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(54) **Title**: METHODS FOR DETERMINING THE EFFICACY PROFILE OF A DRUG CANDIDATE

(57) **Abstract**: This patent application relates to 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 based on a dissociation and reseeding step performed on the differentiated NCs. The method includes differentiating human and/or non-human primate neuronal precursor cells (NPCs) to neuronal cells (NCs) followed by dissociating the differentiated NCs from its support and reseeding the differentiated NCs in a high-throughput cell culture format resulting in robust cultures suitable for high-throughput drug screening assays, in particular to screen antisense oligonucleotides.

Methods for determining the efficacy profile of a drug candidate

FIELD OF THE INVENTION

This patent application relates to 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 based on a dissociation and reseeding step performed on the differentiated NCs. The method includes differentiating human and/or non-human primate neuronal precursor cells (NPCs) to neuronal cells (NCs) followed by dissociating the differentiated NCs from its support and reseeding the differentiated NCs in a high-throughput cell culture format 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 for high-throughput screening of drug candidates.

SUMMARY OF THE INVENTION

Provided herein 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 the drug candidate; and
d) determining the *in vitro* efficacy profile of the drug candidate.

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, one of the primate species is human (Homo sapiens).

In one embodiment, one of the primate species is Cynomolgus monkey (Macaca fascicularis).

In one embodiment, the differentiated NCs are derived from induced pluripotent stem cells (iPSCs).

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

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

In one embodiment, step d) additionally comprises monitoring the cell cultures for signs of toxicity.

In one embodiment, step d) comprises monitoring the cell cultures for a phenotypic change indicative of the efficacy of the drug candidate.

In one embodiment, 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 between the first and the second species is high, in particular more than 90%.

In one embodiment, the first species is cynomolgus monkey (Macaca fascicularis) and the second species is human (Homo sapiens).

In one embodiment, the drug candidate comprises a nucleic acid molecule or targets a specific nucleic acid sequence.

In one embodiment, the drug candidate comprises at least one nucleic acid molecule such as a RNAi agent or an antisense oligonucleotide.

In one embodiment, the further development comprises determining the *in vivo* efficacy and/or toxicity profile of the drug candidate.

In one embodiment, provided is a method for determining the potential *in vivo* efficacy of a drug candidate wherein the *in vitro* efficacy profile of a drug candidate is determined as described herein and wherein the *in vitro* efficacy profile is indicative for *in vivo* efficacy.

In one embodiment, the *in vitro* efficacy profile of a drug candidate is indicative for *in vivo* efficacy in human (Homo sapiens).

In one embodiment, the *in vitro* efficacy profile of a drug candidate is indicative for *in vivo* efficacy in cynomolgus monkey (Macaca fascicularis).

In one embodiment, the *in vivo* efficacy profile is determined in at least one species.

In one embodiment, the *in vivo* efficacy profile is determined in cynomolgus monkey (Macaca fascicularis).

In one embodiment, the determined *in vitro* efficacy profile and/or *in vivo* efficacy profile of the drug candidate is indicative for *in vivo* efficacy in human (Homo sapiens).

In one embodiment, the determined *in vitro* efficacy profile and the *in vivo* efficacy profile of the drug candidate as assessed in cynomolgus monkey is indicative for *in vivo* efficacy in human (Homo sapiens).

In one embodiment, the differentiation medium is basal medium supplemented with 20 ng/ml BDNF, 10 ng/ml GDNF, 0.5 mM cAMP, and 100 µM ascorbic acid phosphate.

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

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.

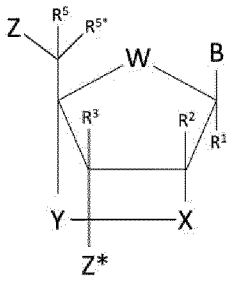
In some embodiments of the invention, the antisense oligonucleotide comprise both 2' sugar modified nucleosides and DNA units. Preferably the antisense oligonucleotide comprise both LNA and DNA units. Preferably, the combined total of LNA and DNA units is 8-30, such as 10 – 25, preferably 12-22, such 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.

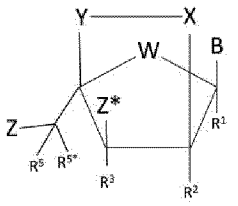
In a preferred embodiment the antisense oligonucleotide has a gapmer design or structure also referred herein merely as "Gapmer". In a gapmer structure the antisense oligonucleotide comprises at least three distinct structural regions a 5'-flank, a gap and a 3'-flank, F-G-F' in '5 -> 3' orientation. 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:



β-D



α-L

or

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. 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 the drug candidate; and
    d) determining the *in vitro* efficacy profile of the drug candidate.

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 one of the primate species is human (Homo sapiens).

4. The method according to any one of embodiment 1 or 2, wherein one of the primate species is Cynomolgus monkey (Macaca fascicularis).

5. The method of any one of embodiments 1 to 4, wherein the differentiated NCs are derived from induced pluripotent stem cells (iPSCs).

6. The method according to any one of embodiments 1 to 5, wherein the differentiated NCs are uniformly distributed over the cell culture area as assessed by cell nucleus staining.

7. The method according to any one of embodiments 1 to 6, wherein the distribution of the differentiated NCs is assessed by DNA staining, in particular by Hoechst staining.

8. The method according to any one of embodiments 1 to 7, wherein step d) additionally comprises monitoring the cell cultures for signs of toxicity.

9. The method according to any one of embodiments 1 to 8, wherein step d) comprises monitoring the cell cultures for a phenotypic change indicative of the efficacy of the drug candidate.

10. The method according to any one of embodiments 1 to 9, 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.

11. 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 of any one of embodiments 1 to 10; and
    (ii) selecting the drug candidate for further development if the efficacy profile of the drug candidate is favourable.

12. The method according to embodiment 11 wherein the genetic similarity between the first and the second species is high, in particular more than 90%.

13. The method according to any one of embodiments 11 or 12, wherein the first species is cynomolgus monkey (Macaca fascicularis) and the second species is human (Homo sapiens).

14. The method according to any one of embodiments 11 to 13, wherein the drug candidate comprises a nucleic acid molecule or targets a specific nucleic acid sequence.

15. The method according to any one of embodiments 11 to 14, wherein the drug candidate comprises at least one nucleic acid molecule such as a RNAi agent or an antisense oligonucleotide.

16. The method according to any one of embodiments 11 to 15, wherein the further development comprises determining the *in vivo* efficacy and/or toxicity profile of the drug candidate.

17. A method for determining the potential *in vivo* efficacy of a drug candidate wherein the *in vitro* efficacy profile of a drug candidate is determined according to any one of embodiments 1 to 10 and wherein the *in vitro* efficacy profile is indicative for *in vivo* efficacy.

