹猴的数量,应尽可能减少。

[0005] 因此,对新药候选物进行功效和毒性筛选的全面布局应该包括体外和体内NHP物种测试、以及使用IPSC来源的人类细胞的平行体外测试。测试顺序应包括,在NHP物种(例如食蟹猴)的体内试验之前,在NHP物种和人的细胞上的体外平行测试。具有较差功效和/或毒性性质的药物候选物应当,在对NHP物种开始体内测试之前,在NHP和/或人类细胞上进行体外评估时在早期阶段就予以排除。事实上,这个顺序可以确保NHP实验室动物的数量可以尽可能低。

[0006] 然而,从干细胞来源的分化NC获得在灵长类物种之间可转化的功效和毒性体外数据,存在许多大的障碍:首先是物种特异性的细胞培养方案和细胞培养条件的不可转化性;其次是分化中的NPCs的不均一分布,导致非最佳的存活条件或受阻的分化效应(由于局部细胞浓度和自分泌和旁分泌信号所致),由此在药物效应的表型评估上造成困难。当培养物必须长期分化以获得所需的细胞分化状态时,这变得最为明显。最值得注意的是,分化的灵长类NC对细胞培养条件天生敏感,不能耐受苛刻的处理,这是用这些细胞产生标准化测定试验的固有障碍。

[0007] 因此,仍然需要一种易于获得且可重现的技术用于从不同灵长类物种(包括人类)产生一致的NC测定试验。

发明内容

[0008] 本文提供了用于自不同灵长类物种产生均匀分布的分化神经元细胞(NC)的标准化细胞培养物的体外方法,所述方法包括以下步骤:

[0009] a)提供神经元前体细胞(NPC);

[0010] b)将NPC区分为NC,包括以下步骤:

[0011] (i)在分化的约20天至约45天后,将分化的NC从其支持物上解离;和

[0012] (ii)以合适的细胞培养形式再接种细胞并继续分化NC约4天至约15天。

[0013] 在一个实施方案中,灵长类物种选自人(Homo sapiens),食蟹猴(Macaca fascicularis)和恒河猴(Macaca mulatta)。

[0014] 在一个实施方案中,灵长类物种选自人(Homo sapiens)和食蟹猴(Macaca fascicularis)。

[0015] 在一个实施方案中,针对2种灵长类物种分开单独产生分化NC的标准化细胞培养物,其中第一灵长类物种是人(Homo sapiens),第二灵长类物种是食蟹猴(Macaca fascicularis)。

[0016] 在一个实施方案中,NPC来源于诱导的多能干细胞(IPSCs)。

[0017] 在一个实施方案中,步骤b)包括用选自Shh,FGF8,BDNF,GDNF,cAMP和抗坏血酸磷酸盐的一种或多种分化剂培养NPCs。

[0018] 在一个实施方案中，步骤b)(i)包括在含有Shh，FGF8和抗坏血酸磷酸盐的基础培养基中培养细胞约5至约10天的时间，接着在含有BDNF，GDNF，cAMP和抗坏血酸磷酸盐的基础培养基中再培养细胞约15天至约35天，然后将分化的NC从其支持物上解离。

[0019] 在一个实施方案中，一种或多种分化剂在基础培养基中的浓度，对于Shh是50-500ng/ml，对于FGF8是25-250ng/ml，对于BDNF是1-50ng/ml，对于BDNF是1-50ng/ml，对于cAMP是0.1-10mM，对于抗坏血酸磷酸盐是20-200μM。

[0020] 在一个实施方案中，一种或多种分化剂在基础培养基中的浓度，对于Shh是200ng/ml，对于FGF8是100ng/ml，对于BDNF是20ng/ml，对于GDNF是10ng/ml，对于cAMP是0.5mM，对于抗坏血酸磷酸盐是100μM。

[0021] 在一个实施方案中，用Shh，FGF8和抗坏血酸磷酸盐的培养期约为7天，用BDNF，GDNF，cAMP和抗坏血酸磷酸盐的培养期为约20至约40天。

[0022] 在一个实施方式中，NPC在Laminin521支持物上提供。

[0023] 在一个实施方案中，步骤b)(i)包括用细胞分离溶液(cell detachment solution)解离细胞。

[0024] 在一个实施方案中，细胞分离溶液是Accutase。

[0025] 在一个实施方案中，步骤b)(ii)包括在Laminin521支持物上以200000个细胞/cm^2重接种细胞。

[0026] 在一个实施方案中，将细胞重接种在96孔或384孔板中。

[0027] 在一个实施方案中，步骤b)(ii)包括在ROCK抑制剂存在下再接种细胞。

[0028] 在一个实施方案中，ROCK抑制剂是Y-27632。

[0029] 在一个实施方案中，步骤b)(ii)包括在再接种细胞后通过在补充了BDNF，GDNF，cAMP和抗坏血酸的基础培养基中培养细胞来继续分化。

[0030] 在一个实施方案中，分化的NC表达MAP2。

[0031] 在一个实施方案中，分化的NC包含MAP2阳性神经突。

[0032] 在一个实施方案中，NPC来自患有神经元病症的个体。

[0033] 在一个实施例中，NPC来自健康的个体。

[0034] 在一个实施方案中，细胞培养物针对不同物种依次产生。

[0035] 在一个实施方案中，如通过细胞核染色所评估的，细胞基本均匀地分布在细胞培养区域上。

[0036] 在一个实施方案中，通过DNA染色，特别是Hoechst染色来评估细胞的分布。

[0037] 在一个实施方案中，提供了通过如本文所述的方法获得的细胞培养系统。

[0038] 在一个实施方案中，提供了根据本文所述的方法获得的细胞培养物用于体外测试药物候选物的毒性的用途。

[0039] 在一个实施方案中，提供了根据本文所述的方法获得的细胞培养物用于体外测试药物候选物的功效的用途。

[0040] 在一个实施方案中，提供了根据本文所述的方法获得的细胞培养物用于体外测试药物候选物的功效的用途，其中在源自患有神经元病症的个体的细胞培养物中以及在源自健康个体的细胞培养物中测试功效。

[0041] 在一个实施方案中，提供了根据本文所述方法获得的细胞培养物用于选择药物候

选物,特别是选择用于进一步开发的药物候选物的用途。

[0042] 在一个实施方案中,提供了细胞培养物用于如本文所述的用途,其中药物候选物包含多核苷酸或靶向多核苷酸的特定序列。

[0043] 在一个实施方案中,提供了如本文所述使用的细胞培养物,其中药物候选物包含至少一个核酸分子,例如RNAi剂或反义寡核苷酸。

[0044] 在一个实施方案中,提供细胞培养物用于如本文所述的用途,其中所述核酸分子包含一个或多个2'糖修饰的核苷,其独立地选自2'-O-烷基-RNA,2'-O-甲基-RNA,2'-O-烷氧基-RNA,2'-O-甲氧基乙基-RNA,2'-氨基-DNA,2'-氟-DNA,阿拉伯糖核酸(arabino nucleic acid,ANA),2'-氟-ANA和LNA核苷。

[0045] 在一个实施方案中,提供了细胞培养物用于如本文所述的用途,其中所述一个或多个2'糖修饰的核苷是LNA核苷。

[0046] 在一个实施方案中,提供了细胞培养物用于如本文所述的用途,其中LNA核苷选自β-D-氧基-LNA,α-L-氧基-LNA,β-D-氨基-LNA,α-氨基-LNA,β-D-硫代-LNA,α-L-硫代-LNA,(S)cET,(R)cET,β-D-ENA或α-L-ENA。

[0047] 在一个实施方案中,提供了细胞培养物用于如本文所述的用途,其中所述核酸分子包含至少一个修饰的核苷间连接。

[0048] 在一个实施方案中,提供了细胞培养物用于如本文所述的用途,其中连续核苷酸序列内的核苷间连接是硫代磷酸酯核苷间连接。

[0049] 在一个实施方案中,提供了细胞培养物用于如本文所述的用途,其中反义寡核苷酸能够募集RNase H.

[0050] 在一个实施方案中,提供了细胞培养物用于如本文所述的用途,其中反义寡核苷酸是gapmer。

[0051] 在一个实施方案中,提供细胞培养物用于如本文所述的用途,其中所述寡核苷酸是式5'-FG-F'-3'的gapmer,其中区F和F'独立地包含1至7个修饰的核苷且G为能够募集RNA酶H的6至16个核苷的区域。

[0052] 在一个实施方案中,提供了基本上如本文所述的方法和用途。

[0053] 附图简述

[0054] 图1:上链举例说明SNORD109B下游的SNHG14转录物的区域(UBE3A-ATS),其中黑框表示测试的小鼠寡核苷酸的位置。下链举例说明UBE3A编码区,其中黑框表示外显子。外显子1位于160kb左右。寡核苷酸被置于外显子9(位于~97kb),外显子10(位于~92kb),外显子13(位于~77kb)和外显子16的5'端(位于~60kb)的反义区中。

[0055] 图2:表示实施例2中测试的寡核苷酸在人神经元细胞培养物中诱导UBE3A重新表达的能力。与SNORT109B和UBE3A编码区上游区域之间(SEQ ID NO:1的位置1至55318)的人SNHG14长非编码RNA区域互补的寡核苷酸用•非重叠表示。与反义于UBE3A pre-mRNA(SEQ ID NO:1的位置55319至141053)的人SNHG14长非编码RNA区域互补的寡核苷酸用▲重叠表示。来自表3的具有对人和恒河猴的保守性的寡核苷酸以▨显示在每个小图的底部。人:恒河猴:鼠之间的保守性标示为■ 。寡核苷酸浓度为0.2,1和5μM,在每个小图的右手侧标出。

[0056] 图3:筛选策略的示意图,在人体上评估药物功效之前,在灵长类动物体外细胞培

养物模型上、然后在灵长类物种体内研究中评估药物候选物的功效。将食蟹猴和人IPSC来源的神经元用作体外模型以评估靶向UBE3A反义物的寡核苷酸的靶结合和功效。在体内研究之前，按优先次序排序具有良好功效特征的候选物。

[0057] 图4：以食蟹猴IPSCs来源的神经祖细胞执行的不同步骤的概览。双重SMAD抑制方案的修改形式用于灵长类动物IPSC系的神经化。MT是指MT培养基；N2B27+SB+LDN是指补充有SB-431542和LDN-193189的N2B27培养基；N2B27+FEB是指补充有FGF，EGF和BDNF的N2B27培养基。

[0058] 图5：Cyno和人神经前体显示相似的Sox2和Nestin染色阳性，并且各自的分化的神经元染色呈Map2阳性。

[0059] 图6：应用的定向分化方法诱导可比的跨物种神经元分化。图6A)Cyno IPSC在补充有BDNF，GDNF，cAMP和抗坏血酸磷酸盐(BGAA)的基础培养基中分化14天。自cyno IPSC和第14天的NC分离RNA，并且通过qPCR分析所示的标志物的表达。将数据相对于持家基因GAPDH进行标化，并且相对于cyno IPSC中的表达水平呈现。图6B)从人PSC和在补充有BGAA的基础培养基中分化14天的人NC中分离RNA，并且通过RNA测序分析所示的标志物的表达。数据以RPKM值表示。图6C)从人PSC和在补充有BGAA的基础培养基中分化14天的人NC中分离RNA，并且通过qPCR分析所示的标志物的表达。将数据相对于持家基因GAPDH标化，并且相对于人PSC中的表达水平呈现。图6D)从在补充有BGAA的基础培养基中分化14天的cyno(左图)和人(右图)NC中分离RNA，通过qPCR分析了UBE3A和UBE3A-ATS转录物的表达。左图：数据相对于持家基因TBP标化并且相对于cyno IPSC呈现。右图：数据相对于持家基因GAPDH标化，并且相对于人PSC呈现。

[0060] 图7：实验设置的示意图，用于比较从cyno神经前体获得适用于化合物筛选的稳健神经元培养物的4种分化方法(A-D)。MI指有丝分裂抑制剂。

[0061] 图8：如图7所述的4种神经元分化方法的基于图像的比较。将NC在分化的第35天固定，并进行品红显示的Sox2染色(Sox2为Glia和NSCs的标志物)和绿色显示的Map2染色(Map2为NC的标志物)。

[0062] 图9：为了测试解离和再接种人iPSC来源的分化NCs的可行性，平行分化两个细胞系。对于每个细胞系，细胞直接分化(即没有重新铺板)6周，或在第21天解离并再接种然后再培养3周。神经元(MAP2)和神经胶质(GFAP)标志物的免疫荧光染色显示，解离和再接种(重新铺板)不妨碍细胞分化的能力。

[0063] 图10：定量分析在有和无分化和再接种(重新铺板)时在两种不同的iPSC来源的细胞系中的分化程度。HuC/D是由神经元表达的标志物，并在总共6周分化的培养物中通过免疫荧光染色来检测，并通过高内涵成像来量化。数据显示重新铺板不会显著改变神经元分化的程度。

[0064] 图11：分析了微管相关蛋白Tau和其两种磷酸化形式的表达。在有和无再接种的两个细胞系中的持续表达和磷酸化程度证明，重新铺板(解离和再接种)不破坏人iPSC来源的NC的细胞骨架特征，表明生理学特征不会被解离和再接种NC而改变。

[0065] 图12：工作流程示意图，该工作流程能够持续提供稳健的灵长类动物神经元培养物用于高通量形式的筛选。SFA是指补充有Shh，FGF8和抗坏血酸磷酸盐的基础培养基；BGAA是指补充有BDNF，GDNF，cAMP和抗坏血酸磷酸盐的基础培养基；箭头表示解离和再接种(重

新铺板)。

[0066] 图13:测试化合物在来自人和食蟹猴的均匀分布的神经细胞(NC)的标准化细胞培养物上的靶结合。测试了两种浓度(低:0.02μM;高:2μM)针对UBE3A-反义转录物(antisense)的反义寡核苷酸,在人中具有已知的靶结合。A和B中来自食蟹猴的NC培养物与C和D中来自人的NC培养物相比,显示出相当的靶结合。在所示浓度下处理cyno和人NCs导致UBE3A反义转录物减少和伴随的UBE3A转录物(有义)上调。根据图12中包括的工作流程产生这些细胞培养物。

[0067] 发明详述

[0068] 如本文所使用的术语"反义寡核苷酸"定义为能够通过与靶核酸,特别是靶核酸上的连续序列,杂交来调节靶基因表达的寡核苷酸。反义寡核苷酸基本上不是双链的,因此不是siRNA。优选地,本发明的反义寡核苷酸是单链的。

[0069] 如本文所用,术语"基础培养基"是指由等体积的DMEM:F12 Glutamax培养基和Neurobasal培养基(Gibco,Invitrogen)组成的成分确定培养基,其中补充有1xB27(Gibco,Invitrogen),1xN2(Gibco,Invitrogen)、0.1mMβ-巯基乙醇(Gibco,Invitrogen)。术语"BGAA"是指补充有20ng/ml BDNF(Peprotech),10ng/ml GDNF(Peprotech),0.5mM cAMP(BIOLOG Life Science)和100μM抗坏血酸磷酸盐(Sigma)的基础培养基。术语"SFA"是指补充有200ng/ml Shh,100ng/ml FGF8和100μM抗坏血酸磷酸盐的基础培养基。

[0070] 如本文所用的术语"连续核苷酸序列"是指与靶核酸互补的寡核苷酸的区域。该术语在本文中与术语"连续核碱基序列"和术语"寡核苷酸基序序列"可互换使用。在一些实施方案中,寡核苷酸的所有核苷酸都存在于该连续核苷酸序列中。在一些实施方案中,寡核苷酸包含连续核苷酸序列并且可以任选地包含其它核苷酸(一个或多个),例如可以用于将官能团连接至连续核苷酸序列的核苷酸接头区。核苷酸接头区可以或可以不与靶核酸互补。

[0071] 如本文所用,术语"成分确定培养基"或"化学成分确定培养基"是指细胞培养基中所有单个组分和它们各自的浓度是已知的。成分确定培养基可以含有重组的和化学确定的成分。

[0072] 如本文所使用的,术语"分化"是指将较低分化细胞转化为较高分化的细胞,特别是有丝分裂后组织特异性细胞类型,的一个或多个步骤,例如将NPC转化为NC。可以例如通过向细胞培养基中加入一种或几种分化剂,诱导NPC向NC的分化。

[0073] 如本文所用,术语"功效谱"或"功效"按本领域技术人员通常理解的定义,包括基于使测试系统(例如细胞培养物或生物体)暴露于(特别是以不同浓度和/或不同施用途径)药物候选物,并随后确定与药物候选物的期望效应相关的所得细胞和/或生理效应,来评估药物候选物的功效。用于确定细胞和/或生理效应的参数在各药物候选物的上下文中定义,包括与药物候选物在测试系统或生物体上的期望表型效应相关的参数。优选地,记录一个以上参数,包括但不限于存活率,细胞活力,形态,特定基因的表达和/或表达水平,和蛋白质合成,以建立药物候选物的功效谱。在本发明的一个方面中,建立效能谱包括评估靶结合。

[0074] "表达标志物"或"标志物"可用于确定细胞类型的身份。细胞特异性基因的某些有信息的DNA序列可以被转录成mRNA,并且通常随后被翻译成在细胞中发挥特定功能的蛋白质(其基因产物)。标志物的表达可以通过本领域已知的方法在RNA水平或蛋白质水平上检

测和定量。IPSC细胞标志物是本领域已知的，并且包括但不限于TRA-1-60，TRA-1-81，Ecat1，Nanog，Oct4/POU5F1，Sox2，Rex1/Zfp-42和UTF1、或其任何组合。NPC细胞标志物在本领域中是已知的，并且包括但不限于Sox2，Nestin，Sox1，Pax6，Dach1。NC细胞标志物是本领域已知的，并且包括但不限于MAP2，β-III-微管蛋白，DCX/Doublecortin，SYN1/Synapsin 1和GPHN/Gephyrin。

[0075] 如本文所用，术语“遗传距离”应理解为两个物种，两个基因组或两个群体之间的遗传分歧的量度。遗传距离，例如不同物种之间的遗传距离，可以通过本领域已知的方法确定，包括但不限于确定Nei标准距离，Goldstein距离或Rynolds/Weir/Cockerham遗传距离。可以使用本领域已知的软件来计算遗传距离，包括但不限于POPTREE2或DISPAN。当遗传距离小时，“遗传相似性”高。

[0076] 如本文所用，使用以下缩写词：成纤维细胞生长因子(FGF)，碱性成纤维细胞生长因子(bFGF)，表皮生长因子(EGF)，音猬因子(sonic hedgehog，shh)，成纤维细胞生长因子8(FGF8)，脑源性神经营养因子(BDNF)，神经胶质细胞来源的神经营养因子(GDNF)，环腺苷单磷酸(cAMP)，Rho相关的卷曲螺旋形成蛋白丝氨酸/苏氨酸激酶(ROCK)。

[0077] 如本文所用，术语“生长因子”意指引起细胞增殖的生物活性多肽或小分子化合物，并且包括生长因子及其类似物。

[0078] 如本文所用，“高通量筛选”应理解为表示，使用本文所述的新试验，可以分析和比较相对大量的不同疾病模型条件和/或化学化合物。典型的这种高通量筛选在多孔微量滴定板中进行，例如在96孔板或384孔板或具有1536或3456孔的板中进行。

[0079] “LNA核苷”是修饰的核苷，其在核苷酸的核糖糖环的C2’和C4’之间包含连接基团(称为二价基(biradicle)或桥)。这些核苷在文献中也称为桥联核酸或双环核酸(BNA)。

[0080] 如本文所用，术语“均匀分布的”，“均匀分布”或“均一分布”是指，本领域技术人员通常理解的，实体在1维，2维或多维空间中的分布，特别是细胞在细胞培养支持物的二维表面上的分布。如果每个区域的平均细胞计数在整个细胞培养表面上基本恒定，则在二维细胞培养表面上建立均匀分布。细胞的分布可以通过例如核染色和使用荧光显微镜确定每个区域的细胞核的数量来评估。细胞非均匀分布的指标包括例如细胞团块，显著数量的重叠细胞核、或显著部分的缺乏细胞的细胞培养区域。一种或多种细胞类型的具有均匀分布的细胞的细胞培养物在本文中被称为“标准化的”。“标准化细胞培养物”或“标准化NC培养物”是指根据本发明产生的细胞培养物，其中细胞的分布基本上是均匀的，即细胞均匀分布，且细胞培养物的特征在于细胞的均匀分布，其中细胞培养物可以包括一种或多种细胞类型。因此，如果细胞显示均匀分布(例如通过核染色和确定每个区域的细胞核数量进行评估)，则认为细胞培养物是标准化的。

[0081] 本文使用的术语“修饰的核苷间连接”按本领域技术人员通常理解定义为，将两个核苷共价连接在一起的、除磷酸二酯(PO)连接以外的连接。具有修饰的核苷间连接的核苷酸也称为“修饰的核苷酸”。与磷酸二酯连接相比，修饰的核苷间连接可以增加寡核苷酸的核酸酶抗性。对于天然存在的寡核苷酸，核苷间连接包括在相邻核苷间产生磷酸二酯键的磷酸基团。修饰的核苷间连接对于稳定用于体内使用的寡核苷酸是特别有用的，并且可用于防止在本发明寡核苷酸中DNA或RNA核苷的区域处(例如在gapmer寡核苷酸的gap区域内以及在修饰的核苷的区域中)的核酸酶切割。

[0082] 如本文所用，术语“MT培养基”是指这样的成分确定培养基，该培养基含有Dulbecco改良Eagle培养基和Ham氏F12营养混合物(DMEM/F12)并具有2.5mM GlutaMAX™，7μg/ml胰岛素，450μM单硫代甘油，1x脂质浓缩物，5mg/ml BSA，14ng/ml亚硒酸钠，1x非必需氨基酸，2mg/ml肝素，15μg/ml转铁蛋白和220μM抗坏血酸-2-磷酸盐。

[0083] 如本文所用，“神经元前体细胞”或“NPC”是指多能细胞的一个子集，其源自IPSC并表达一些神经祖细胞标志物，包括例如Nestin。特别是可以根据Costa等人Cell Rep 2016;15:86-95和Dunkley等人，Proteomics Clin Appl 2015;7-8:684-94(其通过引用整体并入本文)所述的方法或根据本文所述的方法，产生NPC。NPC可以无限扩增并可分化成神经元或神经胶质细胞(例如星形胶质细胞和少突胶质细胞)。术语“患者特异性NPC”是指由已经从患者的体细胞重编程的患者IPSC获得的NPC。如本文所用，“从健康个体获得的NPC”是指从明显健康且未怀疑患有任何病症或疾病的个体的体细胞获得的IPSC分化的NPC。

[0084] 如本文所用，“神经元细胞”或“NCs”是指来自神经元谱系的组织特异性细胞。如本文所述，使用特定的细胞培养条件，例如通过撤除生长因子或通过添加一种或多种分化剂，NC可以在体外从NPCs分化。

[0085] 如本文所用，术语“非人灵长类动物”或“NHP”是指，除智人(Homo sapiens)之外属于灵长类动物的物种。具体而言，根据本发明公开的方法，NHP物种包括但不限于，黑猩猩(Pan troglodytes)，倭黑猩猩(Pan paniscus)，白掌长臂猿(Hylobates lar)，西非大猩猩(Gorilla gorilla)，苏门达腊猩猩(Pongo abelii)，婆罗洲猩猩(Pongo pygmaeus)，青长尾猴(Cercopithecus mitis)，白臀长尾猴(Cercopiticus neglectus)，黑脸绿猴(Chlorocebus aethiops)，绿猴(Chlorocebus sabaeus)，东非黑白疣猴(Colocus guereza)，黑冠白睑猴(Lophocebus aterrimus)，短尾猴(Macaca arctoides)，熊猴(Macaca assamensis)，食蟹猴(Cynomolgus monkey)，日本猴(Macaca fuscata)，猕猴(Macaca mulatta(恒河猴))，豚尾猴(Macaca nemestrina)，狮尾猴(Macaca silenus)，鬼狒(Mandrillus leucophaeus)，山魈(Mandrillus sphinx)，藏酋猴(Macaca thibetana)，东非狒狒(Papio anubis)，草原狒狒(Papio cynocephalus)，阿拉伯狒狒(Papio hamadryas)，几内亚狒狒(Papio papio)，豚尾狒狒(Papio ursinus)，长尾叶猴(Presbytis entellus)，狮尾狒(Theropithecus gelada)，阿氏夜猴(Aotus azarae)，秘鲁夜猴(Aotus nancymaae)，黑夜猴(Aotus nigriceps)，夜猴(Aotus trivirgatus)，巴西夜猴(Aotus vociferans)，白腹蜘蛛猴(Ateles belzebuth)，棕头蜘蛛猴(Ateles fusciceps)，普通狨(Callithrix jacchus)，暗色伶猴(Callicebus moloch)，倭狨(Cebuella pygmaea)，卷尾猴(Cebus apella)，金狮面狨(Leontopithecus rosalia)，白脸僧面猴(Pithecia pithecia)，棕须柽柳猴(Saguinus fuscicollis)，斑柽柳猴(Saguinus geoffroyi)，白唇柽柳猴(Saguinus labiatus)，长须柽柳猴(Saguinus mystax)，绒顶柽柳猴(Saguinus Oedipus)和松鼠猴(Saimiri sciureus)。

[0086] 本文使用的术语“cyno”是食蟹猴的缩写，和/或是指来源于食蟹猴的物质，包括但不限于细胞，组织，器官，血液或由其来源的细胞。

[0087] “核苷酸”是寡核苷酸和多核苷酸的结构单元，为了本发明的目的，包括天然存在的和非天然存在的核苷酸。在自然界中，核苷酸(例如DNA和RNA核苷酸)包含核糖部分，核碱基部分和一个或多个磷酸基团(其在核苷中不存在)。核苷和核苷酸也可以互换地称为“单

元”或“单体”。

[0088] 如本文所用，术语“源自人类基因组的核苷酸序列”是指相应的核苷酸序列来源于人类基因组参考序列，即至少全球人类的一个亚群在基因组中包含该相应核苷酸序列。此外，如本文所用，术语“来源自人类基因组的核苷酸序列”用于这样的序列，所述序列使用NCBI/Blast数据库和算法具有最高的最大得分值而归属于人类基因组(Zheng Zhang, Scott Schwartz,Lukas Wagner,and Webb Miller(2000),"A greedy algorithm for aligning DNA sequences",J Comput Biol 2000;7(1-2):203-14.)。不受理论束缚，认为如果查询序列源自人类基因组的话，则查询序列与人类参考序列的比对得分将高于查询序列与非人类参考序列的比对得分。

[0089] 如本文所用的术语“修饰的核苷”或“核苷修饰”是指，与相应的DNA或RNA核苷相比，通过引入糖部分或(核)碱基部分的一个或多个修饰而修饰的核苷。在优选的实施方案中，修饰的核苷包含修饰的糖部分。术语“修饰的核苷”也可以与术语“核苷类似物”或修饰的“单元”或修饰的“单体”互换使用。

[0090] 术语“修饰的核苷间连接”按本领域技术人员通常理解被定义为，除了磷酸二酯(PO)连接之外的、将两个核苷共价连接在一起的连接。具有修饰的核苷间连接的核苷酸也称为“修饰的核苷酸”。在一些实施方案中，与磷酸二酯连接相比，修饰的核苷间连接增加寡核苷酸的核酸酶抗性。对于天然存在的寡核苷酸，核苷间连接包括在相邻核苷间产生磷酸二酯键的磷酸基团。修饰的核苷间连接对于稳定用于体内使用的寡核苷酸是特别有用的，并且可用于防止本发明寡核苷酸中的DNA或RNA核苷的区域处的核酸酶切割，例如在gapmer寡核苷酸的gap区域内以及在修饰的核苷的区域中的切割。

[0091] 如本文所用，术语“N2B27”是指补充有N2和B27(均来自Gibco,Invitrogen)的、由等体积的DMEM:F12(Gibco,Invitrogen)组成的成分确定培养基。

[0092] 如本文所用，术语“寡核苷酸”如本领域技术人员通常理解地定义，是指包含两个或更多个共价连接的核苷的分子。这种共价结合的核苷也可以被称为核酸分子或寡聚物。寡核苷酸通常通过固相化学合成在实验室中制备并之后进行纯化。当提及寡核苷酸的序列时，指共价连接的核苷酸或核苷的核碱基部分或其修饰的序列或顺序。寡核苷酸可以包含一个或多个修饰的核苷或核苷酸。如本文所使用的术语“反义寡核苷酸”定义为能够通过与靶核酸，特别是靶核酸上的连续序列杂交来调节靶基因表达的寡核苷酸。反义寡核苷酸基本上不是双链的，因此不是siRNA。优选地，反义寡核苷酸是单链的。

[0093] 如本文所用，术语“重编程”是指将体细胞转化为较低分化的细胞所需的一个或多个步骤，例如用于将成纤维细胞，脂肪细胞，角质形成细胞或白细胞转化为NPC的步骤。“重编程的”细胞指通过如本文所述重编程体细胞而产生的细胞。

