(19) LE GOUVERNEMENT
DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère de l'Économie

(11) N° de publication : **92845**

(12) **BREVET D'INVENTION** **B1**

(21) N° de dépôt: 92845

(22) Date de dépôt: 08/10/2015

(51) Int. Cl.:
C12N 5/079, C12N 5/0793, C12N 5/0797, G01N 33/50

(30) Priorité:

(43) Date de mise à disposition du public:

(47) Date de délivrance: 02/05/2017

(73) Titulaire(s):
UNIVERSITE DU LUXEMBOURG CAMPUS BELVAL – 4365 ESCH/BELVAL (Luxembourg)

(72) Inventeur(s):
SCHWAMBORN JENS – 4367
BELVAUX (Luxembourg)MONZEL ANNA – 4367
BELVAUX (Luxembourg)BOLOGNIN SILVIA – 4367
BELVAUX (Luxembourg)

(74) Mandataire(s):
SCHIWECK WEINZIERL KOCH GBR EUROPEAN PATENT ATTORNEYS – 80339 MÜNCHEN (Allemagne)

(54) **Means and methods for generating midbrain organoids.**

(57) The present invention provides means and methods for the generation of midbrain organoids which are useful for studying neurodevelopmental and neurodegenerative diseases. Neuroepithelial stem cells serve as a starting population for the generation of midbrain organoids by contacting them with differentiation medium under agitating conditions in three-dimensional cell culture comprising a matrix.

New LU-patent application
Université du Luxembourg
Our Ref.: LUX15542LU

№92845

92845

Means and methods for generating midbrain organoids

[001] The midbrain or mesencephalon is a portion of the central nervous system associated with vision, hearing, motor control, sleep/wake, arousal (alertness), and temperature regulation. During embryonic development, the midbrain arises from the second vesicle, also known as the mesencephalon, of the neural tube. Unlike the other two vesicles, the forebrain and hindbrain, the midbrain remains undivided for the remainder of neural development.

[002] The midbrain is also that region of the brain, where the majority of the neurotransmitter dopamine (DA) is produced. Dopamine plays, inter alia, a major role in motivation and habituation of species from humans to the most elementary animals such as insects. The regions of DA producing neurons are derived from the tegmentum and are called the substantia nigra pars compacta (SNc, A9 group), and the ventral tegmental area (VTA, A10 group). Especially the mesencephalic dopaminergic (mDA) neurons of the SNc play an important role in the control of multiple brain functions. Their axons ascend rostrally into the dorsolateral striatum of the cortex, where they release the neurotransmitter dopamine (Abeliovich and Hammond, 2007; Gale and Li, 2008). The SNc is of particular interest since mDA neurons of this region selectively undergo degeneration in Parkinson's disease (PD), a progressive neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons in the substantia nigra The loss of mDA neurons leads to a lack of DA in the striatum, which controls voluntary body movements under physiological conditions (Abeliovich and Hammond, 2007). In PD, the degeneration of DA neurons and the subsequent lack of DA in the striatum are associated to motor symptoms including tremor, bradykinesia, and rigidity. Mutation or deletions of several genes have been identified to predispose to the pathology.

[003] Neuronal differentiation towards the dopaminergic lineage is a complex process, which highly relies on the sequential activation of specific transcription factors. In mammals, mDA progenitors start to develop at E7.5. LIM homeobox transcription factor 1α (LMX1A) and forkhead box protein A2 (FOXA2), both induced by SHH signalling, are important determination factors of mDA differentiation. Expression of LMX1A triggers dopaminergic differentiation and recruits MSX1/2, an inhibitor of negative regulators of neurogenesis. It further induces the expression of proneural factors such as Neurogenin 2 (NGN2), which are necessary for the proper development of mDA neurons (Gale and Li, 2008). Upon

maturation, mDA progenitos migrate to exit the proliferative zone at E10.5-E11.5. At this stage, the phenotypic marker of mDA neuons, tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis is induced and mDA progenitors start to express the early neuronal marker βIII Tubulin (TUJ1) (Abeliovich and Hammond, 2007). 92845

[004] Many of the underlying studies of CNS development and specification have been carried out in mouse and chick embryos. Even though it is likely that many of the mechanisms are conserved between mammalian species, there are strong differences between murine and human neurodevelopment. For instance, the size of the cortex is remarkably increased in humans. Some regions, such as the outer subventricular zone or the inner fibre layer are completely absent in mice but present in humans. Notably, rodents are naturally not susceptible to neurodegenerative disorders such as PD and Alzheimer's disease (AD). In addition to differences in neurodevelopment, another obvious but striking difference is the ability to speak.