18. The method according to embodiment 17 wherein the *in vitro* efficacy profile of a drug candidate is indicative for *in vivo* efficacy in human (Homo sapiens).

19. The method according to embodiment 17, wherein the *in vitro* efficacy profile of a drug candidate is indicative for *in vivo* efficacy in cynomolgus monkey (Macaca fascicularis).

20. The method according to embodiment 17 to 19, wherein the *in vivo* efficacy profile is determined in at least one species.

21. The method according to embodiment 17 to 20, wherein the *in vivo* efficacy profile is determined in cynomolgus monkey (Macaca fascicularis).

22. The method according to any one of embodiments 17 to 21, wherein the determined *in vitro* efficacy profile and/or *in vivo* efficacy profile of the drug candidate is indicative for *in vivo* efficacy in human (Homo sapiens).

23. The method according to embodiment 21, wherein the determined *in vitro* efficacy profile and the *in vivo* efficacy profile of the drug candidate as assessed in cynomolgus monkey is indicative for *in vivo* efficacy in human (Homo sapiens).

24. The method according to any one of embodiments 1 to 23, wherein the differentiation medium is basal medium supplemented with 20 ng/ml BDNF, 10 ng/ml GDNF, 0.5 mM cAMP, and 100 µM ascorbic acid phosphate.

25. The method according to any one of embodiment 1 to 24, wherein the cell cultures are produced sequentially for different species.

26. 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	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	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. 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 the drug candidate; and
   d) determining the *in vitro* efficacy profile of the drug candidate.

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 of any one of claims 1 or 2, wherein the differentiated NCs are derived from induced pluripotent stem cells (iPSCs).

4. The method according to any one of claims 1 to 3, wherein the differentiated NCs are uniformly distributed over the cell culture area as assessed by cell nucleus staining.

5. The method according to any one of claims 1 to 4, wherein step d) additionally comprises monitoring the cell cultures for signs of toxicity.

6. The method according to any one of claims 1 to 5, wherein step d) comprises monitoring the cell cultures for a phenotypic change indicative of the efficacy of the drug candidate.

7. The method according to any one of claims 1 to 6, 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.

8. 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 of any one of claims 1 to 7; and
   (ii) selecting the drug candidate for further development if the efficacy profile of the drug candidate is favourable.

9. The method according to claim 8, wherein the first species is cynomolgus monkey (Macaca fascicularis) and the second species is human (Homo sapiens).

10. The method according to any one of claims 8 or 9, wherein the drug candidate comprises a nucleic acid molecule or targets a specific nucleic acid sequence.

11. The method according to any one of claims 8 to 10, wherein the drug candidate comprises at least one nucleic acid molecule such as a RNAi agent or an antisense oligonucleotide.

12. The method according to any one of claims 8 to 11, wherein the further development comprises determining the *in vivo* efficacy and/or toxicity profile of the drug candidate.

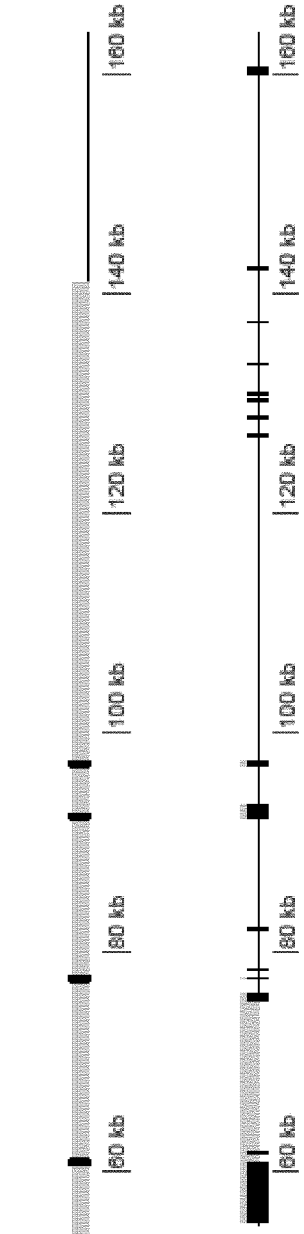
13. A method for determining the potential *in vivo* efficacy of a drug candidate wherein the *in vitro* efficacy profile of a drug candidate is determined according to any one of claims 1 to 7 and wherein the *in vitro* efficacy profile is indicative for *in vivo* efficacy.

14. The method according to claim 13, wherein the *in vivo* efficacy profile is determined in cynomolgus monkey (Macaca fascicularis).

15. The method according to claim 14, wherein the determined *in vitro* efficacy profile and/or *in vivo* efficacy profile of the drug candidate is indicative for *in vivo* efficacy in human (Homo sapiens).

16. The methods and uses essentially as described herein.

Figure 1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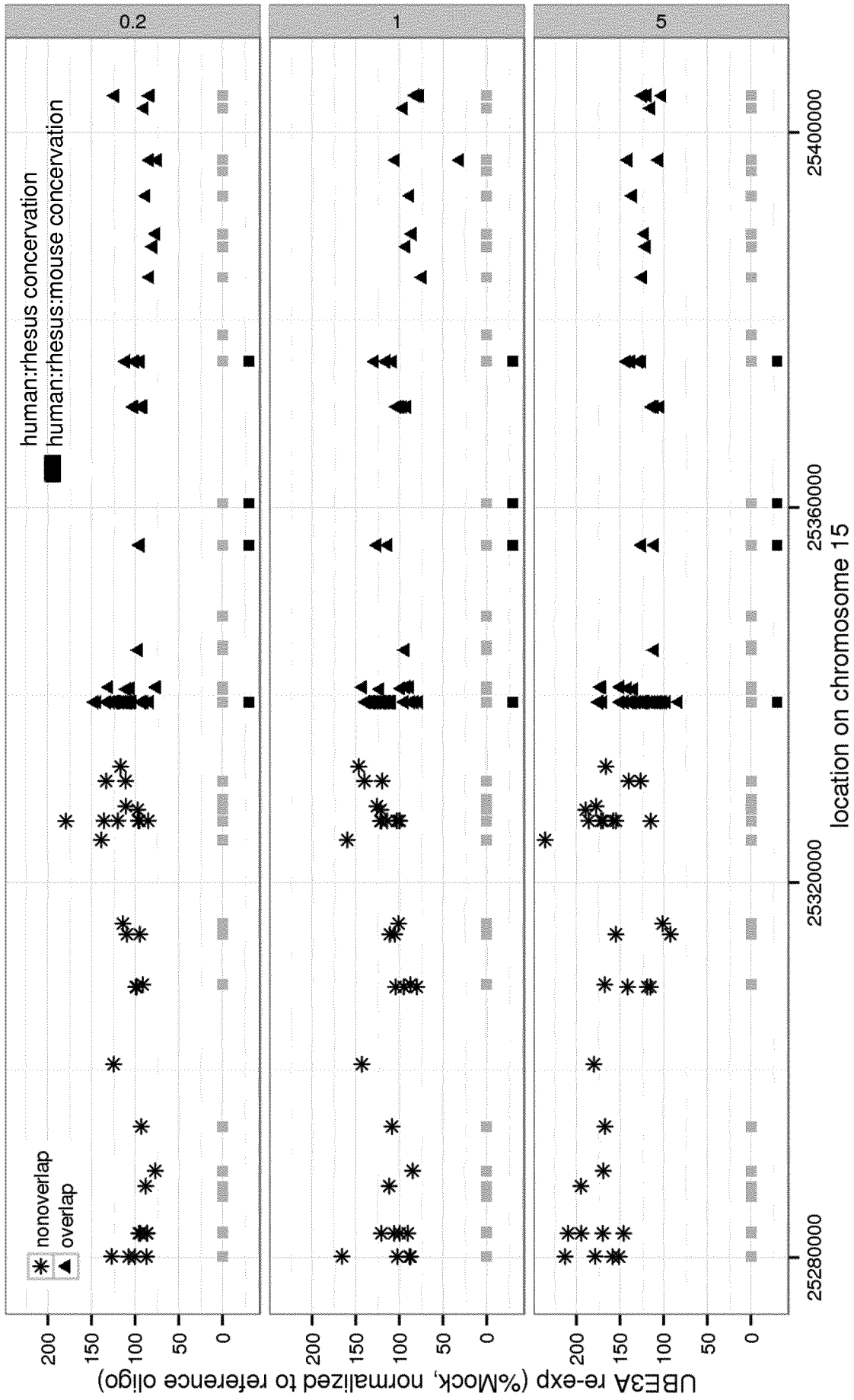
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human:rhesus:mouse concervation
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overlap
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location on chromosome 15
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25360000
25400000
0
50
100
150
200