[0094] 本文使用的术语“小分子”、或“小化合物”、或“小分子化合物”是指，合成的或天然存在的有机或无机分子，其分子量通常小于10,000克/摩尔，任选小于5,000克/摩尔，并且任选小于2,000克/摩尔。

[0095] 如本文所用，术语“体细胞”是指，非生殖细胞(例如，精子和卵子，产生精子和卵子的细胞(配子体细胞))和未分化干细胞的任何形成生物体的细胞。

[0096] 如本文所用，术语“干细胞”是指具有自我更新和分化能力的细胞。如本文所用的“未分化的干细胞”是指未经历分化的干细胞。如本文所用，“多能干细胞”或“PSC”是指可产

生三个胚层(内胚层,外胚层,中胚层)的细胞类型以及生殖系的干细胞。多能干细胞(PSCs)包括但不限于“胚胎干细胞”(“ESCs”)和“诱导的多能干细胞”(“IPSCs”)。术语“hIPSC”和“cIPSC”分别指源自人类细胞的IPSC和源自食蟹猴细胞的IPSCs。

[0097] 如本文所用,术语“细胞活力的实质性丧失”是指,在应用不同的细胞培养条件、或在规定的方法中操作细胞时(特别是与从细胞培养支持物上解离细胞有关的操作),细胞活力的降低。在一个实施方案中,细胞活力的实质性丧失意味着,5%以上的细胞变成无活性和/或经历细胞凋亡。在进一步的实施方案中,细胞活力的实质性丧失意味着,10%以上,15%以上,20%以上,或25%以上的细胞变成无活性和/或经历细胞凋亡。因此,在一个实施方案中,术语“基本上保持活力”是指95%以上的细胞保持活力。在进一步的实施方案中,基本上保持活力是指90%以上,85%以上,80%以上或75%以上的细胞保持活力。

[0098] 如本文所用,“合适的分化培养基”也被描述为“分化培养基”,是指用于将NPC分化成NC的任何化学上确定的培养基。如本文所述的分化培养基含有至少一种“分化剂”。分化剂包括但不限于导致细胞分化的生物活性多肽或小分子化合物。

[0099] “靶”是指期望调节的蛋白质。“靶核酸”是本发明的寡核苷酸与之杂交的预期靶标,并且可以例如是基因,RNA,非编码RNA,长非编码RNA,mRNA和pre-mRNA,成熟的mRNA或cDNA序列。在一些实施方案中,靶核酸是非编码RNA或长非编码RNA或其子序列。对于特定的体内或体外应用,本发明的寡核苷酸能够降低SNORD109B下游SNHG14转录物的水平,从而在预期的靶细胞中缓解亲本UBE3A转录物的抑制。如在整个寡核苷酸的长度上测量的,本发明的寡核苷酸的连续核碱基序列与靶核酸互补,任选地除一个或两个错配以外,以及任选地排除基于核苷酸的接头区域(其可以将寡核苷酸连接至任选的官能团如缀合物上)。寡核苷酸包含与靶核酸分子的子序列互补或杂交的连续核苷酸序列。

[0100] 如本文所用的术语“靶序列”是指存在于靶核酸中的核苷酸序列,其包含与本发明的寡核苷酸互补的核碱基序列。在一些实施方案中,靶序列由靶核酸上与本发明寡核苷酸的连续核苷酸序列互补的区域组成。在一些实施方案中,靶序列比单个寡核苷酸的互补序列长,并且可以例如是可以被本发明的几个寡核苷酸靶向的靶核酸的优选区域。本发明的寡核苷酸包含与靶核酸例如靶序列互补的连续核苷酸序列。寡核苷酸可以包含至少8个核苷酸的、与靶核酸分子中存在的靶序列互补或杂交的、连续核苷酸序列。连续的核苷酸序列(以及因此靶序列)包含至少8个连续的核苷酸,例如9,10,11,12,13,14,15,16,17,18,19,20,21,22,23,24,25,26,27,28,29或30个连续核苷酸,例如12-25个,例如14-18个连续核苷酸。

[0101] 如本文所用的术语“靶细胞”是指表达靶核酸的细胞。在一些实施方案中,靶细胞可以在体内或体外。在一些实施方案中,靶细胞是哺乳动物细胞,诸如啮齿动物细胞,诸如小鼠细胞或大鼠细胞,或灵长类动物细胞诸如猴细胞或人类细胞。在一个实施方案中,靶细胞是神经元细胞(NC)。

[0102] 如本文所用,术语“毒性谱”或“毒性”按本领域通常理解定义为包含潜在有害或无害物质的毒理学评估,所述评估基于将测试系统(例如细胞培养物或细胞培养物)暴露于该物质,特别是以不同的浓度和/或不同的施用途径,随后确定所产生的细胞和/或生理效应,例如细胞存活或健康。确定细胞和/或生理效应的参数在本领域中是熟知的,包括但不限于存活,细胞活力,形态,某些基因的表达和/或表达水平、和蛋白质合成。优选地,记录一个以

上参数以建立毒性未知的物质的毒性谱。本领域中使用药物候选物的毒性谱来选择药物候选物用于进一步开发，例如，在灵长类动物或人上进行体内测试。

[0103] 本发明提供了用于从包括人在内的不同灵长类物种产生可重现和标准化的分化NC培养物的新方法，所述培养物可用于药物候选物的体外高通量测试。该方法包括提供灵长类NPC，将NPC分化为NC，并通过解离分化的灵长类NCs、以合适的细胞培养形式再接种细胞而不实质性丧失细胞活力、以及继续进行NC分化来使细胞培养物能够用于药物候选物的高通量筛选。灵长类NPC可以来源于IPSCs或转分化细胞，两者都可以从体细胞产生。优选地，所述体细胞是灵长类动物细胞，包括人体细胞。

[0104] 在一个实施方案中，提供了用于自不同灵长类物种产生均匀分布的分化神经元细胞(NC)的细胞培养物的体外方法，所述方法包括以下步骤：

[0105] a)提供神经元前体细胞(NPC)；和

[0106] b)将NPC分化为NC，包括步骤：

[0107] (i)在分化的第20天和第45天之间将分化的NC从其支持物上解离；和

[0108] (ii)以合适的细胞培养形式再接种细胞并继续NC分化4天至15天。

[0109] 为了实现这里描述的本发明方法，必须绕过NC培养的一些现有限制。人们普遍接受的观点是，与NPC可以以贴壁或漂浮培养物形式无限扩增不同，分化中的NCs严格依赖于与基质的相互作用，基质通常由细胞培养板的生物聚合物涂层组成。此外，分化的NC对细胞应激(cellular stress)变得敏感，因此，分化细胞的传代被认为对细胞有害。根据本发明的创新方法公开了细胞培养条件，由此可以从不同的灵长类物种产生均一的NC培养物，其中NC在分化的第10天和第40天之间从其支持物解离而没有实质性的活力丧失。NC可以以合适的细胞培养形式再接种并继续分化。解离和再接种导致分化的NC在细胞培养区域的均匀分布。此外，本发明的方法允许在NC分化的晚期改变细胞培养形式。在进一步的实施方案中，步骤b)(i)包括在分化的约第25天和约40天之间，约28天和约30天之间，约28天或约30天时，将分化的NC从其支持物上解离。

[0110] 因此，本发明的一个方面是如本文所述的产生均一的分化NC培养物的方法。在一个实施方案中，步骤b)包括将NPC分化成神经细胞(NC)，包括以下步骤：(i)在分化的第20和45天之间将分化的NC从其支持物上解离；和(ii)以合适的细胞培养形式再接种细胞并继续NC分化4天至10天，其中NC基本上保持活力。所述解离和再接种步骤，对于获得在细胞培养表面区域(例如细胞培养孔)上均匀分布的分化灵长类动物NCs、和/或对于收获分化的灵长类动物NC而没有实质性的丧失活力，是关键的。因此，根据本发明产生的分化灵长类NC培养物测定试验，与没有解离和再接种时产生的细胞培养物相比，可以显示出更均匀分布的细胞，并且更适用于高通量测定。重要的是，细胞培养形式可以在NC分化的后期阶段改变。这与本领域的方法形成对比，在所述方法中最终的细胞培养形式必须在早期时间点予以使用。事实上，能够改变细胞培养形式可以提供相当大的供给灵活性(logistic flexibility)。

[0111] 灵长类NC的高度可重现的细胞培养物是充分标准化的，并且可用于化合物筛选测定，包括但不限于药物候选物的体外功效评估。此外，如本文所述的细胞培养物可以用于选择药物候选物，特别是用于选择药物候选物用于如本文所述的进一步开发。在一个实施方案中，根据本发明的细胞培养物用于确定如本文所述的药物候选物的体外功效谱。在另一

个实施方案中，药物候选物的体外功效谱在测定本文所述的体内功效谱之前确定。在另一个实施方案中，根据本发明的细胞培养物用于确定如本文所述的药物候选物的体外毒性谱。

[0112] 因此，在一个实施方案中，分化的灵长类动物NC从细胞培养容器上解离，其中NC基本上保持活力。解离的NC可以以所需细胞培养物形式以优化的细胞密度再接种，其中所述细胞密度经优化符合给定细胞培养物测定的需要。在一个实施方案中，本发明中描述的解离和再接种条件可以应用于不同的灵长类物种。在一个实施方案中，根据本发明的方法可以用于药物候选物的功效谱的物种间比较，其中细胞培养物是从至少两个物种的细胞单独产生的，其中将基本上相同的条件应用于所有物种的培养物，并且其中对于所有物种，确定并比较效力谱。本发明的范围涵盖，使用基本上相同的细胞培养条件产生源自至少一种灵长类动物物种，至少两种灵长类动物物种或至少三种灵长类动物物种的标准化细胞培养物测定试验，并且比较和整合按照该测定试验读数所测定的结果，以确定至少一种药物候选物的综合效能谱。

[0113] 在一个实施方案中，NPC由源自重编程的体细胞的IPSC产生。将体细胞重编程为IPSC可以通过引入参与维持IPSC性质的特定基因来实现。适合将体细胞重编程为IPSC的基因包括但不限于Oct4，Sox2，Klf4和C-Myc及其组合。在一个实施方案中，用于重编程的基因是Oct4，Sox2，Klf4和C-Myc。WO2012/022725中描述了用于将体细胞转分化成NPC的基因组合，其通过引用并入此处。

[0114] 内脏，皮肤，骨骼，血液和结缔组织均由体细胞组成。用于产生IPSC的体细胞包括但不限于成纤维细胞，脂肪细胞和角质形成细胞，并且可以从皮肤活检物获得。其他合适的体细胞是从血液样品获得的白细胞、成红细胞、或上皮细胞、或从血液或尿样品获得的其他细胞，并通过本领域已知的方法和本文所述重编程为IPSC。体细胞可以从健康个体或患病个体获得。如本文所述的用于重编程的基因通过本领域已知的方法引入体细胞，通过重编程载体递送至细胞中或通过小分子激活所述基因。用于重编程的方法包括例如逆转录病毒，慢病毒，腺病毒，质粒和转座子，微RNA，小分子，修饰的RNA和重组蛋白。在一个实施方案中，使用慢病毒来递送如本文所述的基因。在另一个实施方案中，使用仙台病毒(Sendai virus)颗粒将Oct4，Sox2，Klf4和C-Myc递送至体细胞。此外，体细胞可以在至少一种小分子存在下培养。在一个实施方案中，所述小分子包含Rho相关卷曲螺旋形成蛋白丝氨酸/苏氨酸激酶(ROCK)家族的蛋白激酶的抑制剂。ROCK抑制剂的非限制性例子包括：fasudil(1-(5-异喹啉磺酰基)高哌嗪)，Thiazovivin(N-苄基-2-(嘧啶-4-基氨基)噻唑-4-甲酰胺)和Y-27632((+)-(R)-反式-4-(1-氨基乙基)-N-(4-吡啶基)环己烷甲酰胺二盐酸盐)。由此产生的IPSCs可被诱导分化成NPC。在一个实施方案中，诱导IPSC分化成NPC。

[0115] 在一个实施方案中，灵长类动物NPC通过双重SMAD抑制从IPSC产生。在一个实施方案中，通过使细胞与SB-431542(Calbiochem)和LDN-193189(Calbiochem)接触而由IPSC产生NPC。在一个具体实施方案中，通过使细胞接触5ng/ml FGF(Peprotech)，10μM SB-431542(Calbiochem)和100nM LDN-193189(Calbiochem)，由IPSC产生NPC。产生的灵长类NPC可以在补充有FGF，EGF和BDNF的基础培养基中扩增。在一个实施方案中，使NPC在补充有10ng/ml FGF(Peprotech)，10ng/ml EGF(RnD)和20ng/ml BDNF(Peprotech)的基础培养基中扩增。在补充有FGF，EGF和BDNF的基础培养基中继续传代产生稳定的神经祖细胞系(NPC系)。稳定的

NPC系由其自我更新能力和发育阶段特异性标志物Sox2和Nestin的表达来定义。因此，在一个实施方案中，灵长类NPC表达Sox2和Nestin。

[0116] 为了扩大增殖NPCs，细胞在包含补充有生长因子的无血清培养基的扩增培养基中生长。在一个实施方案中，所述生长因子包含FGF，BDNF和EGF。因此，在一个实施方案中，该方法还包括在适于NPC增殖的条件下孵育步骤a)的细胞，例如，直至达到规定数量的细胞/区域。本文描述了扩增培养基的非限制性示例。在一个实施方案中，扩增培养基补充有10-50ng/ml FGF，10-50ng/ml EGF和1-20ng/ml BDNF。在一个具体的实施方案中，NPC扩增培养基是补充有10ng/ml FGF2，10ng/ml EGF和20ng/ml BDNF的基础培养基。NPC可以以不受限制的量生产，因此最适用于需要大量测定板的高通量细胞培养测定试验。培养在本领域技术人员的能力范围内。

[0117] 在一个实施方案中，在启动分化之前，用合适的缓冲液或培养基洗涤灵长类NPC以去除任何死亡细胞。优选地，在细胞培养方案的每个步骤之间改变培养基，例如通过抽吸或离心细胞并且弃去上清液来去除培养基，然后将用于随后步骤的培养基添加到细胞中。在一个实施方案中，细胞在加入后续步骤的培养基之前用合适的缓冲液或培养基洗涤，以除去任何死细胞和任何残留培养基或在前一步骤中应用的生长因子或细胞因子。本领域已知可用于洗涤细胞的缓冲液或培养基。用于洗涤细胞的合适缓冲液的一个实例是磷酸盐缓冲盐水(PBS)。

[0118] 在一个实施方案中，灵长类NPC培养物以约5000个细胞/cm^2至约100000个细胞/cm^2的密度提供。在进一步的实施方案中，灵长类NPC培养物以约10000个细胞/cm^2至约50000个细胞/cm^2的密度提供。在一个实施方案中，贴壁灵长类NPC培养物以约20000个细胞/cm^2至约40000个细胞/cm^2的密度提供。在一个实施方案中，贴壁灵长类NPC培养物以约30000个细胞/cm^2的密度提供。在一个实施方案中，贴壁灵长类NPC培养物在Laminin521支持物上提供。

[0119] 在一个实施方案中，通过本领域已知和本文所述的方法获得的灵长类动物NPC，在下一步中通过细胞与Shh(音猬因子)，FGF8(成纤维细胞生长因子8)和抗坏血酸磷酸盐接触而诱导分化成NC。在一个实施方案中，将NPC与本文所述的包含Shh，FGF8和抗坏血酸磷酸盐的化学成分确定培养基一起孵育。在一个实施方案中，培养基补充有50-1000ng/ml Shh，25-500ng/ml FGF8和20-200μM抗坏血酸磷酸盐。在进一步的实施方案中，使细胞与Shh，FGF8和抗坏血酸磷酸盐接触约1天，约2天，约3天，约4天，约5天，约6天，约7天，约8天，约9天或约10天。在另一个实施方案中，使细胞与Shh，FGF8和抗坏血酸磷酸盐接触约5天至约10天。在一个具体的实施方案中，灵长类NPC在补充有200ng/ml Shh，100ng/ml FGF8和100μM抗坏血酸磷酸盐的基础培养基中培养约7天。

[0120] 在一个实施方案中，在诱导神经元分化后，细胞以约10000个细胞/cm^2至约80000个细胞/cm^2，约20000个细胞/cm^2至约70000个细胞/cm^2，约30000个细胞/cm^2至约60000个细胞/cm^2，或约40000个细胞/cm^2至约50000个细胞/cm^2重新铺板。在一个具体的实施方案中，在诱导神经元分化后细胞以约45000个细胞/cm^2的密度重新铺板。

[0121] 在一个实施方案中，诱导神经元分化后的细胞在下一步在补充有BDNF，GDNF，cAMP和抗坏血酸磷酸盐的基础培养基中再分化约15天，再分化约16天，再分化约17天，再分化约18天，再分化约19天，再分化约20天，再分化约21天，再分化约22天，再分化约23天，再分化

约24天，再分化约25天，再分化约26天，再分化约27天，再分化约28天，再分化约29天，再分化约30天，再分化约31天，再分化约32天，再分化约33天，再分化约34天或再分化约35天。在一个具体的实施方案中，细胞在补充有20ng/ml BDNF(Peprotech)，10ng/ml GDNF(Peprotech)，0.5mM cAMP(BIOLOG Life Science)和100μM抗坏血酸磷酸盐(Sigma)的基础培养基中再培养约19天至约35天。

[0122] 在一个实施方案中，根据本发明，按本文所述的，将分化的NC从其支持物上解离，并以合适的细胞培养物形式再接种。在进一步的实施方案中，NC在补充有BDNF，GDNF，cAMP和抗坏血酸的基础培养基中分化至少约10天，至少约11天，至少约12天，至少约13天，至少约14天，至少约15天，至少约16天，至少约17天，至少约18天，至少约19天，至少约20天，至少约21天，至少约22天，至少约23天，至少约24天，至少约25天，至少约26天，至少约27天或至少约28天，之后解离并以合适的细胞培养测定试验形式再接种。在进一步的实施方案中，灵长类动物NC在补充有BDNF，GDNF，cAMP和抗坏血酸的基础培养基中分化约15至约30天，约20至约25天，约21至约23天，然后解离并以合适的细胞培养测定试验形式再接种。

[0123] 在本发明的一个方面，通过本文所述的方法获得的分化的NC从细胞培养基质解离而没有实质性的细胞活力丧失。因此，在一个实施方案中，收获分化的NC而没有实质性的细胞活力丧失。细胞数量可根据本领域中使用的常规方法确定，包括但不限于在血细胞计数器中计数细胞数量或使用流式细胞术。细胞活力可以根据常规方法测定，包括但不限于台盼蓝和赤藓红B染色。解离后，根据基于NC的所需测定试验的具体需要或实验参数，细胞可以以合适的细胞密度再接种在合适的细胞培养孔中。

[0124] 因此，在一个实施方案中，通过使用细胞分离溶液，将分化的NC与细胞培养表面分离。在一个实施方案中，将分化的NC从其支持物上解离并以合适的细胞培养形式再接种。在另一个实施方案中，分化的NC在分化的大约第20天和第45天之间从其支持物解离。从分化开始日起计数分化天数，例如与Shh，FGF8和抗坏血酸磷酸一起孵育，其中Shh，FGF8和抗坏血酸的添加日计为第0天。在另外的实施方案中，分化的NC在分化的约第15天和约第50天之间，约第28天和约30天之间，在分化的约第25天，在分化的约第26天，在分化的约第27天，在分化的约第28天，在分化的约第29天，在分化的约第30天，在分化的约第31天，在分化的约第32天，在分化的约第33天，或在约分化的第34天，从其支持物解离。在一个实施方案中，通过用细胞分离溶液孵育细胞，使分化的NC从其支持物解离。在一个实施方案中，细胞分离溶液是Accutase。Accutase是一种具有蛋白水解和胶原蛋白溶解活性的海洋来源酶。在一个实施方案中，将细胞分离溶液加入到分化的NC培养物中并孵育1至5分钟，2至4分钟，优选3分钟。孵育时间结束后，Accutase溶液用培养基，特别是基础培养基稀释。在再接种之前，除去含有Accutase的培养基并补加补充有如本文所述的生长因子的新鲜基础培养基。

[0125] 分离之后，灵长类NC可以在新的细胞培养容器中再接种，例如合适的平板形式的细胞培养孔。NC可以以任何期望的密度再接种，包括低密度，中等密度和高密度。因此，均匀分布的分化NC的培养物可以以确定的和期望的密度产生，包括低密度，中密度和高密度。这与常规的细胞培养方法相反，其中细胞密度在培养开始时固定，并且其中细胞密度由于细胞增殖或细胞死亡可以随时间显著改变。在一个实施方案中，NC以高密度再接种。在进一步的实施方案中，将NC以约50000个细胞/cm^2至约500000个细胞/cm^2的密度，以约75000个细胞/cm^2至约400000个细胞/cm^2的密度，或以约100000个细胞/cm^2至约300000个细胞/cm^2的

密度再接种。在一个具体的实施方案中，将分化的NC以约200000个细胞/cm^2的密度在步骤b)(ii)中再接种。在一个实施方案中，在用BDNF，GDNF，cAMP和抗坏血酸磷酸盐分化后，细胞解离并以约50000个细胞/cm^2至约500000个细胞/cm^2，约75000个细胞/cm^2至约400000个细胞/cm^2，或约100000个细胞/cm^2至约300000个细胞/cm^2的密度再接种。在一个具体的实施方案中，细胞在用BDNF，GDNF，cAMP和抗坏血酸磷酸盐分化后解离并以约200000个细胞/cm^2的密度再接种。在解离和再接种(重新铺板)后，将细胞在补充有BDNF，GDNF，cAMP和抗坏血酸的基础培养基中进一步分化另外约1天，约2天，约3天，约4天，约5天，约6天，约7天，约8天，约9天，约10天，约11天，约12天，约13天，约14天或约15天。

[0126] 根据本发明可以使用解离并再接种的分化NC培养物来测试药物候选物的功效。在一个具体的实施方案中，在将药物候选物应用于细胞之前，细胞在补充有20ng/ml BDNF(Peprotech)，10ng/ml GDNF(Peprotech)，0.5mM cAMP(BIOLOG Life Science)和100μM抗坏血酸磷酸盐(Sigma)的基础培养基中进一步分化约7天。因此，在约10天至约50天，约15天至约45天，约30天至约40天，约35天或约37天的总分化期之后，NC细胞即可用于筛选药物候选物。在一个具体的实施方案中，在约30至约40天的总分化期后，NC即可用于筛选寡核苷酸候选物。

[0127] 在进一步的实施方案中，分化的NC培养物在约28天，约29天，约30天，约31天，约32天，约33天，约34天，约35天，约36天，约37天，约38天，约39天，约40天，约41天，约42天，约43天，约44天，约45天，约46天，约47天，约48天，约49天，约50天，约51天，约52天，约53天，约54天或约55天的总分化期之后，即可用药物候选物处理。在另一个实施方案中，用药物候选物处理可以在本文所述的细胞培养方案的任何步骤进行。

[0128] 在一个具体的实施方案中，灵长类动物NPC在解离和再接种前在包含20ng/ml BDNF，10ng/ml GDNF，0.5mM cAMP和100μM抗坏血酸磷酸盐的无血清分化培养基中分化为NC约21至约23天。在另一个实施方案中，如本文所述再接种后，将分化的NC与如本文所述的分化培养基一起孵育另外约1天，另外约2天，另外约3天，另外约4天，另外约5天，另外约6天，另外约7天，另外约8天，另外约9天，另外约10天，另外约11天，另外约12天，另外约13天，另外约14天，或另外约15天。在一个具体的实施方案中，在再接种后，将分化的NC在含有20ng/ml BDNF，10ng/ml GDNF，0.5mM cAMP和100μM抗坏血酸磷酸盐的无血清分化培养基中孵育约7天。此后，分化的NC即可用于药物候选物处理。

[0129] 在本发明的一个方面中，在将药物候选物添加至细胞培养基之前，继续进行分离和再接种的NC的分化。因此，在进一步的实施方案中，将再接种的NC在如本文所述的分化培养基中孵育约1天，约2天，约3天，约4天，约5天，约6天，约7天，大约8天，大约9天，大约10天，大约11天，大约12天，大约13天，大约14天或大约15天，然后加入药物候选物。在进一步的实施方案中，步骤b)(ii)包括在再接种后用如下基础培养基分化NCs另外约1至约20天，另外约5至约10天，另外约7天，其中所述基础培养基包含1至50ng/ml BDNF，1至50ng/ml GDNF和0.1-10mM cAMP和20至200μM抗坏血酸磷酸盐，然后加入药物候选物。在一个具体的实施方案中，步骤b)(ii)包括，在再接种后用包含20ng/ml BDNF，10ng/ml GDNF和0.5mM cAMP和100μM抗坏血酸磷酸盐的基础培养基再分化NC约7天，之后加入药物候选物。

[0130] 在一个实施方案中，在解离并再接种分化的NC后，将药物候选物添加到细胞培养基中。在进一步的实施方案中，在再接种分化的NC后约1天至约15天，约5天至约10天或约7

天，将药物候选物添加至细胞培养基中。在一个具体的实施方案中，在再接种分化的NC后约7天，将药物候选物添加到细胞培养基中。

[0131] 在一个实施方案中，灵长类动物NPC根据本文所述的方法分化为NC。在一个实施方案中，用与神经元的细胞和/或代谢功能相关的表达标志物评估神经元身份。典型的神经元标志物包括但不限于MAP2，HuC/D，Nestin，β-III-微管蛋白，DCX/Doublecortin，SYN1/Synapsin 1和GPHN/Gephyrin。与原代神经元或神经组织中的表达水平相比，与神经元身份相关的表达标志物可以在源自NPC的NCs中以较低水平表达。与原代神经元或神经组织中相应标志物的表达水平相比，NSC来源的NC中神经元表达标志物的标化表达水平可以低10000倍，或低1000倍，或低100倍，或低10倍，或低2倍。NPC来源的NC和原代神经元之间的神经元表达标志物的表达水平倍数变化，对于不同的表达标志物可以是不同的。标化可以通过将给定标志物的绝对表达水平与合适的持家基因(例如GAPDH或TBP)相关联来进行。

[0132] 在一个实施方案中，本发明的创新方法用于针对包括但不限于人(Homo sapiens)，食蟹猴(Macaca fascicularis)和恒河猴(Macaca mulatta)的不同灵长类物种产生具有均匀NC分布的稳健的分化NC培养物。基本上相同的细胞培养条件可应用于所有灵长类物种。

[0133] 不受理论约束，本发明沿着从多能干细胞至完全分化NC的轴线区分了细胞的两个不同阶段，即NPC和分化的NC。多能NPC可如本文所公开的获得，并可扩增至任何合适的细胞数目，例如用于期望形式的细胞培养测定试验。可以冻融健康个体和患者特定的NPC等分试样。因此，根据本发明，所述NPC可以扩大到合适的细胞数目，冷冻保存或直接分化以产生稳健的分化NC培养测定试验。出乎意料地，本发明人发现，使用本申请中公开的特定条件，可收获分化的灵长类NCs并用作具有固定神经元身份的细胞的来源。这些分化的灵长类NC可以在分化的晚期从细胞培养基质分离而不实质性丧失活力。收获的分化灵长类NC可以以规定的测定试验形式再接种，其中细胞贴附于细胞培养支持物，并且可以在应用待测试的药物候选物之前或平行于该药物候选物应用，继续进行分化。本发明的方法解决了来自不同灵长类物种的NC培养物不均一的问题。因此，用本文描述的方法获得的灵长类NC培养物是筛选有效且安全的药物和阐明各种神经系统疾病的新疗法的有价值模型。

[0134] 因此，在本发明的另一方面，根据本发明产生的NC培养物用于测试至少一种药物候选物的功效。可以在本发明方法的任何阶段将药物候选物添加到细胞培养基中。在一个实施方案中，将药物候选物添加到分化的NC中。在一个实施方案中，将药物候选物添加到解离并再接种的分化NC中以确定药物候选物的功效谱。在进一步的实施方案中，药物候选物在分化的约第1天，约第2天，约第3天，约第4天，约第5天，约第6天，约第7天，大约第8天，大约第9天，大约第10天，大约第11天，大约第12天，大约第13天，大约第14天，大约第15天，大约第16天，大约第17天，大约第18天，大约第19天，大约第20天，大约第21天，大约第22天，大约第23天，大约第24天，大约第25天，大约第26天，大约第27天，大约第28天，大约第29天，大约第30天，大约第31天，大约第32天，大约第33天，大约第34天，大约第35天，大约第36天，大约第37天，大约第38天，大约第39天或大约第40天，加入细胞培养基中。