Figure 2

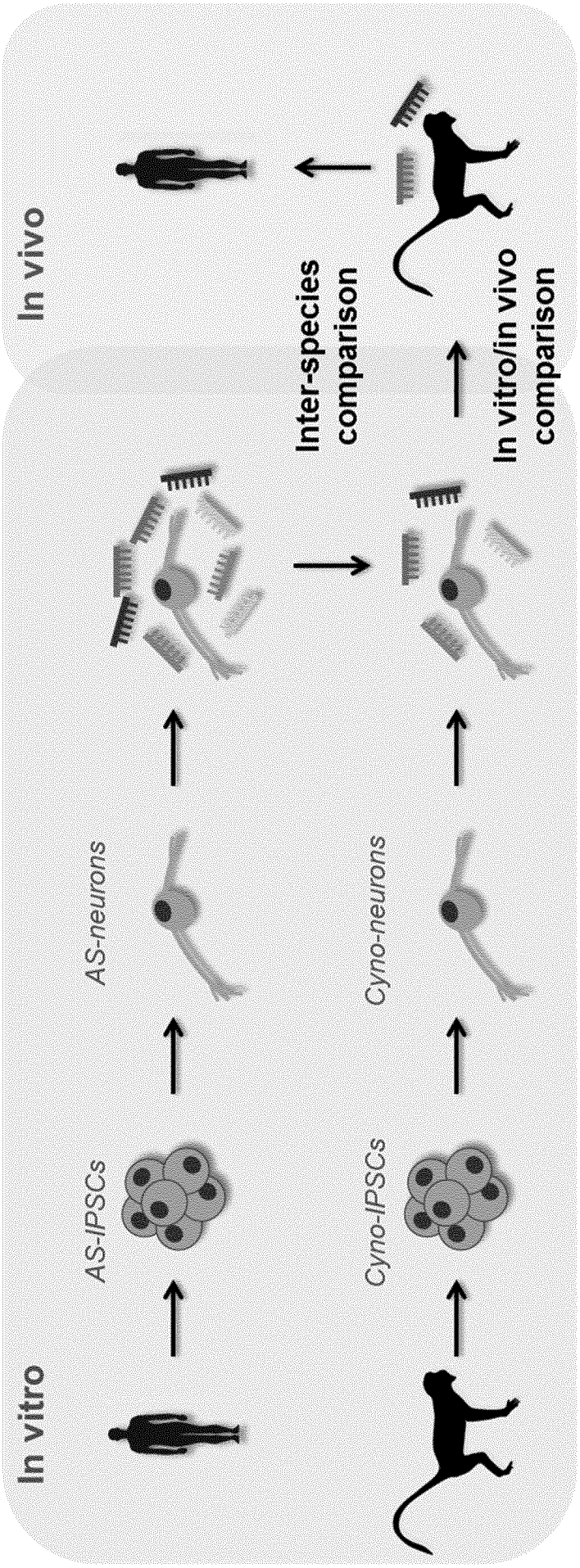

In vivo
Inter-species comparison
In vitro/in vivo comparison
In vitro
AS-neurons
Cyno-neurons
AS-IPSCs
Cyno-IPSCs

Figure 3

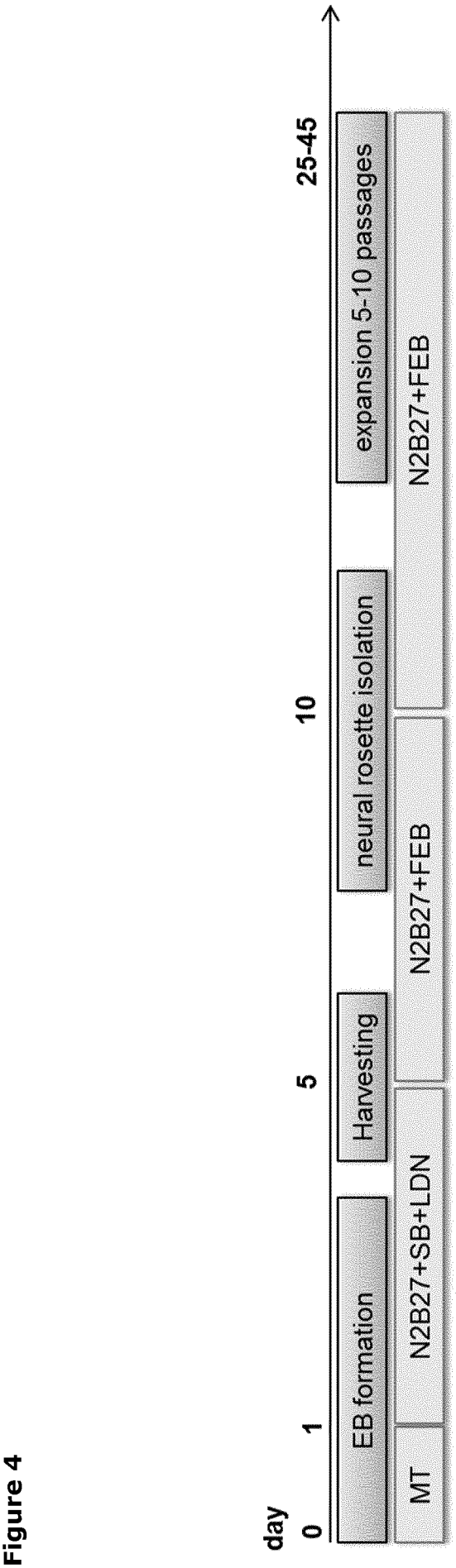

day
0
1
5
10
25-45
EB formation
Harvesting
neural rosette isolation
expansion 5-10 passages
MT
N2B27+SB+LDN
N2B27+FEB
N2B27+FEB

Figure 4

Figure 5B

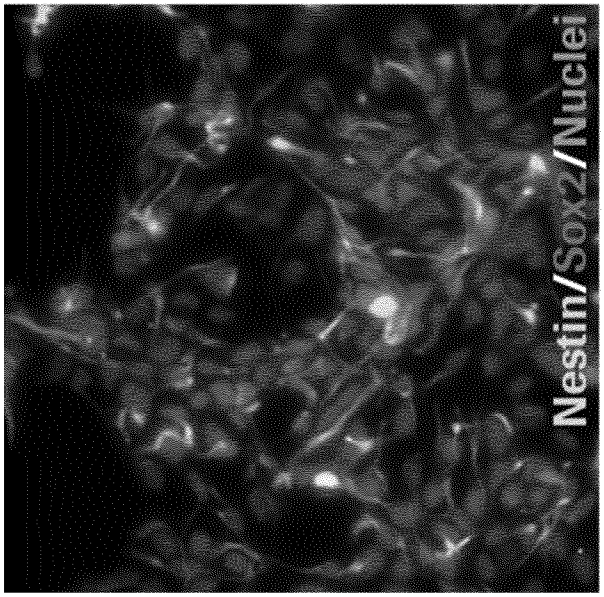


Figure 5A

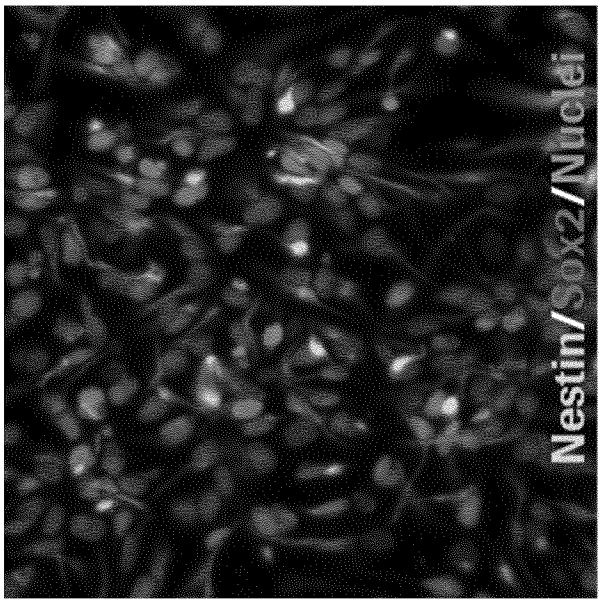


Figure 6A

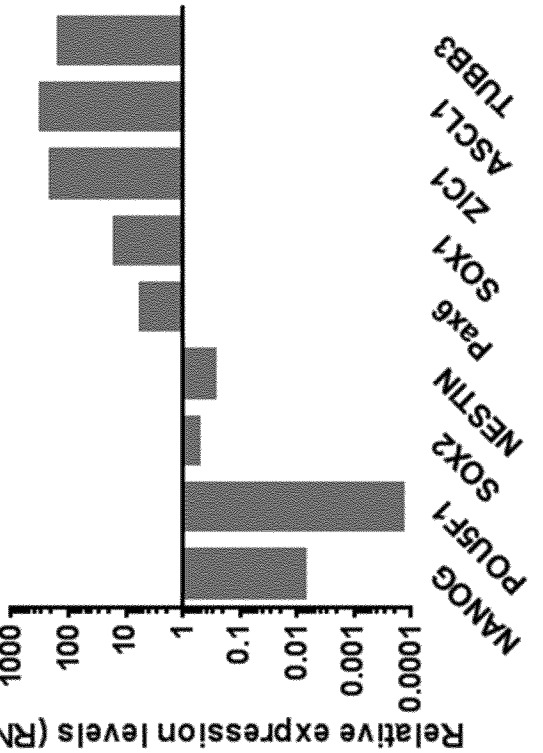


Figure 6B