[0135] 在一个具体的实施方案中，步骤b)(i)包括以该顺序在约28天至约30天后将分化的NC从其支持物解离，并且步骤b)(ii)包括以合适的细胞培养形式再接种细胞，继续分化NC约7天，向细胞培养基添加药物候选物，继续再分化NC约5天，并评估药物候选物的功效

谱。

[0136] 根据本发明的灵长类NC培养物的特征在于均匀的细胞分布，因此测试新药物候选物的功效是直接且充分标准化的。药物候选物的功效可以通过本领域已知的方法确定，包括但不限于测量与药物候选物的功效相关的表型标志物，例如标志物的表达。在一个实施方案中，药物候选物的功效通过确定疾病相关标志物的表达来测试。在一个实施方案中，药物候选物的功效通过测定疾病相关蛋白的表达来测试。在一个实施方案中，药物候选物的功效通过定量实时PCR测定相关标志物的表达来测试。在添加药物候选物后的确定时间点进行功效的确定。在进一步的实施方案中，功效的确定在加入药物候选物后在大约第1天，大约第2天，大约第3天，大约第4天，大约第5天，大约第6天，大约第7天，大约第8天，大约第9天，大约第10天，大约第11天，大约第12天，大约第13天或大约第14天进行。在一个具体的实施方案中，步骤b)(ii)包括在解离和再接种后在大约第7天将药物候选物添加到细胞培养基中，并且在将药物候选物添加到细胞培养基后在约第5天确定药物候选物的功效。可以针对不同的灵长类物种产生根据本发明的稳健且均一的分化NC培养物，并用于测试药物候选物的功效谱。在一个实施方案中，该方法适用于在灵长类物种之间，特别是NHP物种与人之间的功效的物种间比较。

[0137] 本发明的另一方面是通过本文所述方法获得的均匀分布的分化灵长类NCs的用途。在一个优选的实施方案中，通过本发明的方法获得的分化的灵长类NCs用作体外模型以研究CNS疾病的病理生理学。例如，通过本发明的方法获得的分化的灵长类NC可以用于筛选逆转、抑制或预防神经学疾病的化合物。在一个实施方案中，均匀分布的分化灵长类NCs用于筛选逆转、抑制或预防药物例如糖尿病药物的神经副作用的化合物。

[0138] 在一个实施方案中，根据步骤a)至b)的均匀分布的分化灵长类NCs用于高通量筛选选自小分子，蛋白质，肽和核酸的化合物和/或药物候选物。在另一个实施方案中，根据本发明的分化的NC用于核酸分子例如RNAi剂或反义寡核苷酸的高通量筛选。

[0139] 在一个具体的实施方案中，提供了用于选择至少一种药物候选物用于进一步开发的体外方法，其包括分别从人(Homo sapiens)和食蟹猴(Macaca fascicularis)产生均匀分布的分化神经元的细胞培养物，所述方法包括以下步骤：

[0140] a)以约30000个细胞/cm^2的密度单独针对人和食蟹猴提供神经元前体细胞(NPC)，其中所述NPC源自IPSC；

[0141] b)将NPC分化为神经细胞(NC)，包括以下步骤

[0142] (i)将NPC与补充有Shh，FGF8和抗坏血酸磷酸盐的基础培养基一起孵育约7天，以约45000个细胞/cm^2的密度重新铺板细胞，将重新铺板的细胞与补充有BDNF，GDNF，cAMP和抗坏血酸磷酸盐的基础培养基一起孵育约21天至约23天，随后将分化的NC从其支持物上解离；和

[0143] (ii)以约200000个细胞/cm^2的密度单独地以合适的细胞培养形式再接种细胞，并通过将细胞与补充有BDNF，GDNF，cAMP和抗坏血酸的基础培养基一起孵育以继续分化NC约7天，之后将细胞与药物候选物一起孵育约5天，其中将药物候选物添加到补充有BDNF，GDNF，cAMP和抗坏血酸的基础培养基中；和

[0144] 建立药物候选物对人和食蟹猴两者的功效谱，并且如果功效谱是有利的，则选择药物候选物以供进一步开发。在一个实施方案中，建立功效谱包括评估靶结合。在一个实施

方案中，进一步的开发包括在NHP物种中体内测试药物候选物和/或人体内测试。

[0145] 在一个实施方案中，提供了通过任何上述方法产生的分化的灵长类NC群体。在一个实施方案中，解离并再接种分化的灵长类NCs，并进一步分化，以获得分化的NC的均一且标准化的培养物。在一个实施方案中，灵长类NC源自健康个体。在另一个实施方案中，患者来源的灵长类NCs用于产生疾病相关的体外模型以研究CNS疾病的病理生理学。将患者特异性体细胞转化为分化的NC是一种易于获得且可重现的技术，可以产生患者特异性NC来源用于疾病模建或化合物筛选的高通量细胞试验。

[0146] 在一个实施方案中，来自Angelman综合征患者的体细胞用于产生NPC。源自一个或几个患Angelman综合征患者的NPC可用于产生Angelman综合征的疾病模型。通过本领域已知的方法，将病因基因突变引入相应的NHP基因组中，例如通过将相应的突变引入NHP NPC中，可以在相应NHP物种中重现人类单基因疾病模型。

[0147] 在另一个实施方案中，使用本发明细胞测定试验产生的数据旨在用于研究目的以解决神经疾病如神经变性疾病如阿尔茨海默氏病，帕金森氏病，亨廷顿病，肌萎缩性侧索硬化症(ALS/Lou Gehrig's病)中风和脊髓损伤，或用于治疗所述神经学疾病。重要的是，本发明导致一致且可再现的细胞培养试验。事实上，源自灵长类干细胞的先前细胞培养测定试验的主要缺点是分化细胞在细胞培养区域表面的非均匀分布。本发明通过引入解离和再接种步骤解决了这个问题，该步骤出人意料地被分化中的灵长类NCs所耐受，所述细胞保持活力并且适于以确定和均一的细胞密度接种细胞培养测定试验。给定细胞培养容器表面上的均匀细胞分布可以导致改进的和更加可重现的细胞培养试验。因此，提供了产生标准化灵长类NC培养试验的方法，其中根据步骤a)至b)得到的分化NC培养物的特征在于均匀细胞分布，在高通量板孔中均匀分布的细胞，减少的簇和/或团块形成和更相等的细胞分布。因此，所得测定试验显示增加的稳健性，增加的同质性以及试验重复之间减小的变异性。在一个实施方案中，细胞均匀分布在细胞培养区域上，特别是如通过细胞核染色所评估的。

[0148] 一个实施方案是，根据本发明的方法获得的标准化NC培养物用于确定药物候选物功效的用途。在本发明的另一方面，标准化灵长类NC培养物用于体外测试药物候选物的毒性。在本发明的另一方面，标准化灵长类NC培养物用于体外测试药物候选物的功效。培养物可以来源于健康个体和/或来自患病个体，并来自功效和/或毒性的结果整合以预测药物候选物的疾病和/或治疗相关生理学效应。在一个实施方案中，评估药物候选物的体外功效谱，并选择具有有利功效谱的药物候选物用于进一步开发。进一步的开发可以包括在NHP物种中药物候选物的体内测试和/或人体内测试。

[0149] 在一个具体实施方案中，提供了使用来自至少两种灵长类物种的均匀分布的分化神经细胞(NC)的标准化细胞培养物来确定药物候选物的体外功效谱的方法，其中所述分化的NC培养物适用于高通量筛选，该方法包括以下步骤：

[0150] a)在分化约20天至约45天后，将分化的NC从其支持物解离并以高通量细胞培养形式再接种分化的NC；

[0151] b)将再接种的NCs在分化培养基中孵育；

[0152] c)将再接种的NC与药物候选物接触；和

[0153] d)确定药物候选物的体外功效谱。

[0154] 由于NHP物种与人之间的遗传距离很小，因此在人体内进行体内测试之前，在NHP

物种上的体外和/或体内评估功效数据是令人信服的。这与例如与NHP物种相比在遗传学上更远离人类的啮齿动物物种形成对比。当评估靶向人多核苷酸序列的药物候选物时，或当药物候选物本身包含具有或接近源自人基因组的序列的多核苷酸时，NHP物种与人之间的小遗传距离尤其重要。在一个实施方案中，提供如本文所述的方法，其中确定的药物候选物的体外功效谱用于药物候选物的功效谱的种间比较，其中从至少两种灵长类物种的细胞分别产生细胞培养物，其中对所有灵长类物种的培养物应用基本相同的条件，并且其中针对所有灵长类物种，确定并比较功效谱。在一个实施方案中，提供了用于选择用于进一步开发的药物候选物的方法，其包括以下步骤：(i)根据本文所述的方法，确定药物候选物对于第一物种和第二物种的体外功效谱；和(ii)如果药物候选物的功效谱是有利的，则选择药物候选物用于进一步开发。在一个实施方案中，蛋白质编码区在第一和第二物种之间的遗传相似性高。在一个实施方案中，第一和第二物种之间的蛋白质编码区的遗传相似性高于人(Homo sapiens)和小鼠(Mus musculus)之间的遗传相似性。在进一步的实施方案中，第一物种和第二物种之间蛋白质编码区的遗传相似性高于85％，高于90％或高于95％。在一个实施方案中，第一和第二物种之间的蛋白质编码区的遗传相似性高于90％。在一个特定实施方案中，第一物种是食蟹猴(Macaca fascicularis)，第二物种是人(Homo sapiens)。根据本发明的分化的NC培养物可以针对不同物种依次产生。

[0155] 因此，在一个实施方案中，根据本发明来自一种或多种灵长类物种的分化NC的标准化细胞培养物用于药物候选物的体外功效测试，其中药物候选物包含多核苷酸或靶向多核苷酸的特定序列，其中多核苷酸序列来源于人类基因组。在另一个实施方案中，药物候选物包含核酸分子，例如RNAi剂或反义寡核苷酸。

[0156] 在本发明的另一个实施方案中，在体外功效和/或毒性测试中评估的药物候选物包含一个或多个反义寡核苷酸。在其他实施方案中，反义寡核苷酸包含10至30个核苷酸长度或由其组成，与来自人类基因组的序列具有至少90％的同一性，优选100％的同一性。应该理解，可以修饰反义寡核苷酸序列(基序序列)，以例如增加对靶核酸的核酸酶抗性和/或结合亲和力。在一个方面，反义寡核苷酸包含糖修饰的核苷并且还可以包含DNA或RNA核苷。在一些实施方案中，寡核苷酸包含糖修饰的核苷和DNA核苷。另一方面，将修饰的核苷引入寡核苷酸增强寡核苷酸对靶核酸的亲和力。在此情况下，修饰的核苷可以被称为亲和力增强的修饰核苷酸。

[0157] 在一个实施方案中，反义寡核苷酸包含至少1个修饰的核苷，例如至少2个，至少3个，至少4个，至少5个，至少6个，至少7个，至少8个，至少9个，至少10个，至少11个，至少12个，至少13个，至少14个，至少15个或至少16个修饰的核苷。在一个实施方案中，寡核苷酸包含1至10个修饰的核苷，例如2至9个修饰的核苷，例如3至8个修饰的核苷，例如4至7个修饰的核苷，例如6或7个修饰的核苷。在一些实施方案中，修饰的核苷中的至少一个是锁核酸(LNA)，例如至少2个，例如至少3个，至少4个，至少5个，至少6个，至少7个，或至少8个修饰的核苷是LNA。在又一个实施方案中，所有修饰的核苷都是LNA。

[0158] 在一个实施方案中，反义寡核苷酸包含独立地选自这三种类型的修饰(修饰的糖，修饰的核碱基和修饰的核苷间连接)或其组合的修饰。优选地，反义寡核苷酸包含一个或多个糖修饰的核苷，例如2'糖修饰的核苷。优选地，反义寡核苷酸包含一个或多个2'糖修饰的核苷，其独立地选自2'-O-烷基-RNA，2'-O-甲基-RNA，2'-烷氧基-RNA，2'-O-甲氧基乙基-

RNA，2'-氨基-DNA，2'-氟-DNA，阿拉伯糖核酸(ANA)，2'-氟-ANA和LNA核苷。甚至更优选地，一个或多个修饰的核苷是LNA。

[0159] 在另一个实施方案中，反义寡核苷酸包含至少一个修饰的核苷间连接。在一个优选的实施方案中，连续核苷酸序列内的核苷间连接是硫代磷酸酯或硼烷磷酸酯核苷间连接。

[0160] 在一些实施方案中，反义寡核苷酸包含至少一个修饰的核苷，其为2'-MOE-RNA，例如2、3、4、5、6、7、8、9或10个2'-MOE-RNA核苷单位。在一些实施方案中，所述修饰的核苷中的至少一个是2'-氟DNA，如2、3、4、5、6、7、8、9或10个2'-氟-DNA核苷单元。

[0161] 在一些实施方案中，本发明的寡核苷酸包含至少一个LNA单元，诸如1、2、3、4、5、6、7或8个LNA单元，诸如2至6个LNA单元，诸如3到7个LNA单元，4到8个LNA单元，或3、4、5、6或7个LNA单元。在一些实施方案中，所有修饰的核苷都是LNA核苷。在另一个实施方案中，寡核苷酸可以包含β-D-氧基-LNA和一个或多个以下LNA单元：硫代-LNA，氨基-LNA，氧基-LNA和/或ENA，在β-D或α-L构型中或其组合。在另一个实施方案中，所有的LNA胞嘧啶单元是5-甲基-胞嘧啶。在一个优选实施方案中，寡核苷酸或连续核苷酸序列在核苷酸序列的5'末端具有至少1个LNA单元并且在3'末端具有至少2个LNA单元。

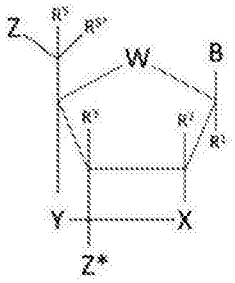
[0162] 在一些实施方案中，反义寡核苷酸包含至少一个LNA单元和至少一个2'取代的修饰的核苷。

[0163] 在本发明的一些实施方案中，反义寡核苷酸包含2'糖修饰的核苷和DNA单元。优选地，反义寡核苷酸包含LNA和DNA单元两者。优选地，LNA和DNA单元的组合总数为8-30，例如10-25，优选12-22，例如12-18，甚至更优选11-16个。在本发明的一些实施方案中，反义寡核苷酸的核苷酸序列，例如连续核苷酸序列，由至少一个或两个LNA单元组成，其余核苷酸单元是DNA单元。在一些实施方案中，反义寡核苷酸仅包含LNA核苷和天然存在的核苷(例如RNA或DNA，最优选DNA核苷)，任选地具有修饰的核苷间连接如硫代磷酸酯。

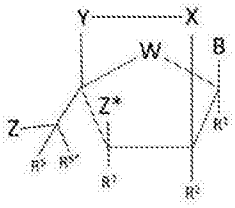
[0164] 在本发明的一个实施方案中，反义寡核苷酸能够募集RNA酶H。

[0165] 在一个优选的实施方案中，反义寡核苷酸具有gapmer设计或结构，在本文中也称为“Gapmer”。在gapmer结构中，反义寡核苷酸以'5→3'方向包含至少三个不同的结构区，即5'-侧翼，gap和3'-侧翼，F-G-F'。在该设计中，侧翼区F和F'(也称为翼区)包含修饰核苷的连续区段，其与靶核酸互补；而gap区G包含连续的核苷酸区段，当反义寡核苷酸与靶核酸形成双链体时，该区段能够招募核酸酶，优选核酸内切酶，例如RNase，例如RNase H。能够募集核酸酶，尤其是RNase H的核苷可选自：DNA，α-L-氧基-LNA，2'-氟-ANA和UNA。位于区域G的5'和3'端侧翼的区域F和F'优选包含非核酸酶募集核苷(具有3'endo结构的核苷)，更优选一个或多个亲和力增强的修饰核苷。在一些实施方案中，3'侧翼包含至少一个LNA核苷，优选至少2个LNA核苷。在一些实施方案中，5'侧翼包含至少一个LNA核苷。在一些实施方案中，5'和3'侧翼区域均包含LNA核苷。在一些实施方案中，侧翼区中的所有核苷都是LNA核苷。在其他实施方案中，侧翼区域可包含LNA核苷和其他核苷(混合侧翼)，如DNA核苷和/或非LNA的修饰核苷，如2'取代的核苷。在这种情况下，gap被定义为至少5个RNase H募集核苷(具有2'endo结构的核苷，优选DNA)的连续序列，其在5'和3'末端侧接增强亲和力的修饰核苷，优选LNA，如β-D-氧基-LNA。因此，与gap区相邻的5'侧翼区和3'侧翼区的核苷是修饰的核苷，优选非核酸酶募集核苷。

[0166] 在一些实施方案中，本发明寡聚物的修饰核苷或LNA核苷具有式I或II的通式结构：

[0167] 

β-D



α-L

式I 或 式II

[0168] 其中W选自-O-，-S-，$-N(R^a)-$，$-C(R^aR^b)-$，例如在一些实施方案中为-O-；

[0169] B表示核碱基或修饰的核碱基部分；

[0170] Z表示与相邻核苷的核苷间连接或5'-末端基团；

[0171] Z*表示与相邻核苷的核苷间连接或3'-末端基团；

[0172] X表示选自$-C(R^aR^b)-$，$-C(R^a)=C(R^b)-$，$-(R^a)=N-$，-O-，$-Si(R^a)_2-$，-S-，$-SO_2-$，$-N(R^a)-$，和>C=Z的基团。

[0173] 在一些实施方案中，X选自：-O-，-S-，NH-，NR^aR^b，$-CH_2-$，CR^aR^b，$-C(=CH_2)-$，和$-C(=CR^aR^b)-$。

[0174] 在一些实施方案中，X是-O-且Y表示选自$-C(R^aR^b)-$，$-C(R^a)=C(R^b)-$，$-C(R^a)=N-$，-O-，$-Si(R^a)_2-$，-S-，$-SO_2-$，$-N(R^a)-$，和>C=Z的基团。

[0175] 在一些实施方案中，Y是选自$-CH_2-$，$-C(R^aR^b)-$，$-CH_2CH_2-$，$-C(R^aR^b)-C(R^aR^b)-$，$-CH_2CH_2CH_2-$，$-C(R^aR^b)C(R^aR^b)C(R^aR^b)-$，$-C(R^a)=C(R^b)-$，和$-C(R^a)=N-$的基团。

[0176] 在一些实施方案中，Y是选自以下的基团：$-CH_2-$，$-CHR^a-$，$-CHCH_3-$，CR^aR^b-，或-X-Y-一起表示二价连接基团(也称为基(radicle))，一起表示由选自以下的1,2,3或4个基团/原子组成的二价连接基：$-C(R^aR^b)-$，$-C(R^a)=C(R^b)-$，$-C(R^a)=N-$，-O-，$-Si(R^a)_2-$，-S-，$-SO_2-$，$-N(R^a)-$，和>C=Z。

[0177] 在一些实施方案中，-X-Y-表示选自由以下组成的组的二价基(biradicle)：$-X-CH_2-$，$-X-CR^aR^b-$，$-X-CHR^a-$，$-X-C(HCH_3)-$，-O-Y-，$-O-CH_2-$，$-S-CH_2-$，$-NH-CH_2-$，$-O-CHCH_3-$，$-CH_2-O-CH_2$，$-O-CH(CH_3CH_3)-$，$-O-CH_2-CH_2-$，$OCH_2-CH_2-CH_2-$，$-O-CH_2OCH_2-$，$-O-NCH_2-$，$-C(=CH_2)-CH_2-$，$-NR^a-CH_2-$，N-O-CH_2，$-S-CR^aR^b-$和$-S-CHR^a-$。

[0178] 在一些实施方案中，-X-Y-表示$-O-CH_2-$或$-O-CH(CH_3)-$，其中Z选自-O-，-S-和$-N(R^a)-$，并且R^a以及，当存在R^b时，各自独立地选自氢、任选取代的C_{1-6}-烷基，任选取代的C_{2-6}-链烯基，任选取代的C_{2-6}-炔基，羟基，任选取代的C_{1-6}-烷氧基，C_{2-6}-烷氧基烷基，C_{2-6}-链烯基氧基，羧基，C_{1-6}-烷氧羰基，C_{1-6}-烷基羰基，甲酰基，芳基，芳氧基-羰基，芳氧基，芳基羰基，杂芳基，杂芳氧基-羰基，杂芳氧基，杂芳基羰基，氨基，单和二(C_{1-6}-烷基)氨基，氨甲酰基，单和二(C_{1-6}-烷基)-氨基-羰基，氨基-C_{1-6}-烷基-氨基羰基，单和二(C_{1-6}-烷基)氨基-C_{1-6}-烷基-氨基羰基，C_{1-6}-烷基-羰基氨基，脲基，C_{1-6}-烷酰基氧基，磺酰基(sulphono)，C_{1-6}-烷基磺酰氧基，硝基，叠氮基，硫烷基(sulphanyl)，C_{1-6}-烷硫基，卤素，其中芳基和杂芳基可以被任选取代，并且其中两个成对取代基R^a和R^b一起可以指任选取代的亚甲基(=CH_2)，其中对于

所有手性中心，不对称基团可以以R或S取向存在，其中R^1，R^2，R^3，R^5和R^{5*}独立地选自：氢，任选取代的C_{1-6}-烷基，任选取代的C_{2-6}-链烯基，任选取代的C_{2-6}-炔基，羟基，C_{1-6}-烷氧基，C_{2-6}-烷氧基烷基，C_{2-6}-链烯基氧基，羧基，C_{1-6}-烷氧基羰基，C_{1-6}-烷基羰基，甲酰基，芳基，芳氧基-羰基，芳氧基，芳基羰基，杂芳基，杂芳氧基-羰基，杂芳氧基，杂芳基羰基，氨基，单和二(C_{1-6}-烷基)氨基，氨甲酰基，单和二(C_{1-6}-烷基)-氨基-羰基，氨基-C_{1-6}-烷基-氨基羰基，单和二(C_{1-6}-烷基)氨基-C_{1-6}-烷基-氨基羰基，C_{1-6}-烷基-羰基氨基，脲基，C_{1-6}-烷酰基氧基，磺酰基(sulphono)，C_{1-6}-烷基磺酰氧基，硝基，叠氮基，硫烷基(sulphanyl)，C_{1-6}-烷硫基，卤素，其中芳基和杂芳基可以被任选取代，并且其中两个成对取代基一起可以指氧代，硫代(thioxo)，亚氨基，或任选取代的亚甲基。

[0179] 在一些实施方案中，R^1，R^2，R^3，R^5和R^{5*}独立地选自C_{1-6}烷基如甲基和氢。

[0180] 在一些实施方案中，R^1，R^2，R^3，R^5和R^{5*}都是氢。

[0181] 在一些实施方案中，R^1，R^2，R^3都是氢，且R^5和R^{5*}之一也是氢，并且R^5和R^{5*}中的另一个不是氢，例如是C_{1-6}烷基如甲基。

[0182] 在一些实施方案中，R^a是氢或甲基。在一些实施方案中，当存在时，R^b是氢或甲基。

[0183] 在一些实施方案中，R^a和R^b中的一个或两个是氢。

[0184] 在一些实施方案中，R^a和R^b中的一个是氢而另一个不是氢。

[0185] 在一些实施方案中，R^a和R^b之一是甲基，另一个是氢。

[0186] 在一些实施方案中，R^a和R^b都是甲基。

[0187] 在一些实施方案中，二价基-X-Y-是-O-CH_2-，W是O，且R^1，R^2，R^3，R^5和R^{5*}都是氢。这样的LNA核苷在WO99/014226，WO00/66604，WO98/039352和WO2004/046160中公开(这些公开都通过引用并入本文)，并且包括通常称为β-D-氧基LNA和α-L-氧基LNA核苷的核苷。

[0188] 在一些实施方案中，二价基-X-Y-是-S-CH_2-，W是O，且R^1，R^2，R^3，R^5和R^{5*}都是氢。这种硫代LNA核苷在WO99/014226和WO2004/046160中公开(这些公开通过引用并入本文中)。

[0189] 在一些实施方案中，二价基-X-Y-是-NH-CH_2-，W是O，且R^1，R^2，R^3，R^5和R^{5*}都是氢。这种氨基LNA核苷在WO99/014226和WO2004/046160中公开(这些公开通过引用并入本文中)。

[0190] 在一些实施方案中，二价基-X-Y-是-O-CH_2-CH_2-或-O-CH_2-CH_2-CH_2-，W是O，并且所有R^1，R^2，R^3，R^5和R^{5*}都是氢。这种LNA核苷公开于WO00/047599和Morita等，Bioorganic&Med.Chem.Lett.12 73-76(其通过引用并入本文)，并且包括通常称为2'-O-4'C-亚乙基桥接的核酸(ENA)的核苷。

[0191] 在一些实施方案中，二价基-XY-是-O-CH_2-，W是O，并且R^1，R^2，R^3全部以及R^5和R^{5*}中的一个是氢，并且R^5和R^{5*}中的另一个不是氢，如C_{1-6}烷基，如甲基。这种5'取代的LNA核苷在WO2007/134181中公开，其通过引用并入本文。

[0192] 在一些实施方案中，二价基-X-Y-是-O-CR^aR^b-，其中R^a和R^b中的一个或两个不是氢，例如甲基，W是O，并且R^1，R^2，R^3全部和R^5和R^{5*}中的一个是氢，并且R^5和R^{5*}中的另一个不是氢，例如C_{1-6}烷基，如甲基。这种双修饰的LNA核苷在WO2010/077578中公开，其通过引入并入本文。

[0193] 在一些实施方案中，二价基-X-Y-表示二价连接基团-O-CH(CH_2OCH_3)-(2'O-甲氧基乙基双环核酸-Seth等人，2010，J.Org.Chem.Vol 75(5)第1569-81页)。在一些实施方案中，二价基-X-Y-表示二价连接基团-O-CH(CH_2CH_3)-(2'O-乙基双环核酸-Seth等人，2010，

J.Org.Chem.Vol 75(5)第1569-81页)。在一些实施方案中,二价基-X-Y-是-O-CHR^a-,W是O,并且R^1,R^2,R^3,R^5和R^{5*}都是氢。这种6'取代的LNA核苷在WO10036698和WO07090071中公开,其在此引入作为参考。

[0194] 在一些实施方案中,二价基-X-Y-是-O-CH(CH_2OCH_3)-,W是O,并且所有R^1,R^2,R^3,R^5和R^{5*}都是氢。这种LNA核苷在本领域中也称为环状MOE(cMOE),并公开在WO07090071中。

[0195] 在一些实施方案中,二价基-X-Y-表示二价连接基团-O-CH(CH_3)-,呈R或S构型。在一些实施方案中,二价基-X-Y-一起表示二价连接基团-O-CH_2-O-CH_2-(Seth等人,2010,J.Org.Chem)。在一些实施方案中,二价基-X-Y-是-O-CH(CH_3)-,W是O,并且所有R^1,R^2,R^3,R^5和R^{5*}都是氢。这种6'甲基LNA核苷在本领域中也称为cET核苷,并且可以是(S)cET或(R)cET立体异构体,参见如WO07090071(β-D)和WO2010/036698(α-L)中的公开(其都通过引用并入本文)。

[0196] 在一些实施方案中,二价基-X-Y-是-O-CR^{a}R^b-,其中R^{a}和R^{b}都不是氢,W是O,且R^1,R^2,R^3,R^5和R^{5*}都是氢。在一些实施方案中,R^{a}和R^{b}都是甲基。这种6'二取代的LNA核苷在WO2009006478中公开,该文献在此引入作为参考。

[0197] 在一些实施方案中,二价基-X-Y-是-S-CHR^a-,W是O,且R^1,R^2,R^3,R^5和R^{5*}都是氢。这种6'取代的硫代LNA核苷在WO11156202中公开,其在此引入作为参考。在一些6'取代的硫代LNA实施方案中,R^{a}是甲基。

[0198] 在一些实施方案中,二价基-X-Y-是-C(=CH_2)-C(R^{a}R^b)-,例如-C(=CH_2)-CH_2-或-C(=CH_2)-CH(CH_3)-W是O,并且R^1,R^2,R^3,R^5和R^{5*}都是氢。这样的乙烯基碳LNA核苷在WO08154401和WO09067647中公开,这两篇文献均通过引用并入本文。

[0199] 在一些实施方案中,二价基-X-Y-是-N(-OR^a)-,W是O,且R^1,R^2,R^3,R^5和R^{5*}全部都是氢。在一些实施方案中,R^{a}是C_{1-6}烷基如甲基。这种LNA核苷也被称为N取代的LNA并公开在WO2008/150729中,其通过引用并入本文。在一些实施方案中,二价基-X-Y-一起表示二价连接基团-O-NR^a-CH_3-(Seth等人,2010,J.Org.Chem)。在一些实施方案中,二价基-X-Y-是-N(R^a)-,W是O,且R^1,R^2,R^3,R^5和R^{5*}全部都是氢。在一些实施方案中,R^{a}是C_{1-6}烷基如甲基。

[0200] 在一些实施方案中,R^5和R^{5*}中的一个或两个是氢,并且当被取代时,R^5和R^{5*}中的另一个是C_{1-6}烷基如甲基。在这样的实施方式中,R^1,R^2,R^3全部可以是氢,并且二价基-X-Y-可以选自-O-CH_2-或-O-C(HCR^a)-,例如-O-C(HCH$_3$)-。

[0201] 在一些实施方案中,所述二价基是-CR^{a}R^b-O-CR^{a}R^b-,例如CH_2-O-CH_2-,W是O,且所有R^1,R^2,R^3,R^5和R^{5*}都是氢。在一些实施方案中,R^{a}是C_{1-6}烷基如甲基。这种LNA核苷也被称为构象限制性核苷酸(CRN),并公开在WO2013036868中,其通过引用并入本文。

[0202] 在一些实施方案中,所述二价基是-O-CR^{a}R^b-O-CR^{a}R^b-,例如O-CH_2-O-CH_2-,W是O,并且所有R^1,R^2,R^3,R^5和R^{5*}都是氢。在一些实施方案中,R^{a}是C_{1-6}烷基如甲基。此类LNA核苷也称为COC核苷酸,并公开于Mitsuoka等人,Nucleic Acids Research 2009 37(4),1225-1238中,其通过引用并入本文。

[0203] 除非另有说明,否则将认识到,LNA核苷可以为β-D或α-L立体异构形。

[0204] 其它gapmer设计在WO2004/046160,WO2007/146511中公开并且通过引用并入。

[0205] 本发明的一个方面是调节猪、灵长类动物或人UBE3A蛋白质表达的水平,特别是增加神经元细胞中,特别是人神经元细胞中的亲本UBE3A表达。人UBE3A蛋白存在几种同种型,

在Uniprot nr.Q05086下列出。母本UBE3A基因中的几个突变可导致Angelman综合征。

[0206] 本发明该方面的寡核苷酸的靶核酸是RNA，特别是长非编码RNA。本发明寡核苷酸靶向的长非编码RNA是人SNHG14(也称为UBE3A-ATS，其Ensembl条目号ENSG00000224078，版本GRCh38.p2)。特别地，靶核酸是SNORD109B下游的区域，对应于染色体15上的位置25278410至25419462(SEQ ID NO:1)。在恒河猴(Macaca mulatta)中，UBE3A阻遏物被定义为SNORD109A下游的区域，使用Ensembl assembly MMUL1.0(SEQ ID NO:2)，对应于染色体7上的第4222848位至第4373084位(正向链)。

[0207] 在一些实施方案中，靶核酸是SEQ ID NO:1，或其天然存在的变体。在某些实施方案中，靶核酸对应于人(SEQ ID NO:1)和恒河猴(SEQ ID NO:2)之间保守的区域。在某些实施方案中，靶核酸对应于人(SEQ ID NO:1)，恒河猴(SEQ ID NO:2)和小鼠(SEQ ID NO:3)之间保守的区域。

[0208] 在某些实施方案中，靶核酸是与UBE3A前mRNA反义的区域，该区域对应于SEQ ID NO:1的55319至141053位。

[0209] 在一些实施方案中，靶核酸存在于体外或体内细胞中，例如哺乳动物细胞，特别是人细胞(靶细胞)中。在某些实施方案中，靶细胞是神经元，优选人神经元细胞。

[0210] 靶序列可以是靶核酸的子序列。在一些实施方案中，寡核苷酸靶向选自UBE3A的外显子9，外显子10，外显子13，外显子14，内含子14，外显子15，内含子15和外显子16的反义区的子序列。在一些实施方案中，寡核苷酸或连续核苷酸序列与选自以下的单链核酸分子杂交或互补：SEQ ID NO:1的位置：55319-76274，77483-77573，92157-93403和97056-97354。在一些实施方案中，寡核苷酸或连续核苷酸序列与选自SEQ ID NO:1的位置：60821-60849，77567-77583，923232-92339和97156-97172的单链核酸分子杂交或互补。

具体实施方案

[0211] 1.用于自不同灵长类物种产生均匀分布的分化神经细胞(NC)的标准化细胞培养物的体外方法，所述方法包括以下步骤：

[0212] a)提供神经元前体细胞(NPC)；

[0213] b)将NPC分化为神经细胞(NC)，包括以下步骤

[0214] (i)在分化的约20天至约45天后，将分化的NC从其支持物解离；和

[0215] (ii)以合适的细胞培养形式再接种细胞并继续分化NC约4天至约15天。

[0216] 2.根据实施方案1所述的方法，其中所述灵长类物种选自人(Homo sapiens)，食蟹猴(Macaca fascicularis)和恒河猴(Macaca mulatta)。

[0217] 3.根据实施方案1所述的方法，其中所述灵长类物种选自人(Homo sapiens)和食蟹猴(Macaca fascicularis)。

[0218] 4.根据实施方案1的方法，其中分化NC的标准化细胞培养物针对2种灵长类物种分别单独地产生，其中第一灵长类物种是人(Homo sapiens)，第二灵长类物种是食蟹猴(Macaca fascicularis)。

[0219] 5.根据实施方案1至4中任一项所述的方法，其中所述NPC源自诱导的多能干细胞(IPSCs)。

[0220] 6.根据实施方案1至5中任一项所述的方法，其中步骤b)包括用选自Shh，FGF8，

BDNF,GDNF,cAMP和抗坏血酸磷酸盐的一种或多种分化剂培养NPCs。

[0221] 7.根据实施方案1至6中任一项所述的方法,其中步骤b)(i)包括在含有Shh,FGF8和抗坏血酸磷酸盐的基础培养基中培养细胞约5至约10天的时间,然后在包含BDNF,GDNF,cAMP和抗坏血酸磷酸盐的基础培养基中再培养细胞约15天至约35天的时间,然后将分化的NC从其支持物解离。

[0222] 8.根据实施方案6或7所述的方法,其中所述一种或多种分化剂在基础培养基中的浓度,对于Shh为50-500ng/ml,对于FGF8为25-250ng/ml,对于BDNF为1-50ng/ml,对于GDNF为1-50ng/ml,对于cAMP为0.1-10mM,对于抗坏血酸磷酸盐为20-200μM。

[0223] 9.根据实施方案6至8中任一项所述的方法,其中所述一种或多种分化剂在基础培养基中的浓度,对于Shh为200ng/ml,对于FGF8为100ng/ml,对于BDNF为20ng/ml,对于GDNF为10ng/ml,对于cAMP为0.5mM,对于抗坏血酸磷酸盐为100μM。

[0224] 10.根据实施方式6至9中任一项所述的方法,其中用Shh,FGF8和抗坏血酸磷酸盐的培养期约为7天,且用BDNF,GDNF,cAMP和抗坏血酸磷酸盐的培养期为约20至约40天。

[0225] 11.根据实施例1至10中任一项所述的方法,其中所述NPC在Laminin521支持物上提供。

[0226] 12.根据实施方案1至11中任一项的方法,其中步骤b)(i)包括用细胞分离溶液解离细胞。

[0227] 13.根据实施方案12所述的方法,其中所述细胞分离溶液是Accutase。

[0228] 14.根据实施方案1至13中任一项的方法,其中步骤b)(ii)包括在Laminin521支持物上以200000个细胞/cm^2再接种细胞。

[0229] 15.根据实施方案1至14中任一项所述的方法,其中所述细胞在96孔或384孔板中再接种。

[0230] 16.根据实施方案1至15中任一项所述的方法,其中步骤b)(ii)包括在ROCK抑制剂存在下再接种所述细胞。

[0231] 17.根据实施方案16所述的方法,其中所述ROCK抑制剂是Y-27632。

[0232] 18.根据实施方案1至17中任一个所述的方法,其中步骤b)(ii)包括在再接种细胞后通过在补充了BDNF,GDNF,cAMP和抗坏血酸的基础培养基中培养细胞来继续分化。

[0233] 19.根据实施例1至18中任一项所述的方法,其中所述分化的NC表达MAP2。

[0234] 20.根据实施方案1至19中任一项的方法,其中所述分化的NC包含MAP2阳性神经突。

[0235] 21.根据实施方案1至20中任一项所述的方法,其中所述NPC源自患有神经元病症的个体。

[0236] 22.根据实施例1至21中任一项所述的方法,其中所述NPC来自健康个体。

[0237] 23.根据实施方案1至22中任一项所述的方法,其中所述细胞培养物针对不同物种依次产生。

[0238] 24.根据实施方案1至23中任一项所述的方法,其中通过细胞核染色评估,细胞在细胞培养区域上基本均匀地分布。

[0239] 25.根据实施方案1至24中任一项所述的方法,其中所述细胞的分布通过DNA染色,特别是Hoechst染色进行评估。

[0240] 26.通过实施方案1至25中任一项的方法获得的细胞培养系统。

[0241] 27.根据实施方案1至26中任一项获得的细胞培养物用于体外测试药物候选物毒性的用途。

[0242] 28.根据实施方案1至27中任一项获得的细胞培养物用于体外测试药物候选物功效的用途。

[0243] 29.根据实施方案28的用途,其中在源自实施方案21的方法的细胞培养物和源自实施方案22的方法的细胞培养物中测试功效。

[0244] 30.根据实施方案1至25中任一项获得的细胞培养物用于选择药物候选物,特别是选择用于进一步开发的药物候选物的用途。

[0245] 31.根据实施方案27至30中任一项的细胞培养物的用途,其中所述药物候选物包含多核苷酸或靶向多核苷酸的特定序列。

[0246] 32.根据实施方案27至31中任一项所述的细胞培养物的用途,其中所述药物候选物包含至少一个核酸分子,例如RNAi剂或反义寡核苷酸。

[0247] 33.根据实施方案32所述的细胞培养物的用途,其中所述核酸分子包含一个或多个2'糖修饰的核苷,所述核苷独立地选自2'-O-烷基-RNA,2'-O-甲基-RNA,2'-烷氧基-RNA,2'-O-甲氧基乙基-RNA,2'-氨基-DNA,2'-氟-DNA,阿拉伯糖核酸(ANA),2'-氟-ANA和LNA核苷。

[0248] 34.根据实施方案33所述的细胞培养物的用途,其中所述一个或多个2'糖修饰的核苷是LNA核苷。

[0249] 35.根据实施方案34所述的细胞培养物的用途,其中所述LNA核苷选自β-D-氧基-LNA,α-L-氧基-LNA,β-D-氨基-LNA,α-L-氨基-LNA,β-D-硫代-LNA,α-L-硫代-LNA,(S)cET,(c)cET,β-D-ENA或α-L-ENA。

[0250] 36.根据实施方案32至35中任一项所述的细胞培养物的用途,其中所述核酸分子包含至少一个修饰的核苷间连接。

[0251] 37.根据实施方案36的细胞培养物的用途,其中所述连续核苷酸序列中的核苷间连接是硫代磷酸酯核苷间连接。

[0252] 38.根据实施例32至37中任一项所述的细胞培养物的用途,其中所述反义寡核苷酸能够募集RNase H.

[0253] 39.根据实施方案32至38中任一项所述的细胞培养物的用途,其中所述反义寡核苷酸是gapmer。

[0254] 40.根据实施方案38或39所述的细胞培养物的用途,其中所述寡核苷酸是式5'-F-G-F'-3'的gapmer,其中区域F和F'独立地包含1至7个修饰的核苷并且G是能够募集RNase H的6至16个核苷的区域。

[0255] 41.基本上如本文所述的方法和用途。

[0256] 这里描述的任何实施方案可以单独使用或组合使用。下面的实施例更详细地解释了本发明。给出以下制备和实施例旨在使本领域技术人员能够更清楚地理解和实践本发明。然而,本发明的范围不受限于示例性实施方案,这些实施方案仅用于举例说明本发明的各单个方面,并且功能上等同的方法在本发明的范围内。事实上,除了本文公开的那些之外,本发明的各种修改对于本领域技术人员而言从前面的描述和附图将变得显而易见。这

些修改旨在落入所附权利要求的范围内。

[0257] 实施例

[0258] 材料和方法

[0259] 表3:列出与SEQ ID NO:1互补的寡核苷酸或连续核苷碱基序列(由SEQ ID NO指出的基序序列)、由这些实施的寡核苷酸设计、以及基于该基序序列设计的具体寡核苷酸化合物(由CMP ID NO指出)。

[0260]

Seq ID NO	基序	设计	化合物	CMP ID NO	ΔG°	起始 SEQ ID NO 1
4	AACTTCATCAATATTTCCC	3-13-3	AACttcatcaatatttCCC	4_1	-23,36	1677
4	AACTTCATCAATATTTCCC	2-15-2	AActtcatcaatatttcCC	4_2	-19,60	1677
5	ACTTCATCAATATTTCCC	3-12-3	ACTtcatcaatatttCCC	5_1	-23,80	1677
5	ACTTCATCAATATTTCCC	2-14-2	ACttcatcaatatttcCC	5_2	-20,24	1677
6	CAACTTCATCAATATTTCCC	2-14-4	CAacttcatcaatattTCCC	6_1	-25,64	1677
6	CAACTTCATCAATATTTCCC	2-16-2	CAacttcatcaatatttcCC	6_2	-22,28	1677
7	CAACTTCATCAATATTTCC	4-13-2	CAACttcatcaatatttCC	7_1	-21,47	1678
7	CAACTTCATCAATATTTCC	2-15-2	CAacttcatcaatatttCC	7_2	-19,46	1678
8	CCAACTTCATCAATATTTCC	3-14-3	CCAacttcatcaatattTCC	8_1	-25,64	1678
9	CCCAACTTCATCAATATTTC	4-14-2	CCCAacttcatcaatattTC	9_1	-25,64	1679
10	ACCCAACTTCATCAATATTT	2-16-2	ACccaacttcatcaatatTT	10_1	-20,05	1680
11	CCCAACTTCATCAATATTT	4-13-2	CCCAacttcatcaatatTT	11_1	-23,96	1680
11	CCCAACTTCATCAATATTT	2-15-2	CCcaacttcatcaatatTT	11_2	-20,28	1680
12	ACCCAACTTCATCAATATT	4-13-2	ACCCaacttcatcaataTT	12_1	-23,64	1681
12	ACCCAACTTCATCAATATT	2-15-2	ACccaacttcatcaataTT	12_2	-19,18	1681
13	CCCAACTTCATCAATATT	4-12-2	CCCAacttcatcaataTT	13_1	-23,09	1681
13	CCCAACTTCATCAATATT	2-14-2	CCcaacttcatcaataTT	13_2	-19,41	1681
14	TACCCAACTTCATCAATAT	2-15-2	TAcccaacttcatcaatAT	14_1	-19,31	1682
15	TACCCAACTTCATCAATA	2-14-2	TAcccaacttcatcaaTA	15_1	-19,14	1683
16	TTACCCAACTTCATCAATA	2-15-2	TTacccaacttcatcaaTA	16_1	-19,74	1683
17	TTTACCCAACTTCATCAAT	4-13-2	TTTAcccaacttcatcaAT	17_1	-21,68	1684
17	TTTACCCAACTTCATCAAT	2-15-2	TTtacccaacttcatcaAT	17_2	-19,22	1684
18	ATACTTTACCCAACTTCAT	3-13-3	ATActttacccaacttCAT	18_1	-23,44	1688
18	ATACTTTACCCAACTTCAT	2-15-2	ATactttacccaacttcAT	18_2	-20,13	1688
19	TACTTTACCCAACTTCAT	3-12-3	TACtttacccaacttCAT	19_1	-22,78	1689
19	TACTTTACCCAACTTCAT	2-14-2	TActttacccaacttcAT	19_2	-19,30	1688
20	TTATACTTTACCCAACTTCA	2-16-2	TTatactttacccaacttCA	20_1	-21,40	1689
21	TCACTGTTCTGACTTT	3-10-3	TCActgttctgacTTT	21_1	-19,11	1712
22	TTCAATCTCTATCTCATCAT	2-16-2	TTcaatctctatctcatcAT	22_1	-19,42	4169
23	CTTCAATCTCTATCTCATCA	4-14-2	CTTCaatctctatctcatCA	23_1	-24,21	4170
23	CTTCAATCTCTATCTCATCA	2-16-2	CTtcaatctctatctcatCA	23_2	-22,04	4170
24	TTCAATCTCTATCTCATCA	2-15-2	TTcaatctctatctcatCA	24_1	-19,44	4170

[0261]

Seq ID NO	基序	设计	化合物	CMP ID NO	ΔG°	起始 SEQ ID NO 1
25	CTTCAATCTCTATCTCATC	2-15-2	CTtcaatctctatctcaTC	25_1	-19,87	4171
26	ACTTCAATCTCTATCTCAT	3-13-3	ACTtcaatctctatctCAT	26_1	-22,36	4172
26	ACTTCAATCTCTATCTCAT	2-15-2	ACttcaatctctatctcAT	26_2	-19,08	4172
27	CACTTCAATCTCTATCTCAT	2-16-2	CActtcaatctctatctcAT	27_1	-20,98	4172
28	ACTTCAATCTCTATCTCA	2-12-4	ACttcaatctctatCTCA	28_1	-21,96	4173
28	ACTTCAATCTCTATCTCA	2-14-2	ACttcaatctctatctCA	28_2	-19,10	4173
29	CACTTCAATCTCTATCTCA	2-13-4	CActtcaatctctatCTCA	29_1	-23,86	4173
29	CACTTCAATCTCTATCTCA	2-15-2	CActtcaatctctatctCA	29_2	-21,00	4173
30	ACACTTCAATCTCTATCTC	2-15-2	ACacttcaatctctatcTC	30_1	-19,38	4174
31	TACACTTCAATCTCTATCTC	2-14-4	TAcacttcaatctctaTCTC	31_1	-23,31	4174
31	TACACTTCAATCTCTATCTC	2-16-2	TAcacttcaatctctatcTC	31_2	-20,53	4174
32	TACACTTCAATCTCTATCT	4-13-2	TACActtcaatctctatCT	32_1	-22,34	4175
33	CTTTGTCTCTCTTTACT	2-13-2	CTttgtctctctttaCT	33_1	-19,36	4374
34	TATACCTTTCTTTAACCC	3-12-3	TATaccttttcttaaCCC	34_1	-24,89	8118
34	TATACCTTTCTTTAACCC	2-14-2	TAtacctttctttaacCC	34_2	-20,83	8118
35	TGTTTATACCCTTTCC	2-12-2	TGtttataccctttCC	35_1	-20,33	9212
36	TCTCCTTTATGACTCC	2-10-4	TCtcctttatgaCTCC	36_1	-23,29	10839
37	CTTCTCCTTTATGACTC	2-13-2	CTtctcctttatgacTC	37_1	-19,26	10840
38	CCATTTATTTCCATTTATT	4-13-2	CCATttatttccatttaTT	38_1	-22,32	15567
38	CCATTTATTTCCATTTATT	2-15-2	CCatttatttccatttaTT	38_2	-19,61	15567
39	CTTTCCATTTATTTCCATTT	2-14-4	CTttccatttatttccATTT	39_1	-23,14	15570
40	TCTTTCCATTTATTTCCATT	2-14-4	TCtttccatttatttcCATT	40_1	-24,62	15571
41	ATTACCCATCCGTTCT	2-12-2	ATtacccatccgttCT	41_1	-21,15	21965
42	GCATTAGGCACATTACAT	3-12-3	GCAttaggcacattaCAT	42_1	-23,96	22211
43	ATTATTATTTAACCTTCCTA	2-16-2	ATtattatttaaccttccTA	43_1	-19,28	30451
44	ACATTATTATTTAACCTTCC	4-14-2	ACATtattatttaaccttCC	44_1	-22,84	30453
44	ACATTATTATTTAACCTTCC	2-16-2	ACattattatttaaccttCC	44_2	-20,13	30453
45	CATTATTATTTAACCTTCC	4-13-2	CATTattatttaaccttCC	45_1	-22,04	30453
45	CATTATTATTTAACCTTCC	2-15-2	CAttattatttaaccttCC	45_2	-19,55	30453
46	CCTCTGCTTATAACTTT	2-13-2	CCtctgcttataacTT	46_1	-19,15	30699
47	CTACTATACTTTCCTCT	2-11-4	CTactatactttcCTCT	47_1	-22,32	30711
48	GTTCTACTATACTTTCC	4-11-2	GTTCtactatacttCC	48_1	-21,69	30714
48	GTTCTACTATACTTTCC	2-13-2	GTtctactatacttCC	48_2	-19,21	30714
49	CACCTGATAACAGACCCT	3-12-3	CACctgataacagacCCT	49_1	-26,38	36068
50	CACCTGATAACAGACC	3-10-3	CACctgataacagACC	50_1	-21,10	36070
51	CCCACCAAAGGATATATT	3-12-3	CCCaccaaaggatatATT	51_1	-23,47	37208
52	ACCAGCTACAGGAACCTC	3-12-3	ACCagctacaggaacCTC	52_1	-26,57	46132
53	CTATATCTCACTCCTATTT	4-13-2	CTATatctcactcctatTT	53_1	-23,07	48143
53	CTATATCTCACTCCTATTT	2-13-4	CTatatctcactcctATTT	53_2	-22,12	48143
54	CTATATCTCACTCCTATT	2-14-2	CTatatctcactcctaTT	54_1	-19,40	48144

[0262]

Seq ID NO	基序	设计	化合物	CMP ID NO	ΔG°	起始 SEQ ID NO 1
54	CTATATCTCACTCCTATT	2-12-4	CTatatctcactccTATT	54_2	-22,28	48144
54	CTATATCTCACTCCTATT	3-12-3	CTAtatctcactcctATT	54_3	-21,44	48144
55	CTACTATATCTCACTCCTAT	2-16-2	CTactatatctcactcctAT	55_1	-22,00	48145
55	CTACTATATCTCACTCCTAT	2-14-4	CTactatatctcactcCTAT	55_2	-25,54	48145
56	TACTATATCTCACTCCTAT	2-13-4	TActatatctcactcCTAT	56_1	-23,29	48145
57	CTACTATATCTCACTCCTA	2-15-2	CTactatatctcactccTA	57_1	-21,91	48146
58	TACTATATCTCACTCCTA	2-14-2	TActatatctcactccTA	58_1	-19,66	48146
58	TACTATATCTCACTCCTA	2-12-4	TActatatctcactCCTA	58_2	-23,59	48146
58	TACTATATCTCACTCCTA	3-12-3	TACtatatctcactcCTA	58_3	-22,62	48146
59	CTACTATATCTCACTCCT	2-14-2	CTactatatctcactcCT	59_1	-21,25	48147
59	CTACTATATCTCACTCCT	4-12-2	CTACtatatctcactcCT	59_2	-23,87	48147
60	CTACTATATCTCACTCC	2-13-2	CTactatatctcactCC	60_1	-20,13	48148
60	CTACTATATCTCACTCC	2-11-4	CTactatatctcaCTCC	60_2	-23,00	48148
60	CTACTATATCTCACTCC	3-11-3	CTActatatctcacTCC	60_3	-22,56	48148
61	CCTACTATATCTCACTC	2-11-4	CCtactatatctcACTC	61_1	-21,93	48149
62	CTCCTACTATATCTCACTC	4-13-2	CTCCtactatatctcacTC	62_1	-25,69	48149
63	TCCTACTATATCTCACTC	3-12-3	TCCtactatatctcaCTC	63_1	-23,88	48149
64	CTCCTACTATATCTCACT	4-12-2	CTCCtactatatctcaCT	64_1	-24,87	48150
64	CTCCTACTATATCTCACT	3-12-3	CTCctactatatctcACT	64_2	-22,93	48150
65	TTTCCTCTCCTACTATATC	2-15-2	TTtcctctcctactataTC	65_1	-21,23	48155
66	ATCCATATCCTTTCCT	3-10-3	ATCcatatccttttCCT	66_1	-24,02	48168
67	CATCCATATCCTTTCCT	4-11-2	CATCcatatccttttcCT	67_1	-24,94	48168
68	ATCATCCATATCCTTTCC	4-12-2	ATCAtccatatccttttCC	68_1	-25,69	48169
69	CATCATCCATATCCTTTC	4-12-2	CATCatccatatccttTC	69_1	-23,32	48170
69	CATCATCCATATCCTTTC	2-14-2	CAtcatccatatccttTC	69_2	-20,72	48170
69	CATCATCCATATCCTTTC	2-12-4	CAtcatccatatccTTTC	69_3	-22,56	48170
70	TACATCATCCATATCCTTTC	2-16-2	TAcatcatccatatccttTC	70_1	-22,45	48170
70	TACATCATCCATATCCTTTC	4-14-2	TACAtcatccatatccttTC	70_2	-25,00	48170
70	TACATCATCCATATCCTTTC	2-14-4	TAcatcatccatatccTTTC	70_3	-24,29	48170
71	ACATCATCCATATCCTTT	3-12-3	ACAtcatccatatccTTT	71_1	-22,11	48171
72	CATCATCCATATCCTTT	2-13-2	CAtcatccatatcctTT	72_1	-19,04	48171
72	CATCATCCATATCCTTT	4-11-2	CATCatccatatcctTT	72_2	-21,64	48171
73	TACATCATCCATATCCTTT	2-15-2	TAcatcatccatatcctTT	73_1	-20,76	48171
73	TACATCATCCATATCCTTT	2-13-4	TAcatcatccatatcCTTT	73_2	-23,36	48171
73	TACATCATCCATATCCTTT	3-13-3	TACatcatccatatccTTT	73_3	-22,88	48171
74	ATACATCATCCATATCCTT	2-15-2	ATacatcatccatatccTT	74_1	-20,80	48172
74	ATACATCATCCATATCCTT	4-13-2	ATACatcatccatatccTT	74_2	-23,12	48172
75	TACATCATCCATATCCTT	2-14-2	TAcatcatccatatccTT	75_1	-19,97	48172
75	TACATCATCCATATCCTT	4-12-2	TACAtcatccatatccTT	75_2	-22,52	48172
76	TATACATCATCCATATCCTT	2-16-2	TAtacatcatccatatccTT	76_1	-21,36	48172

[0263]

Seq ID NO	基序	设计	化合物	CMP ID NO	ΔG°	起始 SEQ ID NO 1
77	ATACATCATCCATATCCT	3-12-3	ATAcatcatccatatCCT	77_1	-24,15	48173
77	ATACATCATCCATATCCT	2-14-2	ATacatcatccatatcCT	77_2	-20,55	48173
77	ATACATCATCCATATCCT	2-13-3	ATacatcatccatatCCT	77_3	-22,92	48173
78	ATATACATCATCCATATCCT	2-16-2	ATatacatcatccatatcCT	78_1	-22,04	48173
79	TACATCATCCATATCCT	2-11-4	TAcatcatccataTCCT	79_1	-23,21	48173
79	TACATCATCCATATCCT	2-13-2	TAcatcatccatatcCT	79_2	-19,71	48173
79	TACATCATCCATATCCT	4-11-2	TACAtcatccatatcCT	79_3	-22,27	48173
80	TATACATCATCCATATCCT	2-15-2	TAtacatcatccatatcCT	80_1	-21,11	48173
80	TATACATCATCCATATCCT	3-13-3	TATacatcatccatatCCT	80_2	-25,15	48173
80	TATACATCATCCATATCCT	4-13-2	TATAcatcatccatatcCT	80_3	-24,01	48173
81	ATACATCATCCATATCC	3-11-3	ATAcatcatccataTCC	81_1	-21,79	48174
82	ATATACATCATCCATATCC	4-13-2	ATATacatcatccatatCC	82_1	-23,73	48174
82	ATATACATCATCCATATCC	2-15-2	ATatacatcatccatatCC	82_2	-20,93	48174
83	TATACATCATCCATATCC	2-14-2	TAtacatcatccatatCC	83_1	-20,00	48174
83	TATACATCATCCATATCC	4-12-2	TATAcatcatccatatCC	83_2	-22,90	48174
84	TATATACATCATCCATATCC	2-16-2	TAtatacatcatccatatCC	84_1	-21,49	48174
84	TATATACATCATCCATATCC	4-14-2	TATAtacatcatccatatCC	84_2	-24,29	48174
85	GCTTCATATTTCTCCA	2-12-2	GCttcatatttctcCA	85_1	-20,44	49345
86	CATCTTGTTCTTTACCT	2-13-2	CAtcttgttctttacCT	86_1	-19,67	49581
87	TATATTCACCATTGCC	2-10-4	TAtattcaccatTGCC	87_1	-22,70	49724
88	CCTTATATTCACCATTG	2-13-2	CCttatattcaccatTG	88_1	-19,44	49726
88	CCTTATATTCACCATTG	2-11-4	CCttatattcaccATTG	88_2	-21,25	49726
89	CCTCCTTATATTCACC	4-10-2	CCTCcttatattcaCC	89_1	-24,64	49730
90	CCCTTCCTTTATTCAA	3-10-3	CCCttcctttattCAA	90_1	-23,86	50189
91	CCTTACTGTTAAATCCT	2-13-2	CCttactgttaaatcCT	91_1	-19,81	50475
92	CAGGCAGATAACCTCCAA	3-12-3	CAGgcagataacctcCAA	92_1	-25,31	52419
93	CAGCAGGCAGATAACCTC	3-12-3	CAGcaggcagataacCTC	93_1	-25,88	52422
94	CGAATCTTGACATACAGG	3-12-3	CGAatcttgacatacAGG	94_1	-21,47	53955
95	CTCATACTTGCTTTAAT	4-11-2	CTCAtacttgctttaAT	95_1	-19,10	60821
95	CTCATACTTGCTTTAAT	2-13-2	CTcatacttgctttaAT	95_2	-16,35	60821
96	ACATCTCATACTTGCTT	2-11-4	ACAtctcatacttGCTT	96_1	-21,31	60825
96	ACATCTCATACTTGCTT	2-13-2	ACAtctcatacttgcTT	96_2	-17,66	60825
96	ACATCTCATACTTGCTT	2-12-3	ACAtctcatacttgCTT	96_3	-19,52	60825
97	ACATCTCATACTTGCT	2-10-4	ACAtctcatactTGCT	97_1	-21,18	60826
97	ACATCTCATACTTGCT	2-12-2	ACAtctcatacttgCT	97_2	-17,70	60826
97	ACATCTCATACTTGCT	2-11-3	ACAtctcatacttGCT	97_3	-19,49	60826
97	ACATCTCATACTTGCT	4-10-2	ACATctcatacttgCT	97_4	-20,48	60826
98	TACATCTCATACTTGCT	2-11-4	TAcatctcatactTGCT	98_1	-22,33	60826
98	TACATCTCATACTTGCT	2-13-2	TAcatctcatacttgCT	98_2	-18,85	60826
98	TACATCTCATACTTGCT	4-11-2	TACAtctcatacttgCT	98_3	-21,40	60826