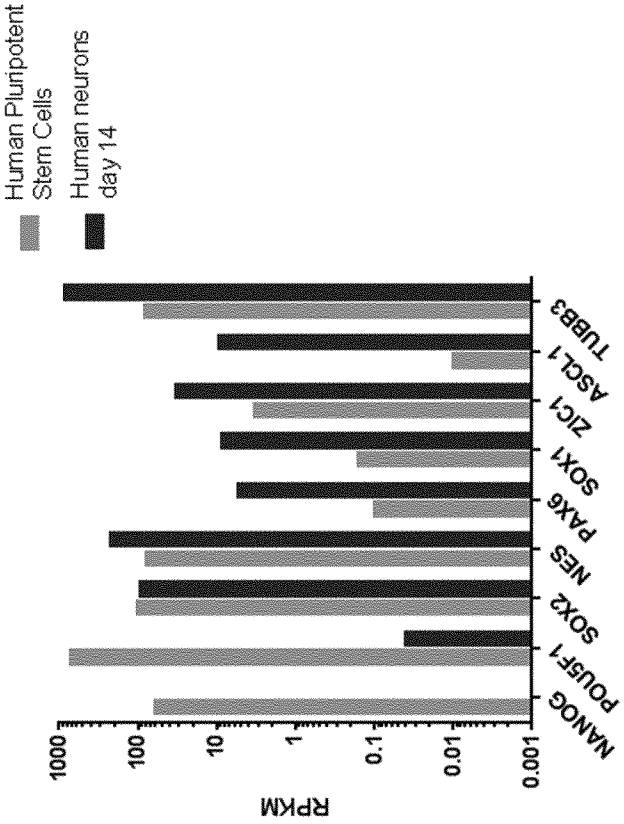


Figure 6C

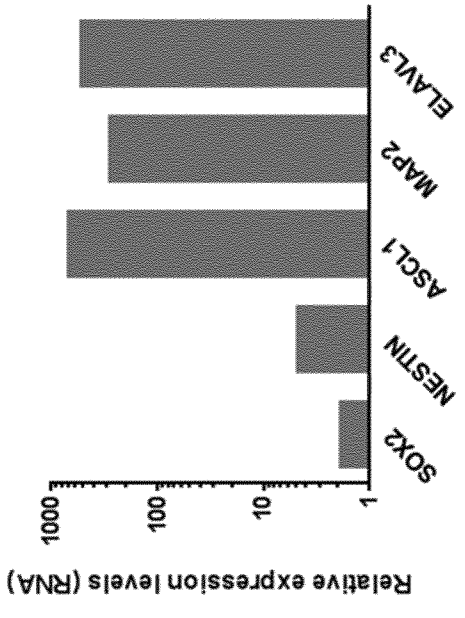


Figure 6D

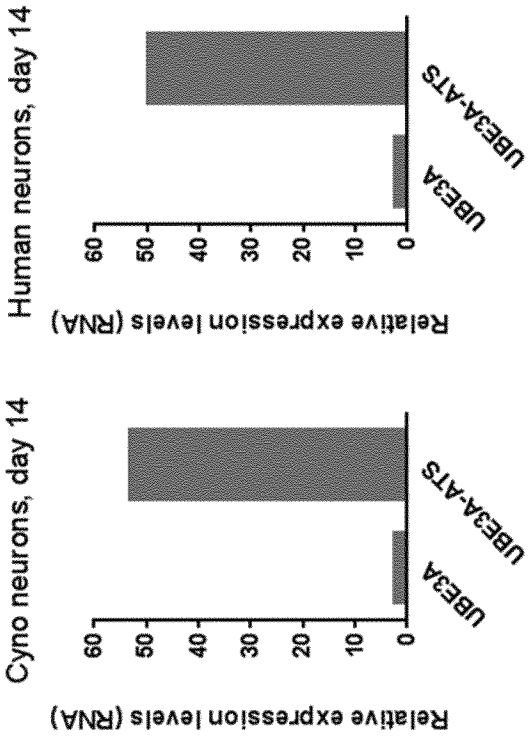
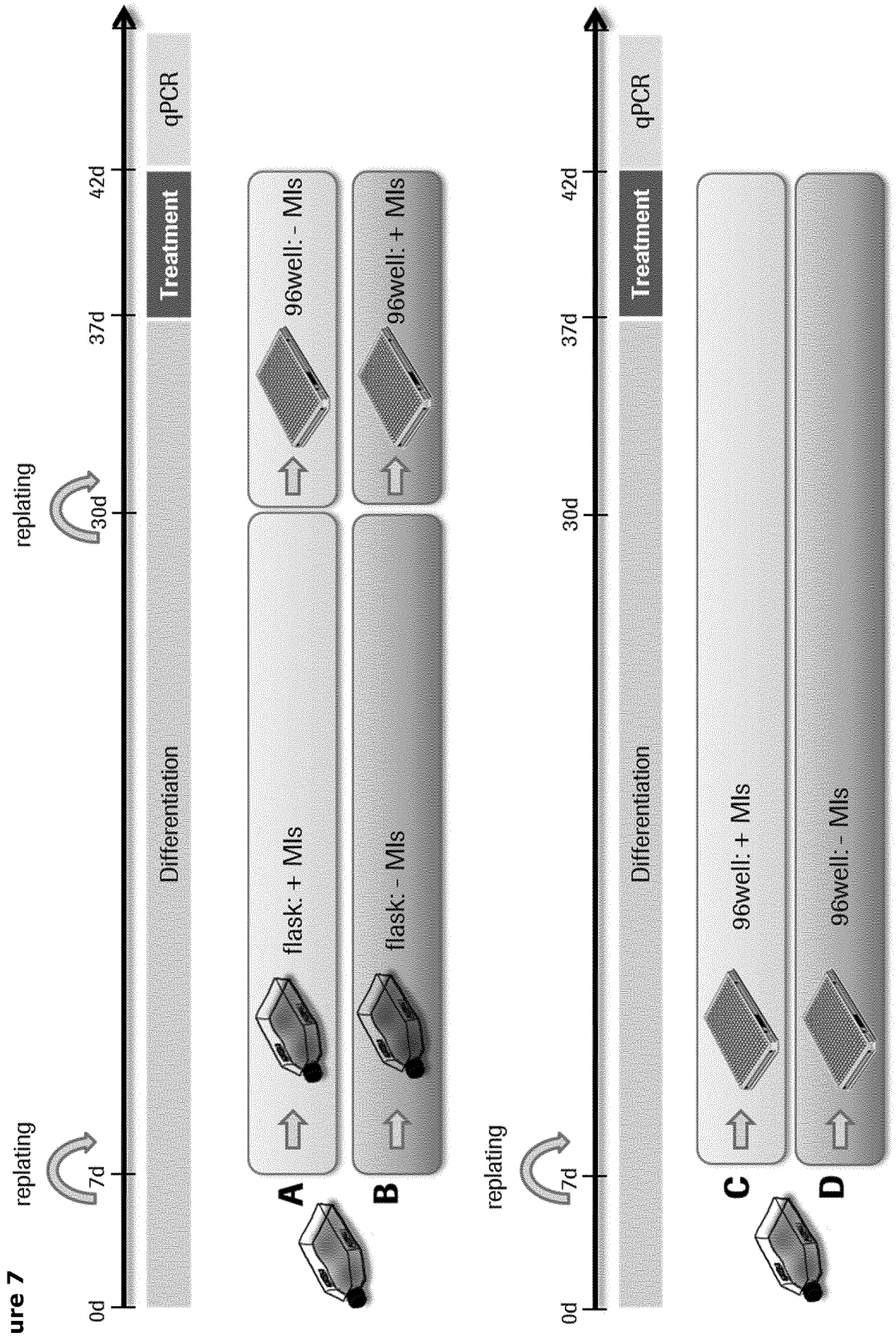


Figure 7


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

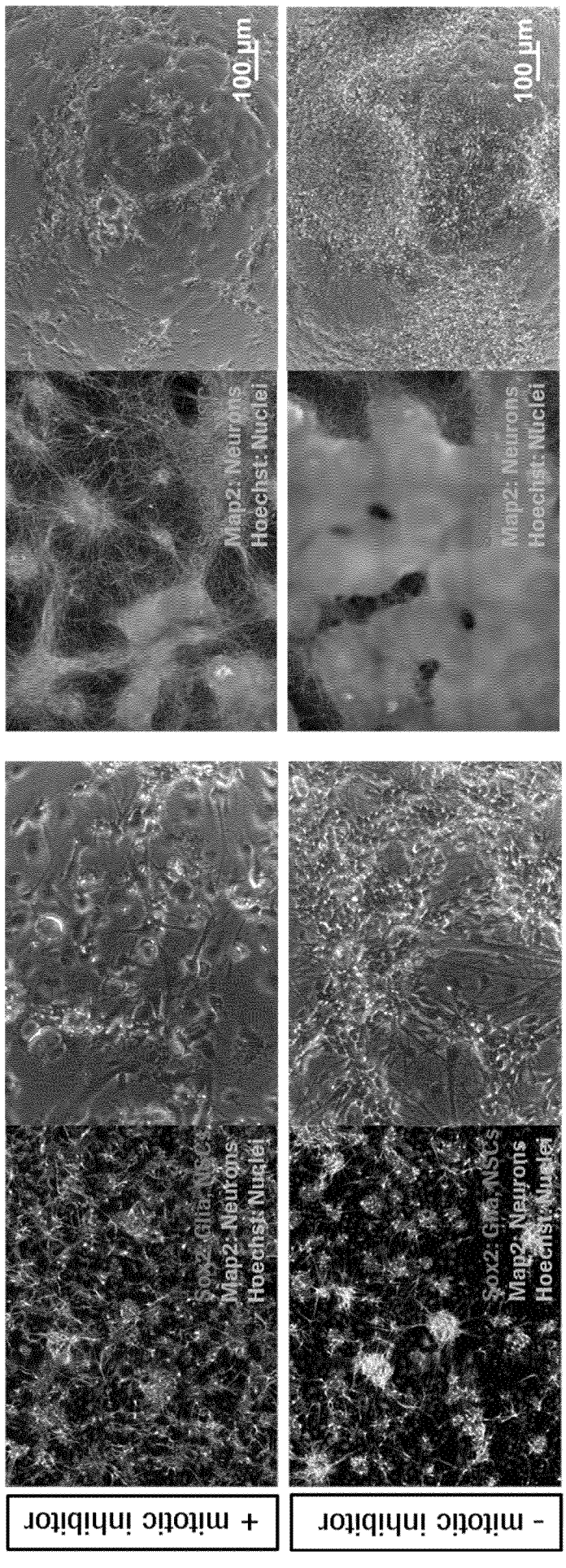

With replating at day 30 of differentiation
NO replating at day 30 of differentiation
+ mitotic inhibitor
- mitotic inhibitor
Sox2: Glia, NSCs
Map2: Neurons
Hoechst: Nuclei
100 µm