[0264]

Seq ID NO	基序	设计	化合物	CMP ID NO	ΔG°	起始 SEQ ID NO 1
99	CCTACATCTCATACTTGC	3-12-3	CCTacatctcatactTGC	99_1	-26,29	60827
99	CCTACATCTCATACTTGC	2-14-2	CCtacatctcatacttGC	99_2	-22,98	60827
99	CCTACATCTCATACTTGC	2-13-3	CCtacatctcatactTGC	99_3	-24,67	60827
99	CCTACATCTCATACTTGC	2-12-4	CCtacatctcatacTTGC	99_4	-25,70	60827
100	CTACATCTCATACTTGC	3-11-3	CTAcatctcatactTGC	100_1	-22,33	60827
100	CTACATCTCATACTTGC	2-13-2	CTacatctcatacttGC	100_2	-19,41	60827
100	CTACATCTCATACTTGC	2-12-3	CTacatctcatactTGC	100_3	-21,10	60827
101	TACATCTCATACTTGC	3-10-3	TACatctcatactTGC	101_1	-19,94	60827
101	TACATCTCATACTTGC	2-12-2	TAcatctcatacttGC	101_2	-17,15	60827
101	TACATCTCATACTTGC	2-11-3	TAcatctcatactTGC	101_3	-18,85	60827
101	TACATCTCATACTTGC	4-10-2	TACAtctcatacttGC	101_4	-19,71	60827
102	CCTACATCTCATACTTG	4-11-2	CCTAcatctcatactTG	102_1	-22,52	60828
102	CCTACATCTCATACTTG	2-13-2	CCtacatctcatactTG	102_2	-19,67	60828
102	CCTACATCTCATACTTG	3-12-2	CCTacatctcatactTG	102_3	-21,29	60828
102	CCTACATCTCATACTTG	3-11-3	CCTacatctcatacTTG	102_4	-22,31	60828
103	ACCTACATCTCATACTT	3-11-3	ACCtacatctcataCTT	103_1	-21,93	60829
103	ACCTACATCTCATACTT	2-13-2	ACctacatctcatacTT	103_2	-17,76	60829
103	ACCTACATCTCATACTT	2-11-4	ACctacatctcatACTT	103_3	-20,03	60829
103	ACCTACATCTCATACTT	3-12-2	ACCtacatctcatacTT	103_4	-20,26	60829
104	CCTACATCTCATACTT	3-10-3	CCTacatctcataCTT	104_1	-21,50	60829
104	CCTACATCTCATACTT	2-12-2	CCtacatctcatacTT	104_2	-18,21	60829
104	CCTACATCTCATACTT	2-10-4	CCtacatctcatACTT	104_3	-20,48	60829
105	TACCTACATCTCATACTT	4-12-2	TACCtacatctcatacTT	105_1	-22,49	60829
105	TACCTACATCTCATACTT	2-14-2	TAcctacatctcatacTT	105_2	-18,81	60829
105	TACCTACATCTCATACTT	2-13-3	TAcctacatctcataCTT	105_3	-20,48	60829
105	TACCTACATCTCATACTT	2-12-4	TAcctacatctcatACTT	105_4	-21,08	60829
106	TTACCTACATCTCATACTT	3-13-3	TTAcctacatctcataCTT	106_1	-22,30	60829
106	TTACCTACATCTCATACTT	2-15-2	TTacctacatctcatacTT	106_2	-19,40	60829
106	TTACCTACATCTCATACTT	2-14-3	TTacctacatctcataCTT	106_3	-21,08	60829
106	TTACCTACATCTCATACTT	2-13-4	TTacctacatctcatACTT	106_4	-21,67	60829
107	ACCTACATCTCATACT	4-10-2	ACCTacatctcataCT	107_1	-21,72	60830
107	ACCTACATCTCATACT	2-12-2	ACctacatctcataCT	107_2	-17,61	60830
107	ACCTACATCTCATACT	3-11-2	ACCtacatctcataCT	107_3	-20,10	60830
107	ACCTACATCTCATACT	2-10-4	ACctacatctcaTACT	107_4	-20,11	60830
108	TACCTACATCTCATACT	4-11-2	TACCtacatctcataCT	108_1	-22,34	60830
108	TACCTACATCTCATACT	2-13-2	TAcctacatctcataCT	108_2	-18,66	60830
108	TACCTACATCTCATACT	3-12-2	TACctacatctcataCT	108_3	-19,85	60830
108	TACCTACATCTCATACT	3-11-3	TACctacatctcatACT	108_4	-20,44	60830
109	TTACCTACATCTCATACT	2-12-4	TTacctacatctcaTACT	109_1	-21,75	60830
109	TTACCTACATCTCATACT	2-14-2	TTacctacatctcataCT	109_2	-19,25	60830

[0265]

Seq ID NO	基序	设计	化合物	CMP ID NO	ΔG°	起始 SEQ ID NO 1
109	TTACCTACATCTCATACT	3-13-2	TTAcctacatctcataCT	109_3	-20,48	60830
109	TTACCTACATCTCATACT	3-12-3	TTAcctacatctcatACT	109_4	-21,08	60830
110	TTACCTACATCTCATAC	3-11-3	TTAcctacatctcaTAC	110_1	-19,50	60831
110	TTACCTACATCTCATAC	2-13-2	TTacctacatctcatAC	110_2	-16,37	60831
111	GTTACCTACATCTCATA	2-11-4	GTtacctacatctCATA	111_1	-21,69	60832
111	GTTACCTACATCTCATA	2-13-2	GTtacctacatctcaTA	111_2	-18,74	60832
111	GTTACCTACATCTCATA	3-12-2	GTTacctacatctcaTA	111_3	-19,98	60832
112	GTTACCTACATCTCAT	3-10-3	GTTacctacatctCAT	112_1	-20,69	60833
112	GTTACCTACATCTCAT	2-12-2	GTtacctacatctcAT	112_2	-17,37	60833
113	ATATACCCAAAGGCACCT	3-12-3	ATAtacccaaaggcaCCT	113_1	-25,99	62200
114	TCTACTCATCCTTTAACTCA	2-14-4	TCtactcatcctttaaCTCA	114_1	-25,63	62251
115	CCTTAATCTGTATCACT	2-13-2	CCttaatctgtatcaCT	115_1	-19,58	62286
116	CCATACACAGCACATA	2-12-2	CCatacacagcacaTA	116_1	-19,04	62424
117	CTCCATACACAGCACAT	2-13-2	CTccatacacagcacAT	117_1	-20,08	62425
118	CAGAATAATTCTCCTCC	2-13-2	CAgaataattctcctCC	118_1	-19,86	62441
119	GTCCTACATATATACC	4-10-2	GTCCtacatatataCC	119_1	-22,09	66380
120	TGCTTCCTTACTAACC	4-10-2	TGCTtccttactaaCC	120_1	-23,93	66701
120	TGCTTCCTTACTAACC	2-12-2	TGcttccttactaaCC	120_2	-20,10	66701
121	CCCTTTGTAATCATCT	4-10-2	CCCTttgtaatcatCT	121_1	-23,44	66838
122	TCCCTTTGTAATCATCT	2-13-2	TCcctttgtaatcatCT	122_1	-19,97	66838
123	CTGCCATCAATACCAT	2-12-2	CTgccatcaataccAT	123_1	-19,14	68918
124	TCACTGCCATCAATACC	2-13-2	TCactgccatcaataCC	124_1	-21,35	68920
125	ATTCTTACTTTATTCCTCA	2-15-2	ATtcttactttattcctCA	125_1	-20,16	70033
126	TCACTTTCCAGATATCA	4-11-2	TCACtttccagatatCA	126_1	-21,61	77567
126	TCACTTTCCAGATATCA	2-13-2	TCactttccagatatCA	126_2	-18,65	77567
127	TCCTTCAAATTCCACATAC	3-13-3	TCCttcaaattccacaTAC	127_1	-24,09	82053
128	ACATGTCCCTTTATATT	4-11-2	ACATgtcccttTataTT	128_1	-20,87	92323
128	ACATGTCCCTTTATATT	2-13-2	ACatgtcccttTataTT	128_2	-17,66	92323
128	ACATGTCCCTTTATATT	3-12-2	ACAtgtcccttTataTT	128_3	-19,13	92323
128	ACATGTCCCTTTATATT	3-11-3	ACAtgtcccttTatATT	128_4	-20,03	92323
129	ACATGTCCCTTTATAT	3-10-3	ACAtgtcccttTaTAT	129_1	-20,11	92324
129	ACATGTCCCTTTATAT	2-12-2	ACatgtcccttTatAT	129_2	-16,74	92324
130	CCAAGAAAGGAGCAAGCT	3-12-3	CCAagaaaggagcaaGCT	130_1	-25,26	97146
131	TCCAAGAAAGGAGCAAGC	3-12-3	TCCaagaaaggagcaAGC	131_1	-24,12	97147
132	CTCATCCCTCCAAGAAA	4-11-2	CTCAtccctccaagaAA	132_1	-22,58	97156
132	CTCATCCCTCCAAGAAA	2-13-2	CTcatccctccaagaAA	132_2	-19,83	97156
132	CTCATCCCTCCAAGAAA	3-12-2	CTCatccctccaagaAA	132_3	-21,11	97156
133	TCATCCCTCCAAGAAA	4-10-2	TCATccctccaagaAA	133_1	-20,41	97156
133	TCATCCCTCCAAGAAA	2-12-2	TCatccctccaagaAA	133_2	-17,63	97156
133	TCATCCCTCCAAGAAA	3-11-2	TCAtccctccaagaAA	133_3	-19,09	97156

[0266]

Seq ID NO	基序	设计	化合物	CMP ID NO	ΔG°	起始 SEQ ID NO 1
133	TCATCCCTCCAAGAAA	3-10-3	TCAtccctccaagAAA	133_4	-19,81	97156
134	CACCTCCCTATTACATAAA	4-13-2	CACCtcccctattacataAA	134_1	-24,18	100018
134	CACCTCCCTATTACATAAA	2-15-2	CAcctcccctattacataAA	134_2	-20,51	100018
135	CACCTCCCTATTACATAA	4-12-2	CACCtcccctattacatAA	135_1	-23,75	100019
135	CACCTCCCTATTACATAA	2-14-2	CAcctcccctattacatAA	135_2	-20,07	100019
136	CCTCCCTATTACATAA	2-12-2	CCtcccctattacatAA	136_1	-18,40	100019
137	CTAAATCTTCCAATTCATA	2-15-2	CTaaatcttccaattcaTA	137_1	-18,12	106139
138	TATCCCTTGATTATCCT	2-13-2	TAtcccttgattatcCT	138_1	-20,68	109406
139	CCTCTTTGTCAAATACT	2-13-2	CCtctttgtcaaataCT	139_1	-19,30	110768
140	CAGCTTATTTACCTCTT	2-13-2	CAgcttatttacctcTT	140_1	-19,30	114828
141	ACTCTTTACCTCTAACACT	4-13-2	ACTCtttacctctaacaCT	141_1	-24,26	117468
142	TTACTCTTTACCTCTAACAC	3-14-3	TTActctttacctctaaCAC	142_1	-23,23	117469
143	CCAACCTAATACCTTAATA	2-15-2	CCaacctaataccttaaTA	143_1	-20,27	118639
144	TACCAACCTAATACCTTAA	2-15-2	TAccaacctaataccttAA	144_1	-18,32	118641
145	CCAATACCCACAAACC	3-10-3	CCAatacccacaaACC	145_1	-23,17	124162
145	CCAATACCCACAAACC	2-12-2	CCaataccccacaaaCC	145_2	-20,85	124162
146	CCATTATTCTACTTTGT	3-11-3	CCAttattctacttTGT	146_1	-21,79	125501
146	CCATTATTCTACTTTGT	2-13-2	CCattattctactttGT	146_2	-18,63	125501
147	CATTTCCTTATCTTCACA	2-14-2	CAttcctlatcttcaCA	147_1	-20,39	125529
148	TCATTTCCTTATCTTCACA	4-13-2	TCATttccttatcttcaCA	148_1	-24,13	125529
149	AATAATTCCTCATTTCCT	2-14-2	AAtaattcctcatttcCT	149_1	-18,01	125539
150	ACAATAATTCCTCATTTCC	3-13-3	ACAataattcctcattTCC	150_1	-22,71	125540
150	ACAATAATTCCTCATTTCC	2-15-2	ACaataattcctcatttCC	150_2	-20,23	125540

[0267] 设计是指gapmer设计,F-G-F',其中每个数字分别代表连续的修饰核苷(例如2'修饰的核苷)的数目(第一个数字=5'侧翼),接着是DNA核苷的数目(第二个数字=gap区域),接着是修饰核苷(例如2'修饰的核苷)的数目(第三个数字=3'侧翼),任选地之前或之后为DNA和LNA的其它重复区域,所述其它重复区域不一定是与靶核酸互补的连续序列的一部分。

[0268] 对于寡核苷酸化合物,大写字母代表β-D-氧基LNA核苷,小写字母代表DNA核苷,所有LNA C均为5-甲基胞嘧啶,且5-甲基DNA胞嘧啶以"e"呈现,所有核苷间连接均为硫代磷酸酯核苷间连接。

[0269] 寡核苷酸合成

[0270] 寡核苷酸合成在本领域中通常是已知的。以下是可应用的方案。本发明的寡核苷酸可以通过在所使用的装置,支持物和浓度方面略作改变的方法而产生。

[0271] 寡核苷酸在尿嘧啶通用支持物上使用亚磷酰胺方法在MerMade12或Oligomaker DNA/RNA合成仪上以1-4μmol规模合成。在合成结束时,使用氨水在60℃进行5-16小时,将寡核苷酸从固相支持物上切下。通过反相HPLC(RP-HPLC)或通过固相萃取纯化寡核苷酸并通过UPLC表征,并且通过ESI-MS进一步确认分子量。

[0272] 寡核苷酸的延伸:

[0273] 通过使用0.1M 5'-O-DMT-保护的亚酰胺的乙腈溶液和DCI(4,5-二氰基咪唑)的乙

腈溶液(0.25M)作为活化剂,偶联β-氰基乙基-亚磷酰胺(DNA-A(Bz),DNA-G(ibu),DNA-C(Bz),DNA-T,LNA-5-甲基-C(Bz),LNA-A(Bz),LNA-G(dmf),LNA-T或氨基-C6接头)。对于最后的循环,可以使用具有所需修饰的亚磷酰胺,例如,用于连接缀合物基团的C6接头或缀合物基团。通过使用氢化黄原素(xanthane hydride)(0.01M在乙腈/吡啶9:1中)进行硫醇化用于引入硫代磷酸酯键。磷酸二酯键可以使用THF/吡啶/水7:2:1中的0.02M碘引入。其余的试剂是通常用于寡核苷酸合成的试剂。

[0274] 通过RP-HPLC纯化:

[0275] 通过制备型RP-HPLC在Phenomenex Jupiter C18 10μ150×10mm柱上纯化粗化合物。使用0.1M乙酸铵pH8和乙腈作为缓冲液,流速为5mL/min。将收集的级分冷冻干燥,得到纯化合物,典型地为白色固体。

[0276] 缩写:

[0277] DCI:4,5-二氰基咪唑

[0278] DCM:二氯甲烷

[0279] DMF:二甲基甲酰胺

[0280] DMT:4,4'-二甲氧基三苯甲基

[0281] THF:四氢呋喃

[0282] Bz:苯甲酰基

[0283] Ibu:异丁酰基

[0284] RP-HPLC:反相高效液相色谱

[0285] Tm分析

[0286] 将寡核苷酸和RNA靶双链体在500ml无RNA酶的水中稀释至3mM,并与500ml 2×Tm缓冲液(200mM NaCl,0.2mM EDTA,20mM磷酸钠,pH7.0)混合。将溶液加热至95℃3分钟,然后在室温退火30分钟。使用PE Templab软件(Perkin Elmer)在配备Peltier温度编程器PTP6的Lambda 40UV/VIS分光光度计上测量双链体解链温度(Tm)。温度从20℃变温上升到95℃,然后下降到25℃,记录260nm的吸收。使用熔解和退火的一阶导数和局部最大值来评估双链体Tm。

[0287] 小鼠原代皮层神经元细胞培养物的制备

[0288] 根据标准程序从15日龄的小鼠胚胎大脑制备原代皮层神经元培养物。简言之,在室温下用聚-L-赖氨酸(50μg/ml聚-L-赖氨酸,10mM四硼酸钠,pH8缓冲液)包被培养板2-3小时。使用前用1×PBS洗涤平板。解剖收获的小鼠胚胎脑并用剃刀刀片均化并浸入38ml解剖介质(HBSS,0.01M Hepes,青霉素/链霉素)中。然后,加入2ml胰蛋白酶,细胞在37℃温育30分钟并离心。将细胞溶解在20ml DMEM(+10%FBS)中并通过注射器进一步匀浆。随后以500rpm离心15分钟。将细胞溶解于DMEM(+10%FBS)中并接种于96孔板(100μl中0.1×10^6个细胞/孔)中。在接种后神经元细胞培养物即可使用。

[0289] 在小鼠原代皮质神经元细胞培养物中筛选寡核苷酸

[0290] 将细胞在96孔板中在生长培养基(Gibco Neurobasal培养基,B27补充物,Glutamax,青霉素-链霉素)中培养,并与寡核苷酸一起以所需浓度孵育3天。从细胞中分离总RNA,并使用来自Quanta Bioscience(95134-500)的qScript™ XLT One-Step RT-qPCR ToughMix®Low ROX™试剂盒,通过qPCR分析测量敲低(know-down)效力。使用来自Thermo

Fisher Scientific的商业taqman测定法来测量Ube3a_ATS,包括GAPDH在内用于标准化。

[0291] 产生人原代神经元细胞培养物

[0292] 在任何所需时间点的任何细胞系在37℃,5%CO 2浓度和95%相对湿度下温育。

[0293] 人诱导的多能干细胞(hIPSC)培养物

[0294] 从诊断有Angelman综合征的患者获得人全血样品。原代外周血单核细胞(PBMC)的后续培养物富集成红细胞。用CytoTune-iPS Sendai重编程试剂盒(Thermo Fisher Scientific)重编程成红细胞,产生患者特异性IPSC系。使用hESC合格的Matrigel(Corning),在mTESR1(STEMCELL Technologies)中,进行每日培养基替换,将来源的IPSC细胞系保持在无饲养细胞条件下。达到汇合时,使用温和细胞解离试剂(STEMCELL Technologies)将集落解离成50-200μm大小的细胞簇,并在10μM Y-27632(Calbiochem)的存在下以1:10-1:20的比例进行传代培养。

[0295] 分化成神经祖细胞(NPC)

[0296] 在诱导神经分化后,将IPSC来源的细胞维持在基础培养基中,所述基础培养基由等体积DMEM:F12Glutamax培养基和Neurobasal培养基(Gibco,Invitrogen)组成,补充有1x B27(Gibco,Invitrogen),1xN2(Gibco,Invitrogen),0.1mMβ-巯基乙醇(Gibco,Invitrogen)和所示补充物。

[0297] 神经祖细胞(NPC)由hIPSC通过双重SMAD抑制并根据稍修改的公开程序而得到(Chambers等,2009Nat Biotechnol.Vol.3pp.275-80,Boissart等,2013Transl Psychiatry.3:e294)。将HIPSC用Accutase(Innovative Cell Technologies Inc.)解离成单细胞悬浮液,并重悬于进一步补充有10μM Y-27632(Calbiochem),5ng/ml FGF(Peprotech),10μM SB-431542(Calbiochem)和100nM LDN(Calbiochem)的MT培养基中。将单细胞悬浮液转移至AggreWell800板(STEMCELL Technologies),使得能够形成由8000个细胞组成的聚集体。5天后,将神经聚集体转移到用聚-L-鸟氨酸(Sigma)和层粘连蛋白(Roche)包被的平板上,并允许在补充有FGF的基础培养基中在持续的双重SMAD抑制(SB-431542和LDN)下形成神经玫瑰花结。使用STEMdiff™神经玫瑰花结选择试剂(STEMCELL Technologies)选择性分离神经玫瑰花结,将其重铺于包被有聚-L-鸟氨酸和Laminin521(BioLamina)的培养皿上,并在补充有10ng/ml FGF(Peprotech),10ng/ml EGF(RnD)和20ng/ml BDNF(Peprotech)的基础培养基中扩增。当达到汇合时,用0.05%胰蛋白酶/EDTA(Gibco,Invitrogen)将细胞酶促解离并继代培养。在补充有FGF,EGF和BDNF的基础培养基中持续传代,在10至20代传代中产生稳定的神经祖细胞系(NPC系)。稳定的神经祖细胞系由其自我更新能力和发育阶段特异性标志物Sox2和Nestin的表达来定义。在特定刺激下,NPC分化成神经元(MAP2+,Tau+,HuC/D+)和星形胶质细胞(GFAP+)后代(Dunkley等,2015Proteomics Clin Appl.Vol.7-8pp.684-94)。

[0298] NPC培养

[0299] 先前已经描述了NPC培养的条件并且在稍作修改下使用(Boissart等,2013 Transl Psychiatry.3:e294)。简言之,将细胞维持在用Laminin521(BioLamina)包被的培养皿中,并在基础培养基[由等体积的DMEM:F12Glutamax培养基和Neurobasal培养基(Gibco,Invitrogen)组成,补充有1x B27(Gibco,Invitrogen),1x N2(Gibco,Invitrogen),0.1mMβ-巯基乙醇(Gibco,Invitrogen)]并补充有10ng/ml FGF(Peprotech),

10ng/ml EGF(RnD)和20ng/ml BDNF(Peprotech)。

[0300] 分化成神经元细胞培养物

[0301] 为了诱导NPC的神经元分化,将细胞用0.05%胰蛋白酶/EDTA(Gibco,Invitrogen)解离成单细胞悬浮液并以12.000个细胞/cm^2的密度接种到Laminin521(BioLamina)包被的培养皿上,并维持在补充有200ng/ml Shh(Peprotech),100ng/ml FGF8(Peprotech)和100μM抗坏血酸磷酸盐(Sigma)的基础培养基中7天。随后,将细胞在补充有20ng/ml BDNF(Peprotech),10ng/ml GDNF(Peprotech),0.5mM cAMP(BIOLOG Life Science)和100μM抗坏血酸磷酸盐(Sigma)的基础培养基中重铺,密度为45000个细胞/cm^2并分化21天。在分化的第21天,将分化的神经元培养物重新铺在筛选兼容的平板形式上。通过将培养物用Accutase(Innovative Cell Technologies Inc.)解离成单细胞悬液来进行重新铺板。在存在10μM Y-27632(来自Calbiochem的细胞可透过的、可逆的Rho激酶抑制剂)的情况下,将细胞以200,000个细胞/cm ^{2}的密度接种到384孔微量滴定板中,用于最终的寡核苷酸筛选测定试验。在补充有20ng/ml BDNF(Peprotech),10ng/ml GDNF(Peprotech),0.5mM cAMP(BIOLOG Life Science)和100μM抗坏血酸磷酸盐(Sigma)的基础培养基中再分化神经元培养物7天。分化培养基每周更换两次。在35天的总分化期后,神经元细胞培养物即可用于寡核苷酸处理。

[0302] 在人神经元细胞培养物中筛选寡核苷酸

[0303] 为了筛选,在384孔微量滴定板(化合物板)中将寡核苷酸原液用水预先稀释至指定浓度。平板布局用作处理模板。将2微升来自每个孔的寡核苷酸稀释液从化合物平板转移到相应的培养板中。所有液体处理均在无菌条件下使用半自动实验室机器人系统(Beckmancoulter)在层流中完成。将神经元细胞培养物与寡核苷酸一起温育5天而不改变培养基。随后,将神经元培养物裂解并用RealTime ready Cell lysis and RNA Virus Master试剂盒(Roche)进行qPCR分析。使用半自动实验室机器人系统(Beckmancoulter)进行液体处理。样品通过Lightcycler480实时PCR系统(Roche)进行分析。

[0304] 使用以下引物和探针通过qPCR监测UBE3A的转录物丰度来评估寡核苷酸的活性:

[0305] UBE3a-有义:正向引物:ATATGTGGAAGCCGGAATCT,

[0306] 反向引物:TCCCAGAACTCCCTAATCAGAA,

[0307] 用染料FAM标记的内部探针:ATGACGGTGGCTATACCAGG

[0308] 将RT-qPCR与持家基因PPIA(肽基脯氨酰异构酶A)复合用于标化。PPIA引物和染料VIC标记的探针购自Thermo Fisher Scientific(试验ID Hs99999904_m1)。每个平板包括作为阴性对照的非靶向寡核苷酸(模拟物)(TTGaataagtggaTGT)和参考寡核苷酸CMP ID NO:41_1,导致UBE3A mRNA的上调。

[0309] 寡核苷酸的选择性通过反筛选位于染色体15上SNORD109B上游的SNORD 115转录物来验证。使用以下引物和探针通过qPCR监测SNORD115的表达

[0310] 正向引物:GGGTCAATGATGAGAACCTTAT,

[0311] 反向引物:GGGCCTCAGCGTAATCCTATT,

[0312] 染料FAM标记的内部探针:TTCTGAAGAGAGGTGATGACTTAAAA

[0313] 寡核苷酸处理后,RT-qPCR与PPIA(Thermo Fisher Scientific)复合。

[0314] 使用以下引物和探针通过RT-qPCR测量SNORD109B(也称为UBE3A阻遏物)下游的

SNHG14转录物的减少：

[0315] 正向引物:ATCCGAGGCATGAATCTCAC,

[0316] 反向引物:CAGGCCAAAACCCTTGATAA,

[0317] 染料FAM标记的内部探针:TTGCTGAGCATTTTTGCATC

[0318] RT-qPCR与PPIA(Thermo Fisher Scientific)复合。

[0319] 数据表示为在所有平板上相对于模拟物的平均%表达,并且针对参考寡核苷酸进行标化以解释板与板的变异性。

[0320] 在人类神经元细胞培养物中筛选寡核苷酸--96孔系统

[0321] 为了筛选,在96孔微量滴定板(化合物板)中将寡核苷酸原液用水预稀释至指定浓度。平板布局用作处理模板。将2微升来自每个孔的寡核苷酸稀释液从化合物平板转移到相应的培养板中。所有液体处理均在无菌条件下使用半自动实验室机器人系统(Beckman Coulter)在层流中完成。将神经元细胞培养物与寡核苷酸一起温育5天而不改变培养基。随后,裂解神经元培养物并使用RNA纯化试剂盒Pure Link Pro96(12173011A) LifeTechnologies进行RNA纯化。使用半自动实验室机器人系统(Beckmancoulter)进行液体处理。使用来自Quanta的qScript™ XLT 1-Step RT-qPCR ToughMix Low ROX(95134-50),在ViiA TM 7实时PCR系统Thermo Fisher Scientific上进行Ube3a和Ube3a-ATS的qPCR分析。

[0322] 使用以下引物和探针：

[0323] qPCR UBE3a-有义:

[0324] 正向引物:ATATGTGGAAGCCGGAATCT,

[0325] 反向引物:TCCCAGAACTCCCTAATCAGAA,

[0326] 染料FAM标记的内部探针:ATGACGGTGGCTATACCAGG

[0327] SNORD109B下游的qPCR SNHG14转录物(也称为UBE3A阻遏物)：

[0328] 商业上可获得的来自Thermo Fisher的引物和探针组:Hs01372957_m1。这些引物扩增Genbank转录物AF400500.1中87bp外显子-外显子跨越序列

[0329] QPCR GAPDH转录物：

[0330] 商业上可获得的来自Thermo Fisher的引物和探针组:Gene Symbol:具有以下试验细节:RefSeq:NM_002046.3,探针外显子位置:3,扩增子大小:122bp。相应的TaqMan Assay ID:Hs99999905_m1。

[0331] 用于Ube3a和Ube3a-ATS两者的RT-qPCR与作为持家基因的GAPDH复合以进行标化。每个平板包括作为阴性对照的非靶向寡核苷酸(模拟物)(TTGaataagtggaTGT)和参考寡核苷酸CMP ID NO:21_1,导致UBE3A mRNA的上调。此外,还包括了不靶向Ub3a或SNORD109B下游的SNHG14转录物(也称为UBE3A阻遏物)的对照寡核苷酸的组,以监测试验噪音和检测假阳性的风险。这些随机分布在板上。

[0332] 对照寡核苷酸：

[0333] 序列

[0334] CGAaccactgaaCAA

[0335] CGAaccactgaacAAA

[0336] CGAagtgcacaCG

[0337] GCGtaaagagaGGT

[0338] GAGAaggcacagaCGG

[0339] GCGaagtgcacaCGG

[0340] GAGaaggcacagaCGG

[0341] CGAaccactgAACA

[0342] GAAccactgaacAAA

[0343] caGCGtaaagagaGG

[0344] GCgtaaagagAGG

[0345] CGAaccactgaAC

[0346] CGAAccactgaaCAAA

[0347] AGCgaagtgcacaCGG

[0348] AGGtgaagcgaAGTG

[0349] TAGTaaactgagCCA

[0350] AGAaggcacagaCGG

[0351] CCGcagtatggaTCG

[0352] 产生食蟹猴原代神经元细胞培养物

[0353] 任何所述时间点的任何细胞系在37℃,5%CO2浓度和95%相对湿度下温育。

[0354] 食蟹猴诱导的多能干细胞(cIPSC)培养物

[0355] 所有的动物操作均按照美国国立卫生研究院的指南进行,并由与美国新泽西州Roche 340 Kingsland Street Nutley NJ 07110(2014年关闭)有关的机构动物护理和使用委员会(IACUC)批准。食蟹猴IPSC使用携带Yamanaka因子(Oct4,Sox2,Klf4和C-Myc)的仙台病毒颗粒(CytoTune-iPS仙台重编程试剂盒,Thermo Fisher Scientific)从14岁雌性毛里求斯食蟹猴的肾成纤维细胞建立(Takahashi,K.&Yamanaka,S.,2006)。转染后五天,将细胞以不同密度传代到丝裂霉素C灭活的饲养细胞上,并在hESC培养基(敲除DMEM:F12,补充有20%敲除血清替代物,0.1mM非必需氨基酸,2mM L-谷氨酰胺,0.1mM 2-巯基乙醇和8ng/ml bFGF,全部来自Life Technologies)中培养。转染后20天选择具有ES样形态的克隆用于进一步传代。通过人工解离集落,每3-4天常规传代细胞。在至少10次传代后,基于Ono等,2014的修改方法,使cIPSC适应无饲养细胞的培养条件(补充有15ng/ml FGF2(Peprotech)和10ng/ml ActivinA(Peprotech)的MT培养基),其中所述修改为在Matrigel(BD Bioscience)包被的平板上培养细胞以及当细胞处于单细胞悬浮液中时使用Y-27632(Calbiochem)代替Thiazovivin。MT培养基是成分确定培养基,含有Dulbecco改良Eagle培养基和Ham's F12营养混合物(DMEM/F12),具有2.5mM GlutaMAX™,7μg/ml胰岛素,450μM单硫代甘油,1x脂质浓缩物,5mg/ml BSA,14ng/ml亚硒酸钠,1x非必需氨基酸,2mg/ml肝素,15μg/ml转铁蛋白和220μM抗坏血酸-2-磷酸盐。使用温和细胞解离试剂(Stem Cell Technologies)将细胞每2-4天传代一次。选择一个克隆用于进一步实验并用于以下所有测定试验。

[0356] 分化成神经祖细胞(NPC)

[0357] 在诱导神经分化后,将cIPSC来源的细胞(参见图4:第1天)维持在由等体积的DMEM:F12Glutamax培养基和Neurobasal培养基(Gibco,Invitrogen)组成的基础培养基中,

其中补充有1x B27(Gibco,Invitrogen),1×N2(Gibco,Invitrogen),0.1mMβ-巯基乙醇(Gibco,Invitrogen)和所示补充物。

[0358] 神经祖细胞(NPC)由cIPSC通过双重SMAD抑制作用并根据轻微修饰的已公布的程序而得到(Chambers等,2009 Nat Biotechnol.Vol.3pp.275-80,Boissart等,2013 Transl Psychiatry.3:e294)。将CynoIPSC用Accutase(Innovative Cell Technologies Inc.)解离成单细胞悬浮液并重悬于补充有10μM Y-27632(Calbiochem)的MT培养基中。将单细胞悬浮液转移至AggreWell800平板(STEMCELL Technologies),以使得可以形成由12000个细胞组成的聚集体(参见图4:EB形成)。对于接下来的连续五天,每个AggreWell中的1.5ml培养基用补充有5ng/ml FGF(Peprotech),10μM SB-431542(Calbiochem)和100nM LDN-193189(Calbiochem)的基础培养基替代。5天后,将神经聚集物转移到用聚-L-鸟氨酸(Sigma)和层粘连蛋白(Roche)包被的平板上并且允许在补充有FGF的基础培养基中在持续的双重SMAD抑制(SB-431542和LDN-193189)下形成神经玫瑰花结(见图4:收获)。通过手动解剖选择性分离神经玫瑰花结,然后重新铺板在包被有聚-L-鸟氨酸和来自BioLamina的Laminin521的培养皿上(参见图4:神经玫瑰花结分离),并在补充有10ng/ml FGF(Peprotech),10ng/ml EGF(RnD)和20ng/ml BDNF(Peprotech)的基础培养基中扩增。当达到汇合时,用0.05%胰蛋白酶/EDTA(Gibco,Invitrogen)将细胞酶促解离并继代培养。在补充有FGF,EGF和BDNF的基础培养基中继续传代,在5至10代中产生稳定的神经祖细胞系(NPC系)(参见图4,第24-45天)。稳定的神经祖细胞系由其自我更新能力和发育阶段特异性标志物Sox2和Nestin的表达来定义。在特定的刺激下,cyno NPCs分化成神经元MAP2+细胞。

[0359] NPC培养

[0360] 先前已经描述了NPC培养的条件并且稍作修改予以使用(Boissart等,2013 Transl Psychiatry.3:e294)。简言之,将细胞维持在用Laminin521(BioLamina)包被的培养皿中,并在补充有10ng/ml FGF(Peprotech),10ng/ml EGF(RnD)和20ng/ml BDNF(Peprotech)的基础培养基[由等体积的DMEM:F12 Glutamax培养基和Neurobasal培养基(Gibco,Invitrogen)组成,补充有1x B27(Gibco,Invitrogen),1xN 2(Gibco,Invitrogen),0.1mMβ-巯基乙醇(Gibco,Invitrogen)]培养。

[0361] 分化成神经元细胞培养物

[0362] 为了诱导NPC的神经元分化,将细胞用0.05%胰蛋白酶/EDTA(Gibco,Invitrogen)解离成单细胞悬浮液并以30,000个细胞/cm^2的密度接种到Laminin521(BioLamina)包被的培养皿上,并保持在补充有200ng/ml Shh(Peprotech),100ng/ml FGF8(Peprotech)和100μM抗坏血酸磷酸盐(Sigma)的基础培养基中7天。随后,将细胞在补充有20ng/ml BDNF(Peprotech),10ng/ml GDNF(Peprotech),0.5mM cAMP(BIOLOG Life Science)和100μM抗坏血酸磷酸盐(Sigma)的基础培养基中重铺,密度为45000个细胞/cm^2并分化21天。在分化的第21天,将分化的NC解离并再接种到筛选兼容的平板形式上。通过将培养物用Accutase(Innovative Cell Technologies Inc.)分离成单细胞悬液来进行重铺板。在10μM Y-27632(来自Calbiochem的细胞可透过的、可逆的Rho激酶抑制剂)存在下,将细胞以200000个细胞/cm^2的密度接种到384孔微量滴定板中用于最终的寡核苷酸筛选测定试验。在补充有20ng/ml BDNF(Peprotech),10ng/ml GDNF(Peprotech),0.5mM cAMP(BIOLOG Life Science)和100μM抗坏血酸磷酸盐(Sigma)的基础培养基中再分化神经元培养物7天。分化

培养基每周更换两次。在35天的总分化期后，神经元细胞培养物即可用于寡核苷酸处理。

[0363] 在cyno神经元细胞培养物中筛选寡核苷酸--96孔系统

[0364] 为了筛选，在96孔微量滴定板(化合物板)中将寡核苷酸原液用水预稀释至指定浓度。平板布局用作处理模板。将2微升来自每个孔的寡核苷酸稀释液从化合物平板转移到相应的培养板中。所有液体操作均在无菌条件下使用半自动实验室机器人系统(Beckmancoulter)在层流中完成。将神经元细胞培养物与寡核苷酸一起温育5天而不改变培养基。随后，使用PureLink® Pro 96总RNA纯化试剂盒(Thermo Fisher)将神经元培养物裂解和纯化，并随后使用qScript XLT One-Step RT-qPCR Tough Mix,Low ROX(Quanta Biosiences)进行qPCR测定。液体操作使用半自动实验室机器人系统(Integra Assist and Integra Viaflo)进行。样品通过Lightcycler480实时PCR系统(Roche)进行分析。

[0365] 使用以下引物和探针，通过qPCR监测UBE3A-有义和UBE3A-反义的转录物丰度来评估寡核苷酸的活性：

[0366] UBE3a-有义:HS00166580_VIC Taqman试验(Thermo Fisher)

[0367] UBE3A-反义:HS01372957_VIC Taqman试验(Thermo Fisher)

[0368] RT-qPCR与持家基因TBP(TATA盒结合蛋白)复合以进行标准化。TBP引物和FAM标记的探针购自Roche(REF.:05532957001,Assay Id.101145)。每个平板包括非靶向寡核苷酸(模拟物)作为阴性对照(TTGaataagtggaTGT)。

[0369] 数据表示为相对于模拟物处理条件的平均百分比表达。

[0370] 免疫细胞荧光染色

[0371] 细胞用4%PFA固定并用0.1%TritonX(Sigma)在PBS中(含有Ca2+和Mg2+)透化。使用补充有0.1%TritonX的SuperBlock溶液(Thermo Fisher Scientific)进行封闭。细胞用以下一级抗体染色：抗SOX2(Milipore AB5603)，抗GFAP(Dako Z033401)，抗HuC/D(Invitrogen A21271)，抗Nestin(Millipore mab5326)和抗Map2(Neuromics CH22103)。随后，洗涤细胞并用缀合于Alexa488,Alexa555或Alexa647(全部Molecular Probes)的第二抗体染色。细胞核用Hoechst 1:1000(Molecular Probes)染色。使用Axiovert显微镜(Zeiss)或Operetta高内涵成像系统(Perkin Elmer)使细胞成像。使用ImageJ软件分析图像。

[0372] qPCR分析

[0373] 自cIPSC，食蟹猴分化的NC，hESC和人分化的NC，使用miRNeasy Mini试剂盒(QIAGEN)分离RNA，并进行qPCR分析(对于人类细胞，使用Ag-Path-ID One-Step RT-PCR试剂盒，Ambion；对于食蟹猴细胞，使用Transcriptor第一链cDNA合成试剂盒Roche进行逆转录，使用LightCycler480 Probes Master,Roche进行qPCR)。使用以下引物和探针：对于人类细胞：NES Hs04187831_g1；MAP2 Hs00258900_m1；ASCL1 Hs00269932_m1；SOX2 Hs01053049_s1；ELAVL3 Hs00154959_m1；GAPDH 4352665，全部来自Thermo Fisher/Applied Biosystems；UBE3A-ATS Fw引物：CAA ATG CCT CAC CCA CTC TT,RV引物：CCA GCT GTC AAC ATG TGC TT,内部寡聚物：AAG TGC GCT CCT GTG AAA AG；UBE3A Fw引物：TCT GGG AAA TCG TT CATT CA,RV引物：TGT AGG TAA CCT TTC TGT GTC TGG,内部寡聚物TAC AAC GGG CAC AGA CAG AG。对于食蟹猴细胞：NANOG测定ID 700103,POU5F1测定ID 113034,SOX2测定ID 111867,Nestin测定ID 138150,PAX6测定ID 136139,SOX1测定ID 136988,ZIC1测

定ID 112077,ASCL1测定ID 700027,TUBB3测定ID 700047,GAPDH Cat N.04694333001,探针#147;TBP测定ID 101145,全部从罗氏公司购买。UBE3A-ATS Fw引物:AATGCAAAGGCAGCAGTACA,Rv引物:TTGGGGAGTTGGTTATTGGA,内部寡聚物TGACACCACCAGAAGAACACA;

[0374] UBE3A有义:Fw引物:ATATGTGGAAGCCGGAATCT,

[0375] Rv引物:TCCCAGAACTCCCTAATCAGAA,

[0376] 内部寡聚物:ATGACGGTGGCTATACCAGG。

[0377] RNA测序分析

[0378] 使用miRNeasy Mini试剂盒(QIAGEN)进行RNA分离。使用Ribo-Zero Gold depletion和TruSeq Stranded Total RNA LT试剂盒(Illumina)进行文库制备。使用cBOT仪器和HiSeq PE Cluster kit v4进行簇生成。使用以下试剂在HiSeq 2500仪器上进行测序,以匹配末端Single-indexed测序运行进行(2×125个循环):HiSeq SBS试剂盒v4 250个循环(Illumina)。

[0379] Western Blot分析

[0380] 对于蛋白质表达研究,通过刮取收集细胞并快速冷冻。使用完成了磷酸酶和蛋白酶抑制剂和DNAse(均来自Roche)的Cytobuster(Millipore)裂解沉淀。通过BCA测定(Thermo Scientific)测量提取物中的蛋白质浓度,并将该溶液加载到聚丙烯酰胺凝胶(Invitrogen)上。电泳后,使用iBlot系统(Invitrogen)将蛋白质印迹在硝酸纤维素膜上。用于检测感兴趣蛋白质的抗体是抗hTau HT7(Pierce MN1000),抗pTauS422(Roche)抗pTauS404(Abcam ab92676);这些通过合适的HRP缀合的二抗检测。使用成像仪(Biorad),使用SuperSignal West Dura Extended Duration Substrate(Thermo Fisher)通过发光检测复合物。

[0381] 参考文献

[0382] Ono,T.et al.A single-cell and feeder-free culture system for monkey embryonic stem cells.PLoS One 9,e88346,doi:10.1371/journal.pone.0088346(2014).

[0383] Takahashi,K.&Yamanaka,S.Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors.Cell 126,663-676,doi:10.1016/j.cell.2006.07.024(2006).

[0384] 实施例1-在小鼠原代神经元细胞培养物中的寡核苷酸活性

[0385] 靶向与UBE3A前mRNA反义的SNHG14长非编码RNA的一部分(SEQ ID NO:1的位置55319至141053)的寡核苷酸,测试其降低SNHG14长非编码RNA转录物(在数据表中也称为UBE3A阻遏物或UBE3A-SUP)阻止UBE3A表达的能力、以及它们在小鼠原代皮质神经元细胞培养物中诱导UBE3A mRNA重新表达的能力,如上文“材料和方法”部分所述获得细胞培养物。寡核苷酸浓度为5μM。

[0386] 根据在“材料和方法”部分中描述的用于在小鼠皮层神经元细胞培养物中筛选的方案,筛选寡核苷酸。结果如表4所示。

[0387] 表4:原代小鼠神经元细胞培养物中的寡核苷酸活性。

[0388]

CMP ID NO	寡核苷酸	%模拟物 UBE3A_SUP	sd	%模拟物 UBE3A	sd
95_1	CTCAtacttgctttaAT	3,6	0,1	154,1	15,1
95_2	CTcatacttgctttaAT	15,9	2,6	119,8	12,4
96_1	ACatctcatacttGCTT	4,0	0,5	149,9	11,5
96_2	ACatctcatacttgcTT	9,3	3,9	139,9	36,4
96_3	ACatctcatacttgCTT	3,1	0,2	143,2	3,9
97_1	ACatctcatactTGCT	4,0	1,5	154,5	10,0
97_2	ACatctcatacttgCT	6,1	1,7	141,1	14,1
97_3	ACatctcatacttGCT	3,7	0,6	162,7	15,0
97_4	ACATctcatacttgCT	5,2	0,4	156,7	24,4
98_1	TAcatctcatactTGCT	5,0	0,9	159,0	15,6
98_2	TAcatctcatacttgCT	15,5	5,3	130,4	3,4
98_3	TACAtctcatacttgCT	4,7	0,4	140,3	38,2
101_1	TACAtctcatactTGC	2,6	0,5	152,6	10,2
101_2	TAcatctcatacttGC	19,2	6,0	112,0	15,0
101_3	TAcatctcatactTGC	3,5	0,4	117,2	13,7
101_4	TACAtctcatacttGC	3,0	0,7	140,5	12,4
100_1	CTAcatctcatactTGC	5,4	0,8	160,4	4,1
100_2	CTacatctcatacttGC	9,6	3,7	159,2	14,5
100_3	CTacatctcatactTGC	3,0	0,1	133,2	5,9
99_2	CCtacatctcatacttGC	7,8	1,4	150,7	11,0
99_3	CCtacatctcatactTGC	3,2	0,6	134,7	12,5
99_4	CCtacatctcatacTTGC	2,7	0,2	145,2	4,7
102_1	CCTAcatctcatactTG	5,8	1,7	127,0	24,5
102_2	CCtacatctcatactTG	20,2	6,6	129,7	9,2
102_4	CCTacatctcatacTTG	4,0	0,6	140,2	7,2
102_3	CCTacatctcatactTG	3,9	1,0	133,3	10,0
104_1	CCTacatctcataCTT	6,6	1,5	136,5	8,7
104_3	CCtacatctcatACTT	3,5	0,4	131,4	6,0
103_1	ACCtacatctcataCTT	5,8	1,4	130,8	0,7
103_2	ACctacatctcatacTT	11,4	2,2	123,6	12,4
103_3	ACctacatctcatACTT	5,8	0,8	132,2	4,5
105_1	TACCtacatctcatacTT	5,2	0,8	152,3	7,2
106_1	TTAcctacatctcataCTT	13,3	3,0	140,1	17,5
106_2	TTacctacatctcatacTT	21,0	1,4	116,9	15,0

[0389]

CMP ID NO	寡核苷酸	% 模拟物 UBE3A_SUP	sd	% 模拟物 UBE3A	sd
107_1	ACCTacatctcataCT	6,2	0,9	119,2	3,4
107_2	ACctacatctcataCT	14,3	7,4	142,9	13,7
108_1	TACCtacatctcataCT	5,6	1,0	127,0	10,7
108_2	TAcctacatctcataCT	21,4	12,5	117,1	8,5
109_1	TTacctacatctcaTACT	4,4	0,4	138,9	1,2
109_2	TTacctacatctcataCT	22,9	3,3	117,1	13,0
110_1	TTAcctacatctcaTAC	8,7	2,1	133,2	5,1
110_2	TTacctacatctcatAC	21,0	5,1	111,4	11,1
111_1	GTtacctacatctCATA	8,0	2,4	143,8	14,8
111_2	GTtacctacatctcaTA	19,0	2,3	115,4	4,1
112_1	GTTacctacatctCAT	6,6	1,4	145,5	16,8
112_2	GTtacctacatctcAT	15,8	4,5	120,3	8,1
126_1	TCACtttccagatatCA	8,0	1,9	133,8	5,4
126_3	TCactttccagatatCA	53,4	75,9	112,0	11,4
128_1	ACATgtccctttataTT	16,3	2,5	114,7	11,1
128_2	ACatgtccctttataTT	14,8	1,1	136,9	6,2
129_1	ACAtgtccctttaTAT	11,8	1,9	135,0	14,3
132_1	CTCAtccctccaagaAA	9,1	1,6	131,7	8,4
132_2	CTcatccctccaagaAA	11,2	3,9	159,3	17,7

[0390] 实施例2-在人神经元细胞培养物中的寡核苷酸活性

[0391] 在患者来源的人神经元细胞培养物中(参见“材料和方法”部分中的方案),测试了靶向人染色体15上SNORD109B下游区域中对应于位置25278410至25419462(SEQ ID NO:1)的人SNHG14的寡核苷酸。分析了寡核苷酸降低SNORD109B下游区域中SNHG14转录物(在数据表中也称为UBE3A阻遏物或UBE3A-SUP)而不影响SNORD115表达的能力。此外,分析了诱导UBE3A mRNA重新表达的能力。

[0392] 在根据上文“材料和方法”部分中描述的人神经元细胞培养物中,根据筛选寡核苷酸的方案,筛选寡核苷酸。

[0393] 结果显示在表5中。已经针对所有化合物测量了UBE3A mRNA的表达,但是对于所有化合物,尚没有分析UBE3A阻遏物的敲低和SNORD1115水平的维持。

[0394] 表5:在患者来源的人神经元细胞培养物中的寡核苷酸活性。

[0395]

Start SEQ ID NO 1	CMP ID NO	靶	% 模拟物 Oligo 浓度 0.2 μM	sd	% 模拟物 Oligo 浓度 1.0 μM	sd	% 模拟物 Oligo 浓度 5.0 μM	sd
1678	10_1	UBE3A	107	14	88	10	151	8
1679	12_2	UBE3A	100	9	87	14	158	16
1687	20_1	UBE3A	87	7	102	22	213	44
1712	21_1	UBE3A	127	23	166	6	178	13

[0396]

起始 SEQ ID NO 1	CMP ID NO	靶	%模拟物 Oligo 浓度 0.2 μM	sd	%模拟物 Oligo 浓度 1.0 μM	sd	%模拟物 Oligo 浓度 5.0 μM	sd
1712	21_1	UBE3A-SUP	81	3	82	8	72	12
1712	21_1	SNORD115	115	6	142	24	169	26
4167	22_1	UBE3A	87	5	90	8	146	20
4170	27_1	UBE3A	94	16	106	11	170	10
4171	29_2	UBE3A	86	13	100	12	194	35
4172	30_1	UBE3A	96	6	121	12	209	27
9210	35_1	UBE3A	88	5	112	23	195	27
10638	37_1	UBE3A	77	7	85	9	169	24
15565	38_2	UBE3A	93	11	108	6	167	34
22209	42_1	UBE3A	125	16	143	14	180	17
22209	42_1	UBE3A-SUP	108	14	98	15	85	18
22209	42_1	SNORD115	101	14	93	25	127	21
30449	43_1	UBE3A	99	5	95	13	115	8
30451	44_1	UBE3A	99	15	80	20	141	17
30451	44_2	UBE3A	98	31	104	16	119	7
30697	46_1	UBE3A	91	8	87	5	167	20
36066	49_1	UBE3A	95	6	111	10	155	29
36066	49_1	UBE3A-SUP	76	7	84	24	110	31
36066	49_1	SNORD115	99	14	111	20	94	6
36068	50_1	UBE3A	109	15	105	11	92	14
36068	50_1	UBE3A-SUP	122	24	93	28	73	7
36068	50_1	SNORD115	120	15	113	12	99	6
37206	51_1	UBE3A	114	16	101	7	101	3
37206	51_1	UBE3A-SUP	128	21	67	9	84	13
37206	51_1	SNORD115	140	26	110	9	100	11
46130	52_1	UBE3A	139	3	160	1	236	36
46130	52_1	UBE3A-SUP	135	16	133	26	160	32
46130	52_1	SNORD115	104	8	119	14	100	8
48145	59_1	UBE3A	179	3	122	17	115	NA
48170	76_1	UBE3A	85	16	100	8	155	12
48171	80_1	UBE3A	120	7	114	10	172	20
48171	78_1	UBE3A	136	31	103	20	169	11
48172	82_2	UBE3A	96	11	121	4	186	32
48172	84_1	UBE3A	95	14	100	8	158	14
49343	85_1	UBE3A	97	22	121	10	189	17
49722	87_1	UBE3A	111	9	126	11	177	22
52417	92_1	UBE3A	133	7	140	30	140	8
52417	92_1	UBE3A-SUP	88	14	80	14	82	8
52417	92_1	SNORD115	102	8	114	20	91	9

[0397]

起始 SEQ ID NO 1	CMP ID NO	靶	% 模拟物 Oligo 浓度 0.2 μM	sd	% 模拟物 Oligo 浓度 1.0 μM	sd	% 模拟物 Oligo 浓度 5.0 μM	sd
52420	93_1	UBE3A	111	14	120	9	126	16
52420	93_1	UBE3A-SUP	104	23	82	20	79	8
52420	93_1	SNORD115	110	11	114	17	95	7
53953	94_1	UBE3A	117	12	147	15	166	15
53953	94_1	UBE3A-SUP	92	18	81	5	86	22
53953	94_1	SNORD115	124	33	122	17	106	14
60819	95_1	UBE3A	103	11	131	14	175	7
60819	95_1	UBE3A-SUP	93	13	87	3	74	6
60819	95_1	SNORD115	162	19	158	20	201	11
60819	95_2	UBE3A	147	10	129	20	117	2
60819	95_2	UBE3A-SUP	118	24	87	13	83	8
60819	95_2	SNORD115	104	17	118	10	129	6
60823	96_1	UBE3A	115	16	135	19	174	17
60823	96_1	UBE3A-SUP	104	25	93	32	91	11
60823	96_2	UBE3A	108	7	114	9	115	13
60823	96_2	UBE3A-SUP	99	17	92	19	93	10
60824	97_1	UBE3A	111	12	134	23	169	14
60824	97_1	UBE3A-SUP	110	27	105	33	92	10
60824	97_2	UBE3A	124	13	126	12	124	11
60824	97_2	UBE3A-SUP	113	17	107	33	96	20
60824	98_1	UBE3A	111	16	119	11	138	14
60824	98_1	UBE3A-SUP	118	34	98	23	82	19
60824	98_1	SNORD115	109	11	123	18	114	16
60824	98_2	UBE3A	128	10	109	7	136	12
60824	98_2	UBE3A-SUP	91	15	77	11	110	16
60824	98_2	SNORD115	101	3	110	7	124	11
60825	99_1	UBE3A	125	6	115	5	131	10
60825	99_1	UBE3A-SUP	139	18	121	34	127	45
60825	99_1	SNORD115	110	18	112	12	99	19
60825	99_2	UBE3A	120	21	111	11	135	22
60825	99_2	UBE3A-SUP	96	21	79	15	75	11
60825	99_2	SNORD115	104	34	113	22	131	24
60825	100_1	UBE3A	123	34	139	34	145	21
60825	100_1	UBE3A-SUP	104	37	127	46	99	17
60825	100_2	UBE3A	124	46	138	37	145	31
60825	100_2	UBE3A-SUP	111	36	120	47	92	11
60825	101_1	UBE3A	112	18	123	15	150	13
60825	101_1	UBE3A-SUP	96	18	102	14	88	12
60825	101_2	UBE3A	118	15	138	24	139	32

[0398]

起始 SEQ ID NO 1	CMP ID NO	靶	% 模拟物 Oligo 浓度 0.2 μM	sd	% 模拟物 Oligo 浓度 1.0 μM	sd	% 模拟物 Oligo 浓度 5.0 μM	sd
60825	101_2	UBE3A-SUP	100	29	110	39	92	10
60826	102_1	UBE3A	132	17	120	7	125	9
60826	102_1	UBE3A-SUP	113	16	83	5	88	18
60826	102_1	SNORD115	121	36	131	23	100	9
60826	102_2	UBE3A	90	6	116	23	103	7
60826	102_2	UBE3A-SUP	91	7	90	12	64	18
60826	102_2	SNORD115	116	15	146	27	183	28
60827	103_1	UBE3A	106	8	112	10	115	9
60827	103_1	UBE3A-SUP	99	15	110	28	94	8
60827	103_2	UBE3A	107	14	120	13	112	14
60827	103_2	UBE3A-SUP	97	14	118	38	93	20
60827	104_1	UBE3A	128	14	111	9	111	6
60827	104_1	UBE3A-SUP	111	12	97	9	87	19
60827	104_1	SNORD115	114	10	110	12	109	13
60827	104_2	UBE3A	108	10	111	16	109	10
60827	104_2	UBE3A-SUP	103	13	103	33	89	9
60827	105_1	UBE3A	122	13	121	12	121	4
60827	105_1	UBE3A-SUP	119	7	97	15	93	7
60827	105_1	SNORD115	114	21	128	12	116	9
60827	105_2	UBE3A	123	5	110	9	114	8
60827	105_2	UBE3A-SUP	110	11	89	17	94	21
60827	105_2	SNORD115	102	15	106	16	107	18
60827	106_1	UBE3A	114	17	133	23	125	9
60827	106_1	UBE3A-SUP	112	35	103	15	87	12
60827	106_2	UBE3A	110	12	130	22	123	14
60827	106_2	UBE3A-SUP	105	19	107	27	93	10
60828	107_1	UBE3A	83	11	117	13	112	6
60828	107_1	UBE3A-SUP	86	11	114	16	67	7
60828	107_1	SNORD115	108	17	130	21	137	24
60828	107_2	UBE3A	143	42	117	10	122	11
60828	107_2	UBE3A-SUP	116	12	92	4	100	8
60828	107_2	SNORD115	108	4	127	16	108	14
60828	108_1	UBE3A	120	7	127	31	132	31
60828	108_1	UBE3A-SUP	153	33	118	34	89	17
60828	108_1	SNORD115	114	9	114	9	105	15
60828	108_2	UBE3A	122	18	133	26	128	9
60828	108_2	UBE3A-SUP	101	19	100	28	69	17
60828	109_1	UBE3A	108	10	129	14	128	5
60828	109_1	UBE3A-SUP	106	21	107	24	84	8

[0399]

起始 SEQ ID NO 1	CMP ID NO	靶	% 模拟物 Oligo 浓度 0.2 μM	sd	% 模拟物 Oligo 浓度 1.0 μM	sd	% 模拟物 Oligo 浓度 5.0 μM	sd
60828	109_2	UBE3A	109	11	110	8	111	13
60828	109_2	UBE3A-SUP	95	15	86	14	83	9
60829	110_1	UBE3A	104	6	83	3	101	15
60829	110_1	UBE3A-SUP	100	13	95	12	79	4
60829	110_1	SNORD115	126	21	125	6	182	13
60829	110_2	UBE3A	92	7	87	8	96	7
60829	110_2	UBE3A-SUP	99	7	108	9	81	5
60829	110_2	SNORD115	118	15	139	22	198	39
60830	111_1	UBE3A	110	6	122	13	124	10
60830	111_1	UBE3A-SUP	104	14	90	28	79	11
60830	111_2	UBE3A	115	10	120	15	121	10
60830	111_2	UBE3A-SUP	114	20	89	19	87	9
60831	112_1	UBE3A	93	8	94	13	106	10
60831	112_1	UBE3A-SUP	97	1	68	29	82	7
60831	112_1	SNORD115	116	20	110	13	158	20
60831	112_2	UBE3A	83	8	78	7	83	6
60831	112_2	UBE3A-SUP	106	35	80	23	69	9
60831	112_2	SNORD115	107	6	106	8	159	21
62198	113_1	UBE3A	110	3	122	6	134	9
62198	113_1	UBE3A-SUP	113	20	85	19	79	24
62198	113_1	SNORD115	116	18	123	9	91	9
62284	115_1	UBE3A	105	14	98	19	141	36
62422	116_1	UBE3A	130	19	142	29	172	18
62423	117_1	UBE3A	76	8	93	13	171	17
62439	118_1	UBE3A	75	7	88	9	150	19
66378	119_1	UBE3A	96	14	93	5	110	10
77565	126_1	UBE3A	94	6	113	5	125	14
77565	126_1	UBE3A-SUP	83	17	95	33	85	5
77565	126_1	SNORD115	105	11	123	19	152	15
77565	126_2	UBE3A	95	5	126	9	111	2
77565	126_2	UBE3A-SUP	77	27	106	21	83	15
77565	126_2	SNORD115	115	17	157	13	180	15
92321	128_1	UBE3A	102	7	91	5	111	13
92321	128_1	UBE3A-SUP	115	3	104	25	91	13
92321	128_1	SNORD115	135	9	132	12	196	35
92321	128_2	UBE3A	91	5	96	8	104	8
92321	128_2	UBE3A-SUP	112	20	92	20	79	7
92321	128_2	SNORD115	125	7	111	13	169	12
92322	129_1	UBE3A	101	5	103	2	110	7

[0400]

起始 SEQ ID NO 1	CMP ID NO	靶	% 模拟物 Oligo 浓度 0.2 μM	sd	% 模拟物 Oligo 浓度 1.0 μM	sd	% 模拟物 Oligo 浓度 5.0 μM	sd
92322	129_1	UBE3A-SUP	99	39	113	12	94	13
92322	129_1	SNORD115	124	25	114	6	140	13
92322	129_2	UBE3A	93	2	100	4	113	16
92322	129_2	UBE3A-SUP	109	4	102	22	85	7
92322	129_2	SNORD115	103	11	99	9	152	31
97154	132_1	UBE3A	100	10	128	13	142	13
97154	132_1	UBE3A-SUP	103	9	115	8	109	6
97154	132_1	SNORD115	49	7	90	12	143	25
97154	132_2	UBE3A	111	8	128	17	128	17
97154	132_2	UBE3A-SUP	95	7	116	9	105	13
97154	133_2	SNORD115	86	7	106	9	121	9
97154	133_1	UBE3A	101	3	107	11	124	19
97154	133_1	UBE3A-SUP	112	9	117	7	146	25
97154	133_1	SNORD115	60	7	110	15	141	15
97154	133_2	UBE3A	94	13	116	14	138	12
97154	133_2	UBE3A-SUP	116	6	128	13	148	38
97154	132_2	SNORD115	70	5	108	9	160	34
106137	137_1	UBE3A	83	12	74	11	124	20
109404	138_1	UBE3A	80	20	92	7	120	21
110766	139_1	UBE3A	76	5	85	12	121	17
114826	140_1	UBE3A	87	10	88	11	136	9
118637	143_1	UBE3A	83	7	104	30	141	28
118639	144_1	UBE3A	74	17	31	39	106	33
124160	145_2	UBE3A	89	6	95	10	115	25
125499	146_1	UBE3A	83	13	76	7	124	16
125499	146_2	UBE3A	123	30	79	14	102	23
125538	150_2	UBE3A	82	17	82	7	119	24

[0401] 在测试的187种化合物中，以5微摩尔浓度，与模拟寡核苷酸相比，约90％显示出UBE3A的重新表达。能够诱导UBE3A重新表达的寡核苷酸的数目在SEQ ID NO:1的位置1至55318之间的区域(非重叠区域)中，比在与UBE3A编码区域互补的区域(重叠区域)中更高。图2描述了根据寡核苷酸在15号染色体上的位置以及相对于模拟寡核苷酸的UBE3A mRNA表达而绘制的寡核苷酸分布图。

[0402] 对于已经测试SNORD115的寡核苷酸，在1和5μM，与模拟物相比，没有显著的下调。

[0403] 实施例3-体外系统中cyno神经元分化的产生和验证

[0404] 按照材料和方法部分中以及在图4中描述的方案，诱导Cyno IPSC分化成NPC。该方案允许获得可在补充有FGF，EGF和BDNF的基础培养基中维持和扩增的NPC。为了验证cyno NPC的有效诱导，通过免疫染色评估了神经干细胞标志物SOX2和NESTIN的表达(参见图5)。Cyno NPC表达SOX2和NESTIN，其表达模式与人类NPC高度相似。

[0405] 为了获得分化的NC，将扩增的NPC解离并在SFA培养基中接种一周。之后将细胞暴露于分化培养基(BGAA)。为了评估cyno NPC的神经元分化潜能，进行转录分析并将结果与

分化的人类NPC的转录谱进行比较(参见图6)。在BGAA培养基中分化14天后,与cIPSC相比,食蟹猴培养物显示多能性标志物如NANOG和POU5F1的表达降低以及外胚层和神经元标志物如PAX6,SOX1,ZIC1,ASCL1,TUBB3的表达增加(参见图6A)。类似地,在BGAA中分化14天的人多能干细胞来源的NC的转录分析(图6B中的RNA测序和图6C中的qPCR)揭示了,与hESC相比,在第14天BGAA分化的NC中多能性标志物的表达降低和外胚层和神经元标志物的表达增加。重要的是,UBE3A(涉及Angelman综合征的疾病基因)和UBE3A-ATS(被描述为在神经元中表达的非编码转录物)的表达变化在分化的食蟹猴和人NCs之间是相当的(参见图6D)。这些数据表明,食蟹猴IPCS分化为NCs,特定标志物的表达谱与分化的人NCs相似。

[0406] 实施例4-分化的食蟹猴NCs的解离和再接种

[0407] 干细胞来源的细胞作为药物筛选的体外系统具有很大的潜力,特别是如果相应的原代细胞类型不可得或仅限于有限的量,如神经元的情况。然而,药物筛选需要可用于合适平板形式的稳健的细胞筛选系统。为此,我们测试了不同的策略,将来源于NHP-IPSCs的NPC分化为神经元细胞(NCs)。为了鉴定具有均匀分布的NC的稳健的细胞系统,以两种方式之一将扩增的NPC解离并暴露于分化培养基:将它们直接以最终测定试验形式接种,或者将细胞接种于细胞培养瓶然后在进一步分化后解离并再接种到最终测定试验形式(参见图7)。在方法A和B中,NPC在瓶中分化23天,然后解离并以测定试验形式再接种。在7天的另外的分化期后,用寡核苷酸处理细胞。或者,在方法C和D中,解离NPC并直接铺板到96孔板上。方法A和C中的分化培养基补充有丝分裂抑制剂混合物(尿苷35μg/ml和15μg/ml 5-氟-2'-脱氧尿苷)以抑制细胞增殖。在第35天,将培养物固定并对神经祖细胞和神经胶质细胞标志物SOX2和神经元标志物MAP2进行染色(参见图8)。方法B(有重铺板并且没有有丝分裂抑制剂)导致与其他方法相比更均匀分布的细胞培养物,具有MAP2阳性细胞并具有较少的SOX2阳性细胞。出于这个原因,神经元分化方法B被选择用于随后描述的寡核苷酸处理。

[0408] 实施例5-分化的人NCs的解离和再接种

[0409] 如实施例4所述,我们确立了,重铺板分化中的cyno神经元是使这些细胞适合于筛选目的的必要步骤。因此,我们着手验证是否可以对人IPSC来源的神经元采用类似的程序。为此,来自两个IPSC系(ED4和SFC808)的先前建立的神经祖细胞(NPC)按照材料和方法部分中描述的程序进行分化。简而言之,将扩增的NPC解离并在SFA培养基中接种一周。之后,将细胞以两种方式之一暴露于分化培养基(BGAA):将它们直接铺板在测定试验形式中或细胞培养瓶中。在后一种情况下,细胞分化21天,然后解离并以测定试验形式再接种。在处理之前,两个实验组(即直接分化的或解离并再接种的)分化总共6周(分别为在测定试验形式中6周,或在瓶中3周和在测定试验形式中3周)。

[0410] 对于第一组实验,通过免疫荧光和高内涵成像分析神经元。所使用的标志物对神经祖细胞(SOX2),神经胶质细胞(GFAP)或神经元(MAP2和HuC/D)是特异的。如图9所示例的,重铺板过程似乎不影响人类IPSC来源的神经元的分化或形态。这通过定量染色进一步验证,其显示在直接分化和重铺板组中HuC/D阳性细胞的存在没有显著差异(图10;对于ED80,$p=0.542$,对于SFC808,$p=0.579$)。

[0411] 为了进一步证实可以解离和再接种分化的人IPSC来源的神经元而不改变其特征,我们想确认细胞骨架相关蛋白不被解离过程破坏。我们分析了微管相关蛋白Tau的表达,因为它对于神经元功能是至关重要的,并且直接参与神经变性疾病,例如额颞痴呆和阿尔茨

海默氏病。如上所述培养细胞(有和没有解离和再接种),并通过蛋白质印迹分析蛋白质提取物。如图11所示,Tau及其两种磷酸化形式的恒定表达证明再接种令人惊讶地不破坏人类IPSC来源的神经元的细胞骨架特征,表明生理学特征不会因该过程而改变。

[0412] 这些结果表明,人类IPSC来源的神经元可以在分化过程中经历解离和再接种并保持它们的关键特征,这表明重铺板步骤(解离和再接种)对于灵长类干细胞来源的神经元的分化是可行的。

[0413] 实施例6-使用灵长类IPSC来源的经解离和再接种的分化NC进行高通量筛选

[0414] 上述灵长类IPSC来源的神经元培养物是高度灵活和可重现的系统,适合用于筛选目的。我们开发了一个工作流程,其中用寡核苷酸处理细胞,并通过定量聚合酶链式反应(qPCR)验证靶标和相关基因的表达(见图12)。为此,如上所述处理人或食蟹猴神经祖细胞。为了筛选,细胞在分化3周后解离并以200,000个细胞/cm^2的密度接种在96孔板中。再分化一周后,将细胞用寡核苷酸处理5天,然后如材料和方法部分中所述处理用于表达分析。该方法允许持续获得待以高通量方式测试的待测样品。

[0415] 对于本实施例,我们利用了5种不同的寡核苷酸,它们以8个浓度靶向Ube3A反义(Ube3A_ATS)转录物,从20μM开始并在每一步降低3倍,导致剂量20.000,6.325,2.000,0.632,0.2200,0.063,0.020和0.006μM。我们在从Angelman综合征患者IPSCs(人)或食蟹猴IPSCs(cyno)分化的神经元上测试了这些化合物。

[0416] 处理后,裂解细胞,提取RNA并通过使用市售的TaqMan测定试验进行Ube3A和Ube3A_ATS的qPCR反应;在人类细胞中使用GAPDH,在食蟹猴细胞中使用TBP作为持家基因以对表达数据进行标化。在所有情况下,处理导致了Ube3A_ATS显著降低,这与有义转录物的表达增加相匹配。这种效应以剂量反应方式是明显的;为了方便可视化,我们在图13中报告了高剂量(2μM)和低剂量(0.02μM)时的表达水平。

[0417] 在2μM的剂量下,反义转录物表达为赋形剂(PBS)处理的细胞的水平的~50%;这对于人类和食蟹猴细胞都是如此。Ube3A_ATS的敲低导致了Ube3A的表达增加,对于人和食蟹猴分别为~250%和~130%的赋形剂水平。这种差异并不令人惊讶,因为患者来源的神经元由于Ube3A基因的一个等位基因的缺失故表达非常低水平的有义转录物。

[0418] 这些实验证明在人和cyno神经元中针对Ube3A_ATS的寡核苷酸的靶结合。因此,所描述的方法适用于在灵长类IPSC来源神经元中的治疗性寡核苷酸的高通量筛选。

序列表

<110> 豪夫迈·罗氏有限公司(F. Hoffmann la Roche AG)
<120> 来自灵长类物种的标准化神经元细胞测定试验
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[0072]

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[0115]

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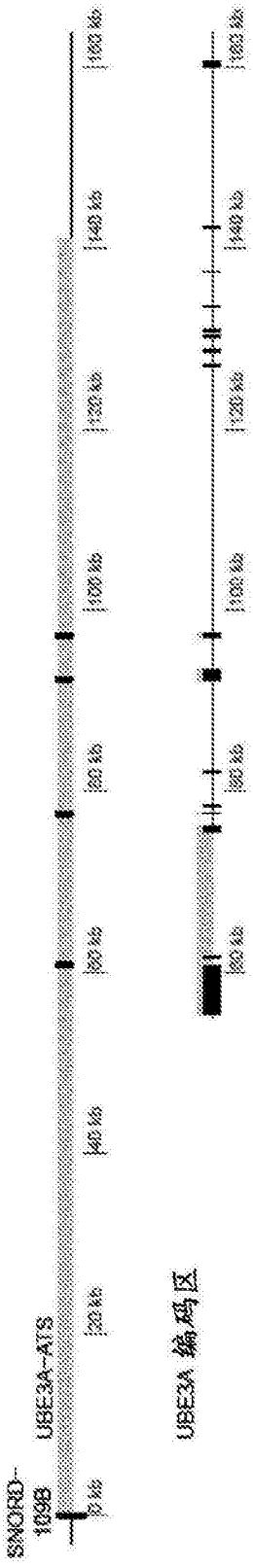

图1

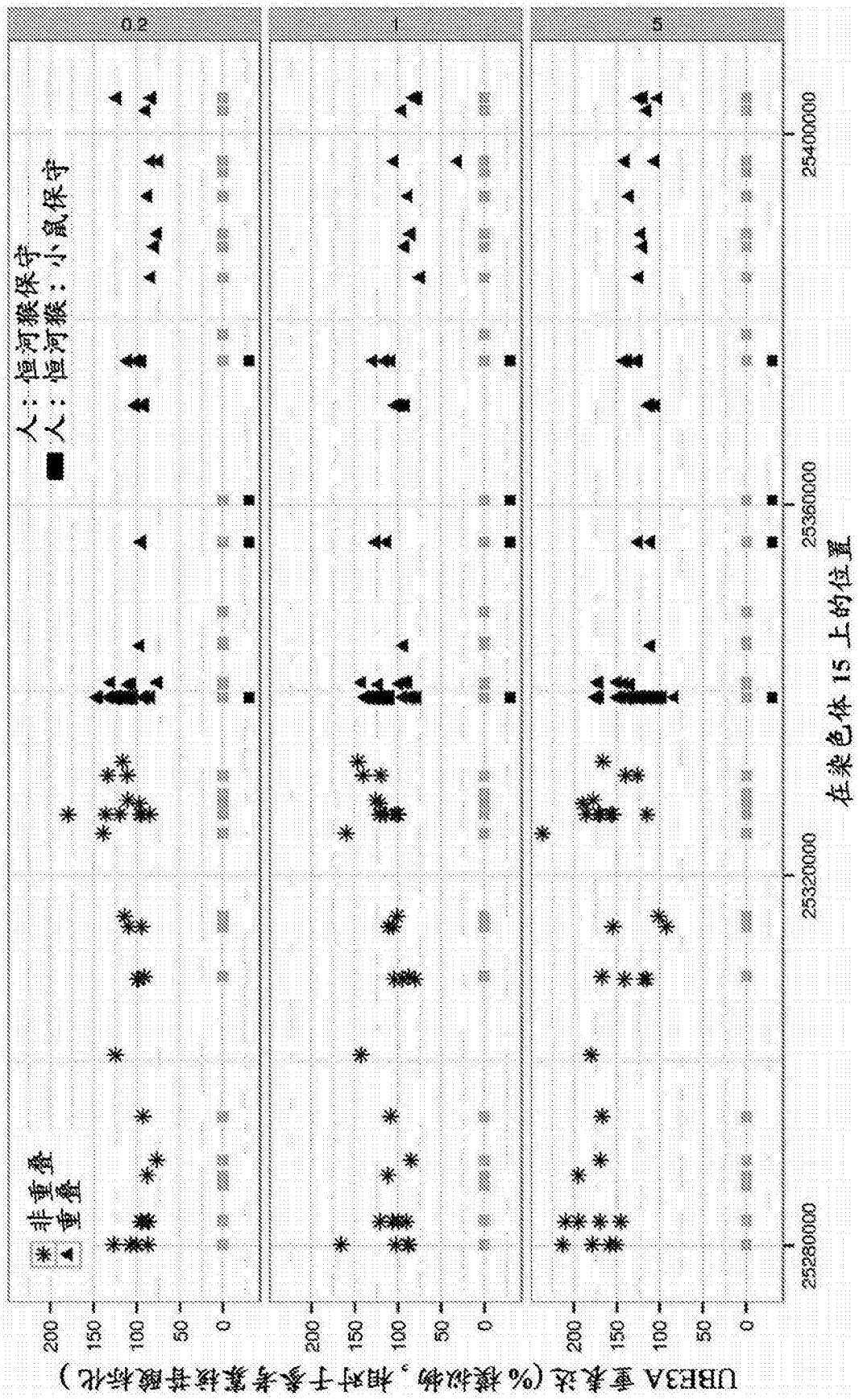
0.2
1
5
非重叠
重叠
人：恒河猴保守
人：恒河猴：小鼠保守
UBE3A 重表达(% 模拟物，相对于参考聚核苷酸标化)
200
150
100
50
0
25280000
25320000
25360000
25400000
在染色体 15 上的位置

图2

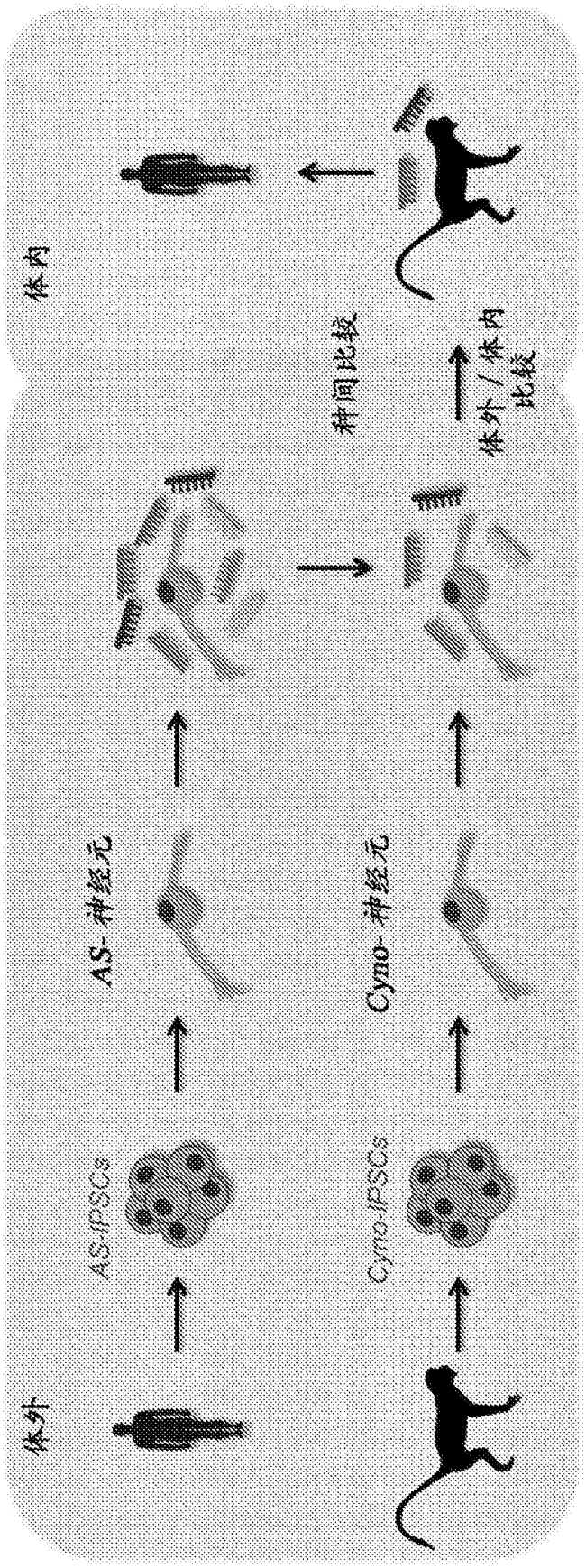

图3

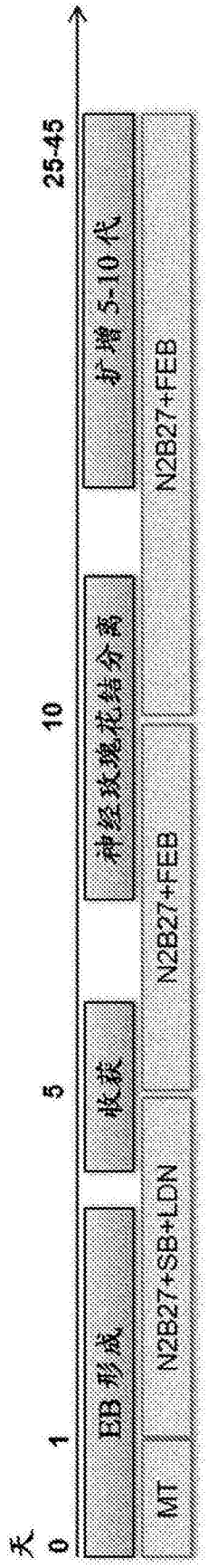
天
0
1
5
10
25-45
EB 形成
MT
N2B27+SB+LDN
收获
N2B27+FEB
神经球团结分离
N2B27+FEB
扩增 5-10 代
N2B27+FEB

图4

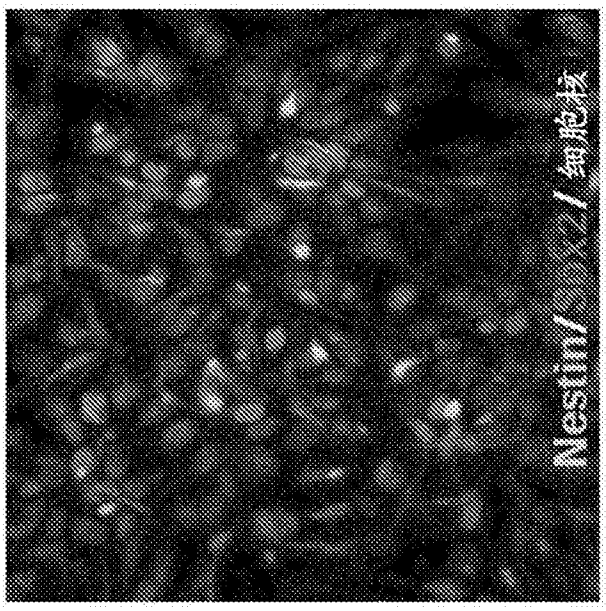

图5A

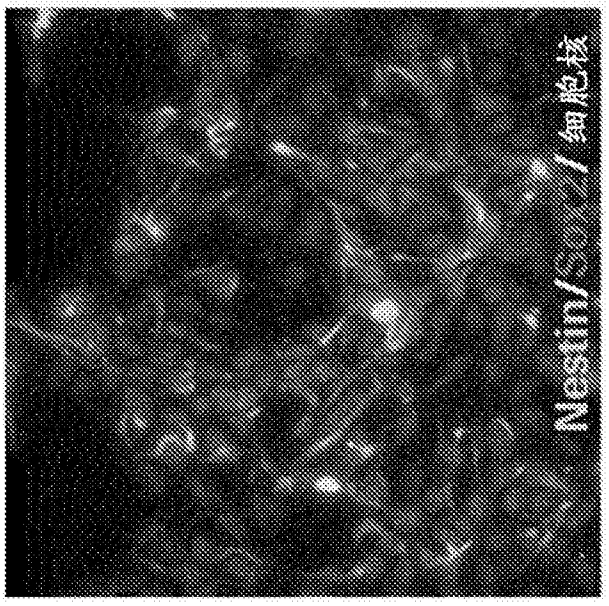

图5B

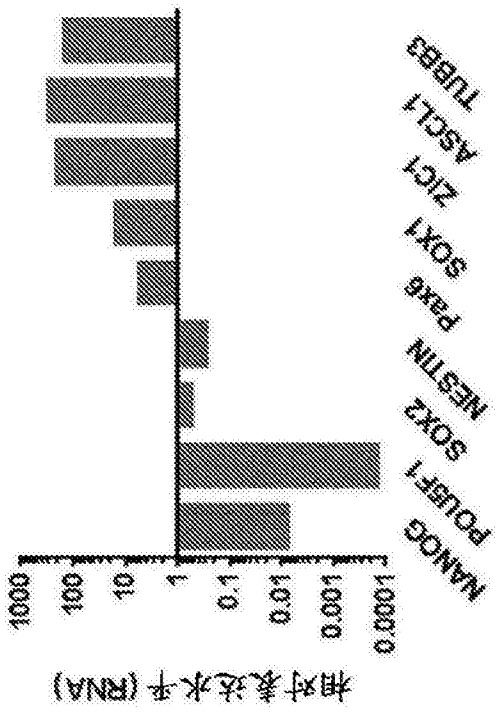
相对表达水平(RNA)
1000
100
10
1
0.1
0.01
0.001
0.0001
NANOG
POUSF1
SOX2
NESTIN
PAX6
SOX1
ZIC1
ASCL1
TUBB3