Figure 8

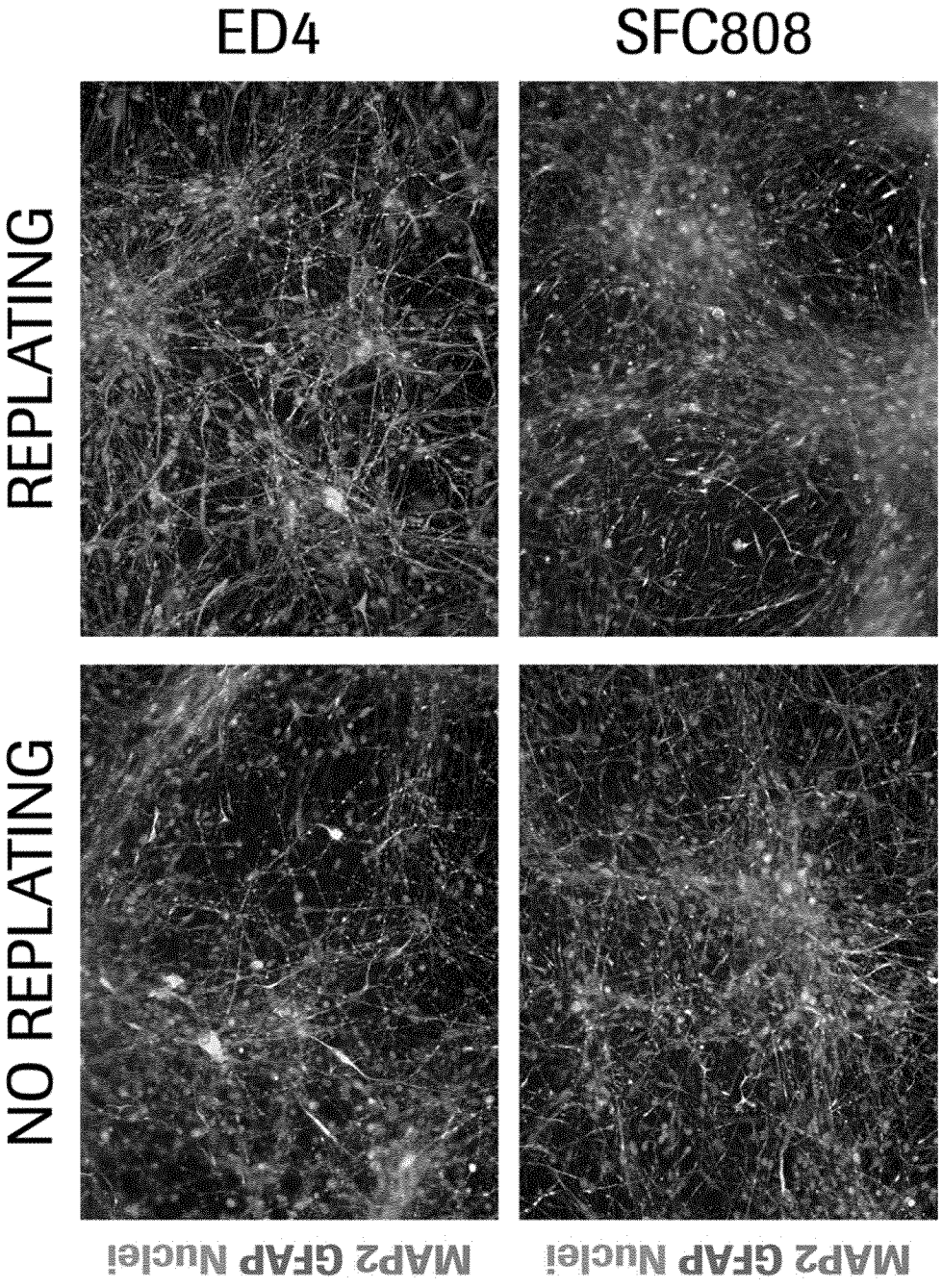

ED4
SFC808
REPLATING
NO REPLATING
MAP2 GFAP Nuclei
MAP2 GFAP Nuclei

Figure 9

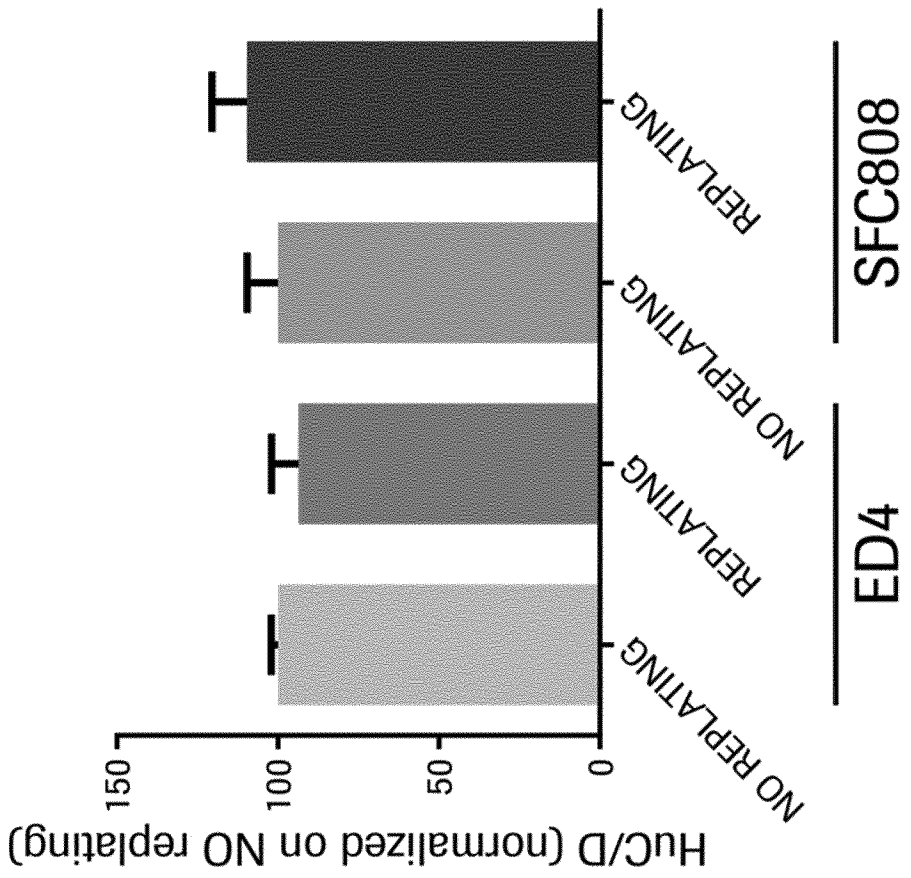

HuC/D (normalized on NO replating)
150
100
50
0
NO REPLATING
REPLATING
NO REPLATING
REPLATING
ED4
SFC808

Figure 10

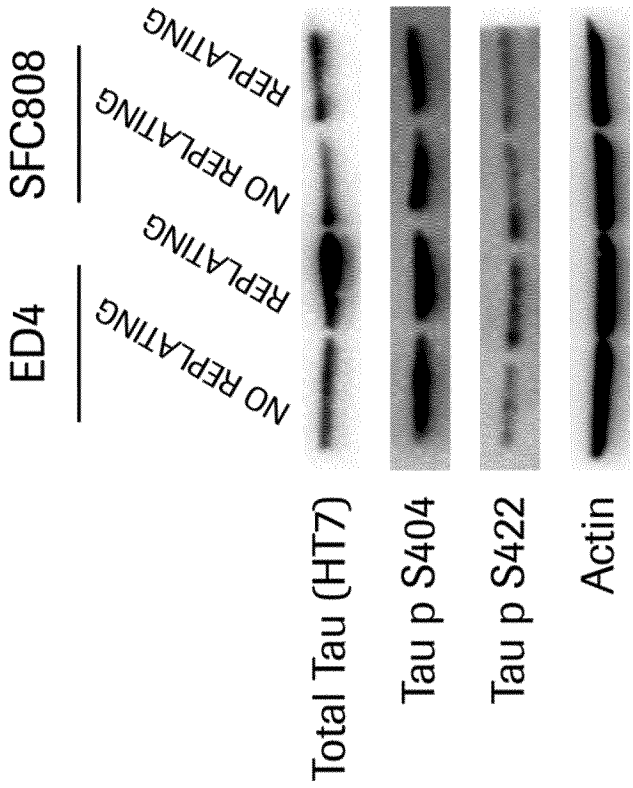
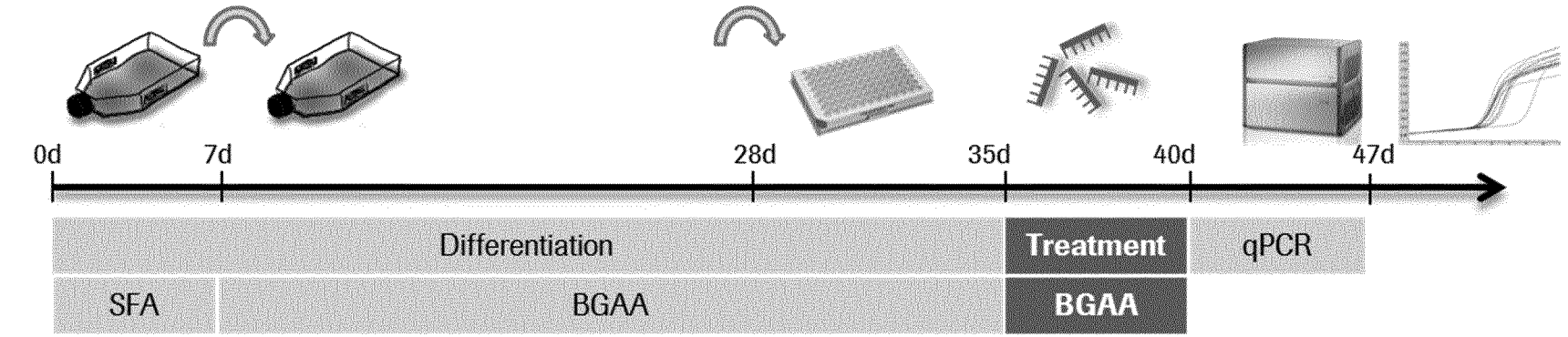

ED4
SFC808
NO REPLATING
REPLATING
NO REPLATING
REPLATING
Total Tau (HT7)
Tau p S404
Tau p S422
Actin

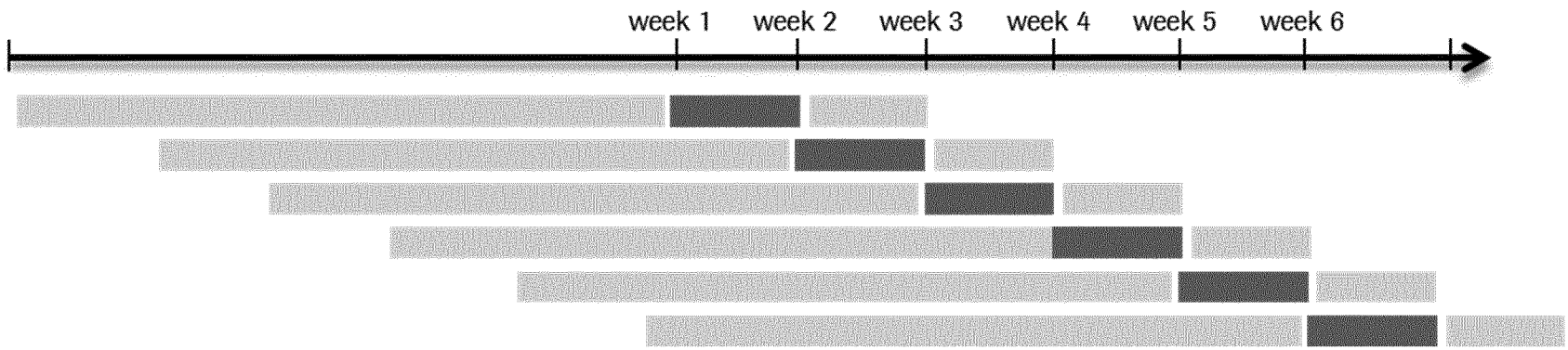
Figure 11

Figure 12



NPCs **NCs** **ANALYSIS**

Throughput of the screening platform



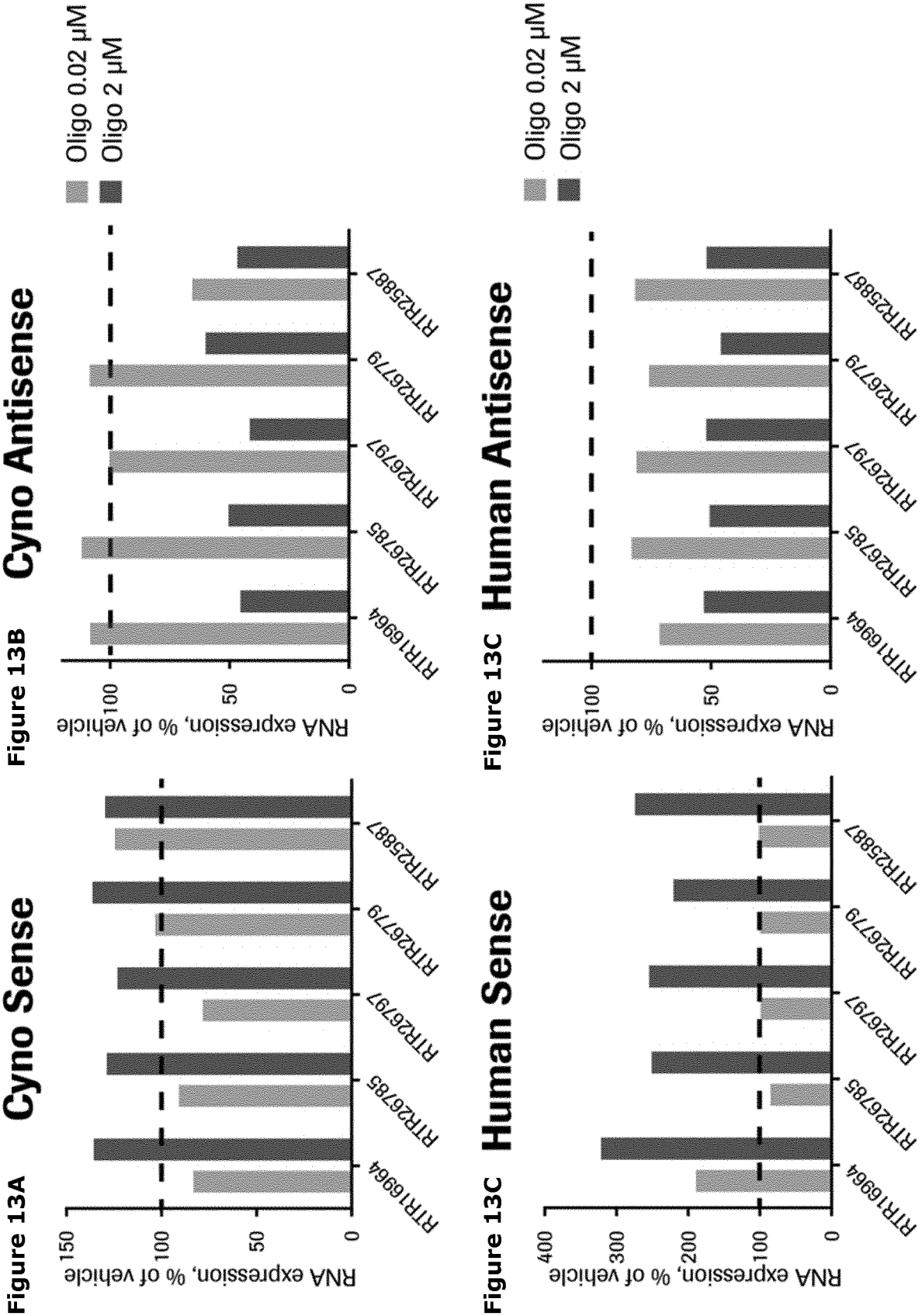


Figure 13A
Cyno Sense
RNA expression, % of vehicle
150
100
50
0
RTR16964
RTR26785
RTR26797
RTR26779
RTR25887
Figure 13B
Cyno Antisense
Oligo 0.02 µM
Oligo 2 µM
RNA expression, % of vehicle
100
50
0
RTR16964
RTR26785
RTR26797
RTR26779
RTR25887
Figure 13C
Human Sense
RNA expression, % of vehicle
400
300
200
100
0
RTR16964
RTR26785
RTR26797
RTR26779
RTR25887
Figure 13C
Human Antisense
Oligo 0.02 µM
Oligo 2 µM
RNA expression, % of vehicle
100
50
0
RTR16964
RTR26785
RTR26797
RTR26779
RTR25887

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/077435

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.
Y	RICHARD L. CARTER ET AL: "Reversal of Cellular Phenotypes in Neural Cells Derived from Huntington's Disease Monkey-Induced Pluripotent Stem Cells", STEM CELL REPORTS, vol. 3, no. 4, 1 October 2014 (2014-10-01) , pages 585-593, XP055345080, United States ISSN: 2213-6711, DOI: 10.1016/j.stemcr.2014.07.011 the whole document ----- -/--	1-16

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

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"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

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 February 2017	22/02/2017

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

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	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	1-16
A	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	1-16
A	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	1-16
Y,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

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INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/077435

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
T	EVA C. THOMA ET AL: "Establishment of a translational endothelial cell model using directed differentiation of induced pluripotent stem cells from Cynomolgus monkey", SCIENTIFIC REPORTS, vol. 6, 25 October 2016 (2016-10-25), page 35830, XP055344067, DOI: 10.1038/srep35830 the whole document -----	1-16

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