图6A

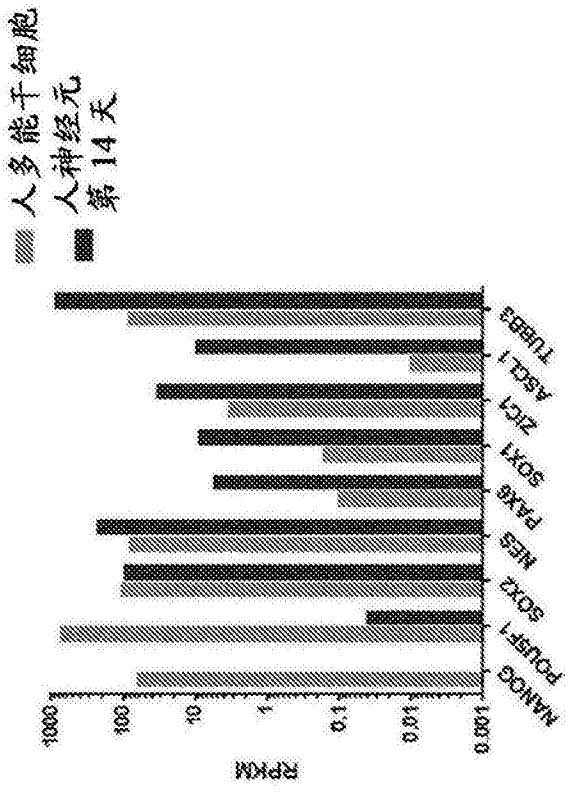
人多能干细胞
人神经元
第14天
RPKM
1000
100
10
1
0.1
0.01
0.001
NANOG
POUSF1
SOX2
NES
PAX6
SOX1
ZIC1
ASCL1
TUBB3

图6B

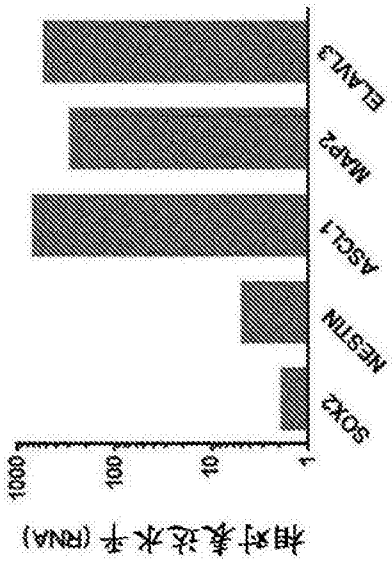
SOX2
NESTIN
ASCL1
MAP2
ELAVL3
1000
100
10
1
相对表达水平 (RNA)

图6C

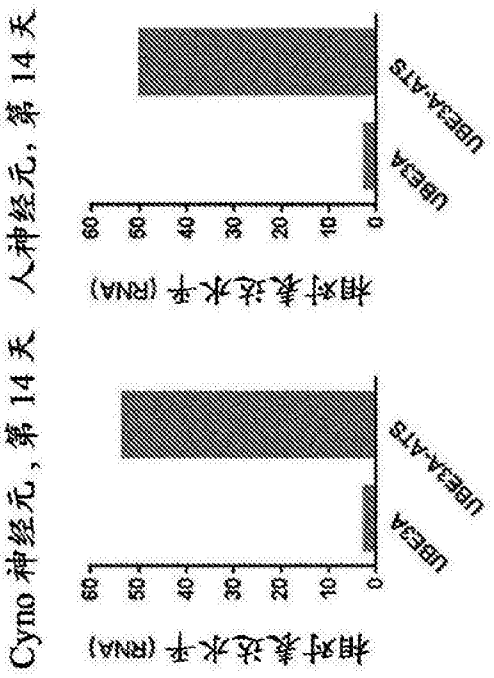
Cyno 神经元，第 14 天 人神经元，第 14 天
UBE3A
UBE3A-ATS
60
50
40
30
20
10
0
相对表达水平 (RNA)

图6D

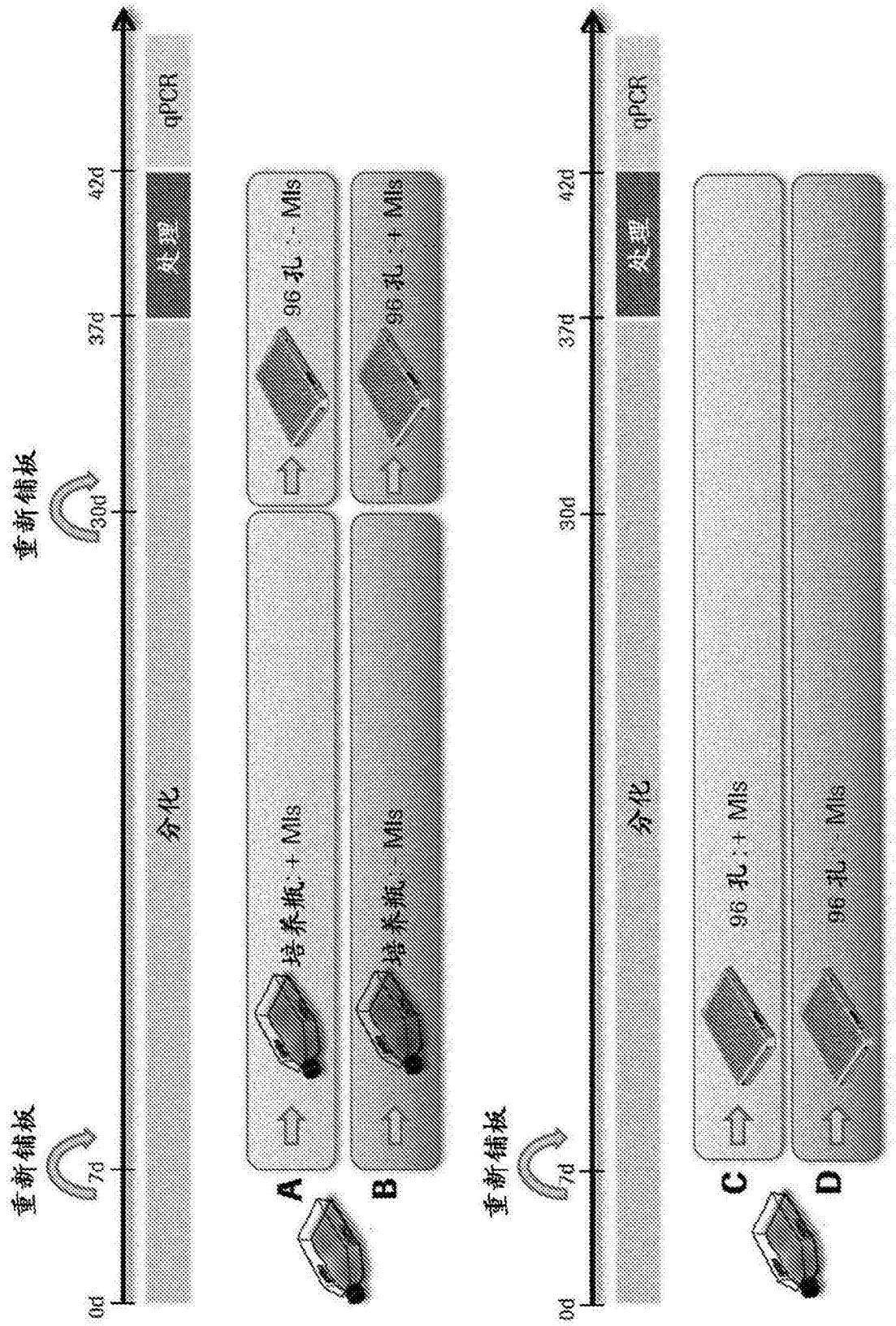
重新铺板
7d
30d
0d
37d
42d
分化
处理
qPCR
A
培养瓶 + Mls
96 孔 - Mls
B
培养瓶 - Mls
96 孔 + Mls
C
96 孔 + Mls
D
96 孔 - Mls

图7

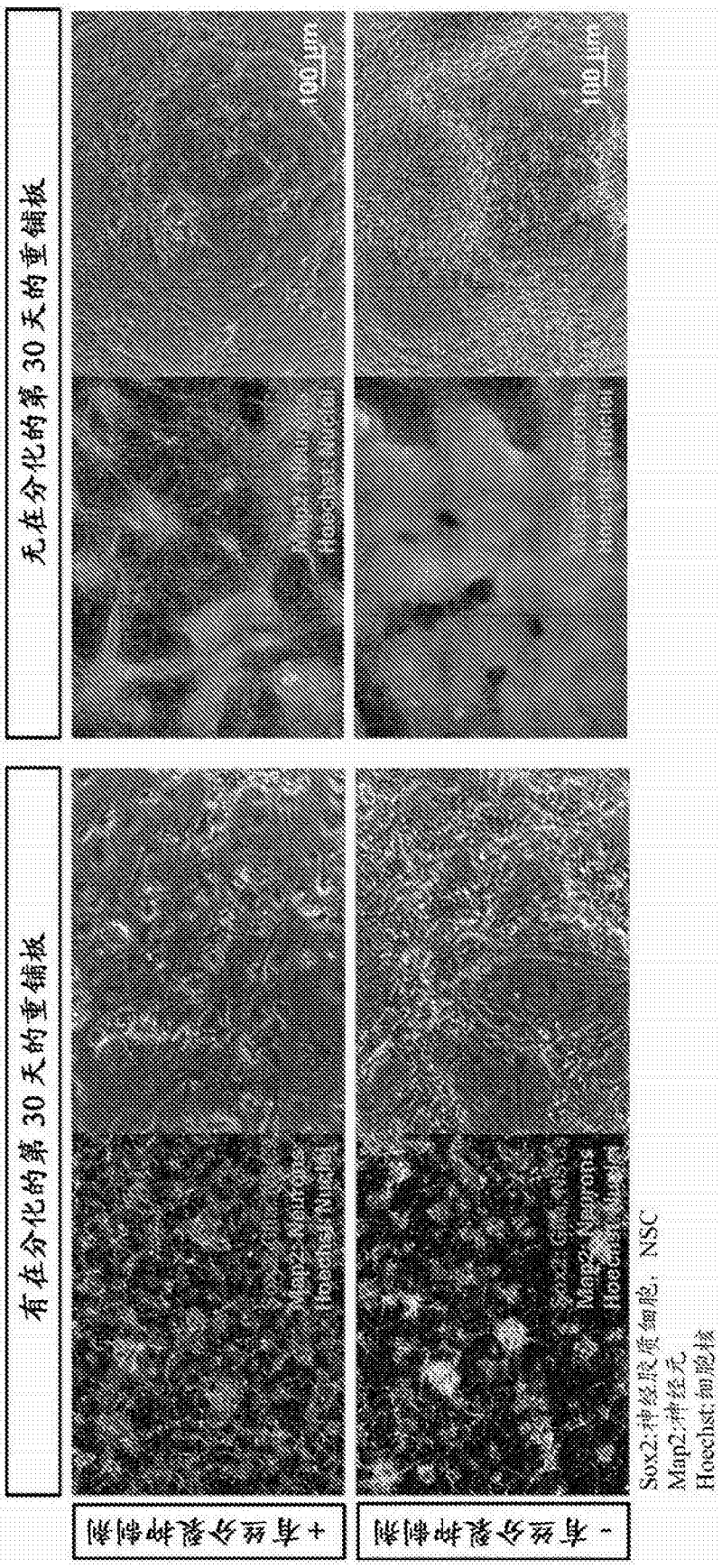
无在分化的第 30 天的重铺板
有在分化的第 30 天的重铺板
- 有丝分裂抑制剂
+ 有丝分裂抑制剂
100 μm
Sox2:神经胶质细胞；NSC
Map2:神经元
Hoechst:细胞核

图8

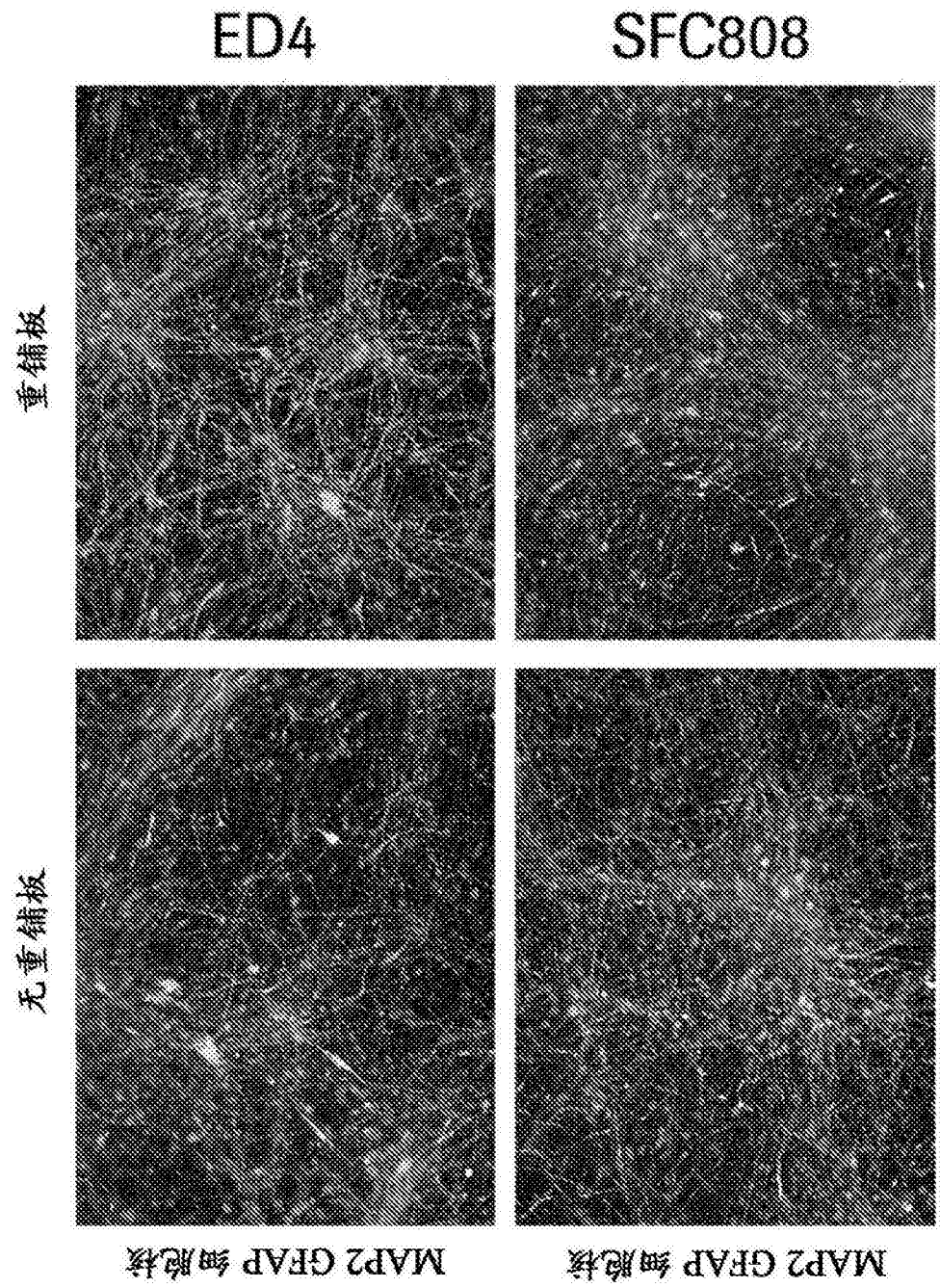

图9

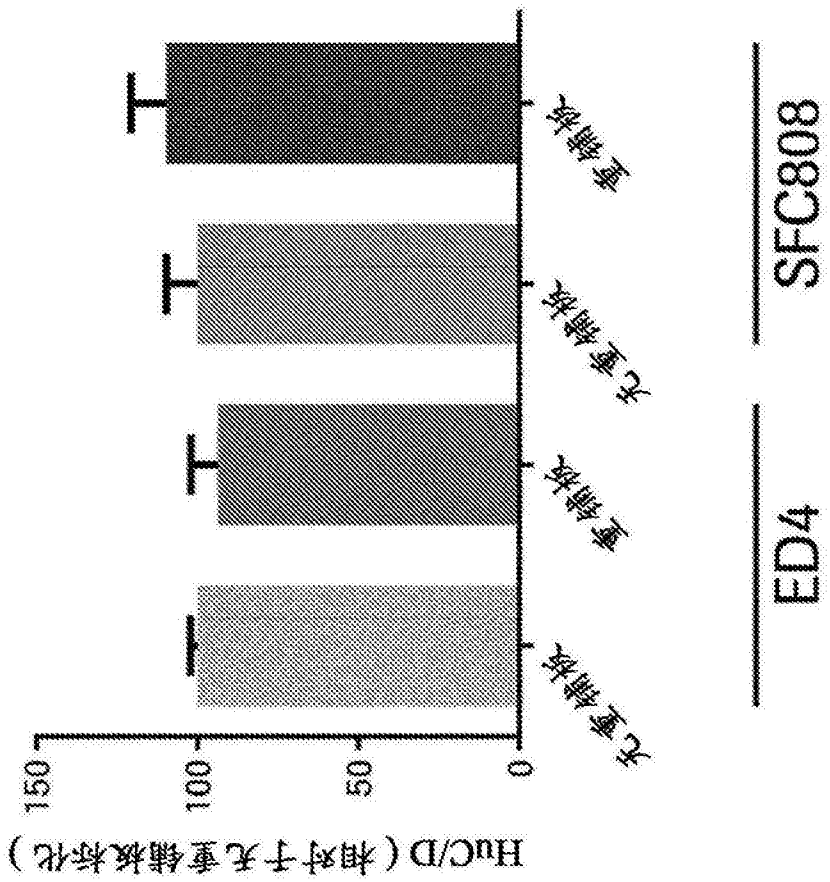

图10

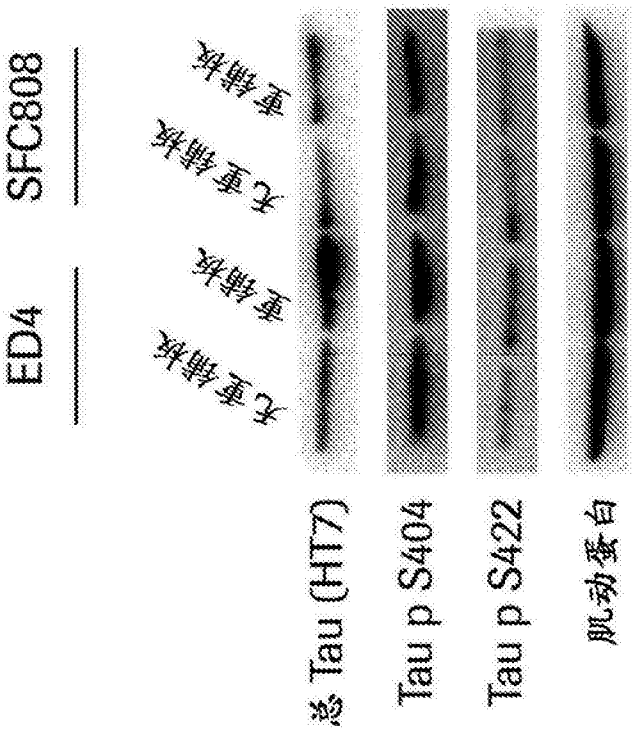

图11

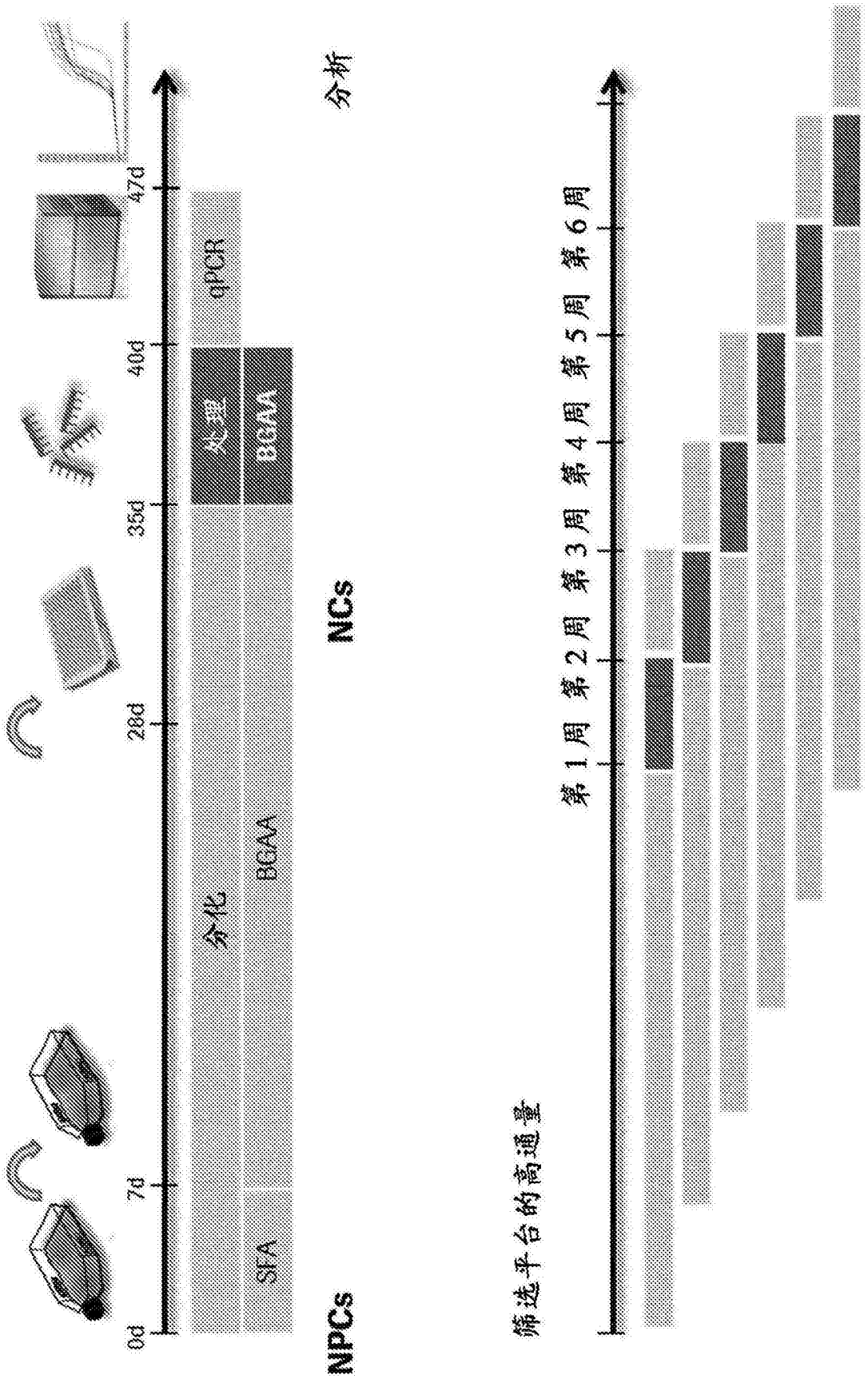
0d
7d
28d
35d
40d
47d
NPCs
NCs
分析
分化
SFA
BGAA
BGAA
qPCR
筛选平台的高通量
第 1 周
第 2 周
第 3 周
第 4 周
第 5 周
第 6 周

图12

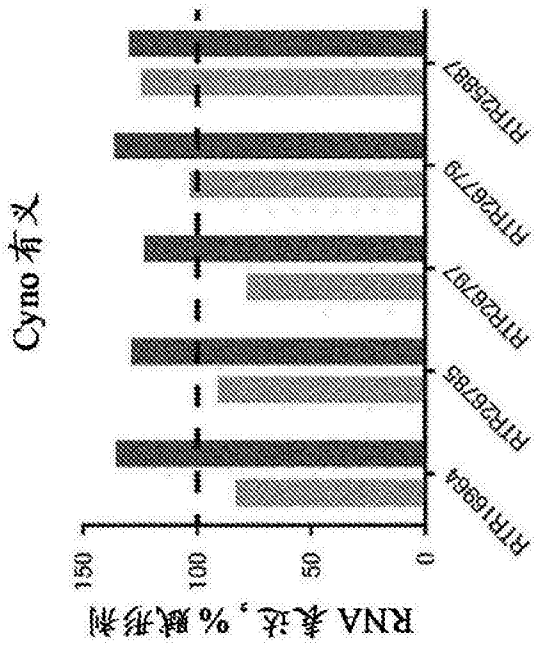
Cyno 有义
RNA 表达，% 媒形剂
150
100
50
0
AM08964
AM28785
AM28797
AM28779
AM28807

图13A

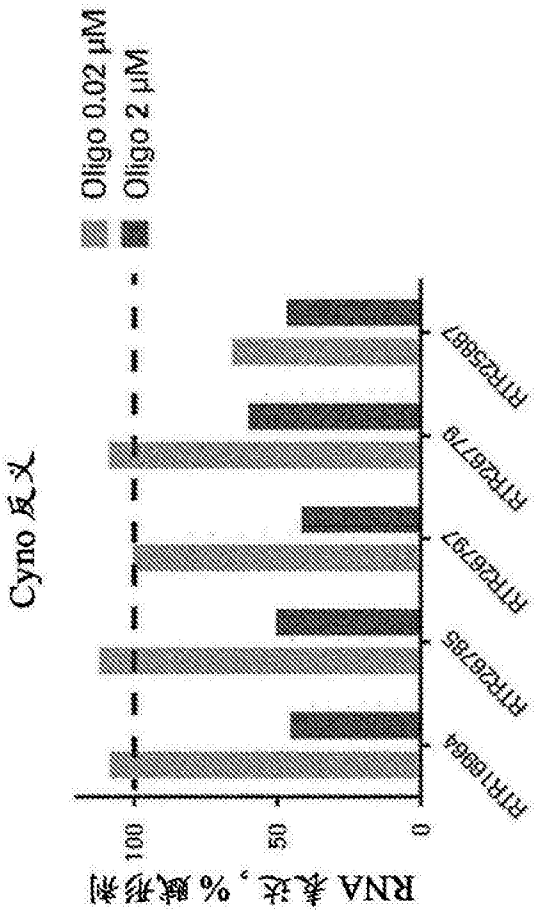
Oligo 0.02 μM
Oligo 2 μM
Cyno 反义
RNA 表达，% 媒形剂
100
50
0
AM08964
AM28785
AM28797
AM28779
AM28807

图13B

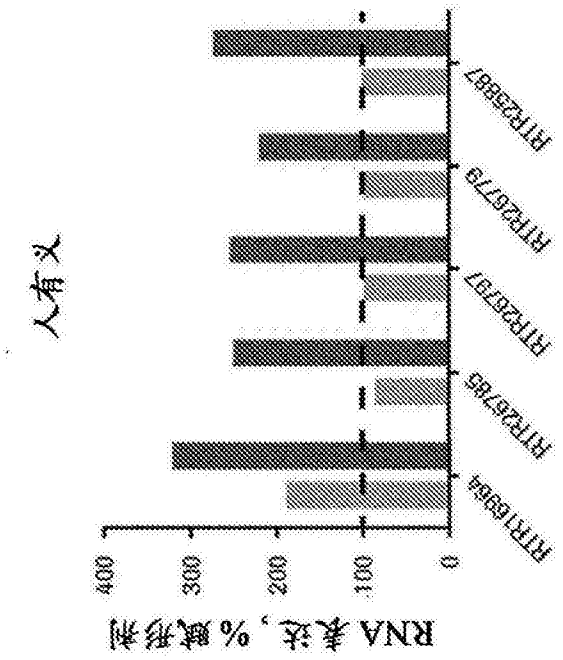
人有义
RTR1696A
RTR2587B5
RTR2587B7
RTR2677B5
RTR2677B7
RTR2677B9
RTR2588B7
0
100
200
300
400
RNA 表达，% 媒形剂

图13C

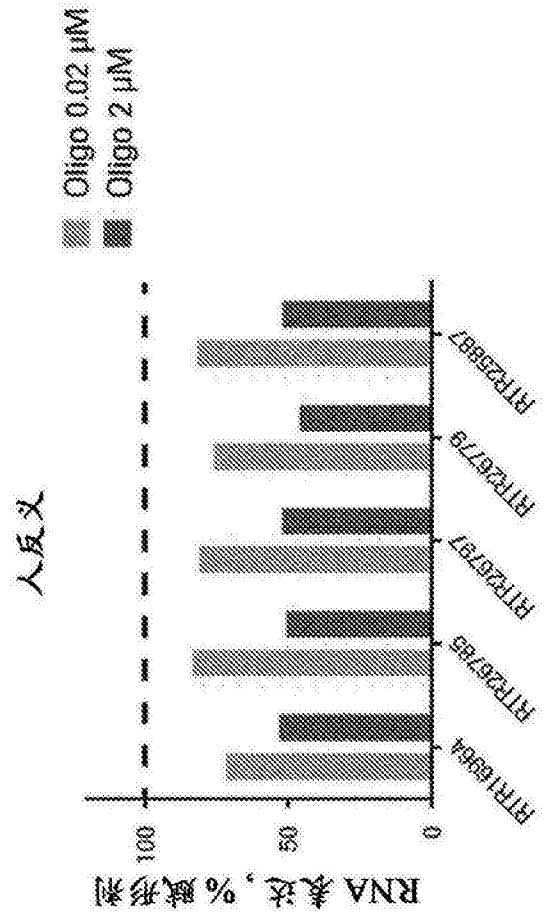
Oligo 0.02 μM
Oligo 2 μM
人反义
RTR1696A
RTR2587B5
RTR2677B7
RTR2677B9
RTR2588B7
0
50
100
RNA 表达，% 媒形剂

图13C