[005] Parkinson's disease is characterized by the progressive loss of midbrain dopaminergic neurons in the substantia nigra pars compacta. The underlying mechanisms that lead to severe cell death remain poorly understood. Mouse models often cannot recapitulate the phenotype seen in vivo, especially in the case of complex pathologies such as neurodegenerative disorders, which do not affect animals. For this reason, given the divergences between humans and mice which are most frequently used as in vivo model systems, as well as the shortcomings of 2D cell cultures, there is an obvious need for robust in vitro models of human brain development, especially in terms of studying neurodevelopmental and neurodegenerative diseases.

[006] The technical problem underlying the present application is to comply with this need. The solution to said technical problem is the provision of means and methods for generating midbrain organoids as reflected in the claims, described herein, illustrated in the Figures and exemplified in the Examples of the present application.

[007] Much to their surprise the present inventors observed that they were able to obtain midbrain organoids by contacting neuroepithelial stem cells with differentiation medium, when they cultured said neuroepithelial stem cells in a three-dimensional cell culture comprising a matrix under agitating conditions. Specifically, the present inventors used a human neural precursor cell line, called human neuroepithelial stem cells (hNESCs), which served as a starting population for the generation of midbrain organoids. A single colony was embedded into a matrix allowing three-dimensional cell culture, said matrix being e.g. a droplet of Matrigel. Matrigel droplets are described, for example, in Lancaster and Knoblich (2014a). A single colony was cultured under agitating conditions, such as continuous

spinning. The medium used for differentiation contained, inter alia, signaling molecules for the induction of midbrain development as described herein. At several time points, midbrain organoids were characterized using immunohistochemical stainings in order to determine whether indeed a differentiation into midbrain-specific structures was detectable. After about 3 weeks of differentiation, midbrain organoids developed several neural cell types, including dopaminergic neurons, and an asymmetric organization, consistent with brain development in vivo. Such midbrain organoids may serve as 3D models which can, for example, be used to study neurodevelopmental and/or neurodegenerative diseases, such as Parkinson's disease (PD), Multiple sclerosis, Batten's disease or Alzheimer's disease.

[008] During their studies, the present inventors observed that NESCs seeded on round-bottom plates with low attachment surfaces formed globular colonies that expanded quickly (**Fig. 4**). However, this involved increasing cell death in the centre of the colonies due to a limited nutrient exchange. Sectioning of early organoids between day 6 and 10 revealed that the majority of the cells in the core were dead. However, surprisingly, cell death significantly decreased when the 3D structures were kept under agitating conditions.

[009] NESCs embedded in a matrix allowing three-dimensional cell culture such as MATRIGEL and kept under agitating conditions grew further and quickly started to develop long processes that migrated through the entire ECM (**Fig. 4 d-f**). These processes expressed the neuronal markers TUJ1 and partially MAP2, indicative of axon formation (**Fig. 6** and **Fig. 7**). Upon neurogenesis, young neurons adopt bipolar morphology with leading and trailing processes, which eventually become axons between embryonic day 11 to 18 (Lewis et al., 2013). Cell bodies were mainly located adjacent to the core. Under maintenance conditions, hNESCs stably express the neural progenitor markers SOX1, SOX2, and NESTIN (**Fig. 3**). This expression pattern is retained in early midbrain organoids that were seeded on ultralow attachment plates (**Fig. 5**). Some SOX2 positive cells remained in the inner part of the organoid after 24 days of differentiation, abutting to TH positive DA neurons (**Fig. 7**). During the course of midbrain development *in vivo*, the neuroepithelium in the midbrain regions thickens and becomes layered. Some neural stem cells remain in the inner layer and retain their proliferative properties, while other cells migrate to an intermediate zone and start to differentiate into mDA neurons. Immature neurons continue to migrate to reach the marginal zone where they become mature neurons. The mDA neuron progenitors express LMX1, FOXA2, TH, and the neuronal marker TUJ1 (Ang, 2009; Gale and Li, 2008). *In vitro*, midbrain organoids seem to develop a similar layered organisation with neural stem cells remaining adjacent to the inner core and mDA neurons migrating basally. DA neurons started to develop after 6 days under differentiation conditions. Interestingly, DA neurons and neural precursors accumulated in a particular area and were not equally distributed across

the tissue at day 30 and 44, suggesting self-patterning of midbrain organoids along the D-V axis (**Fig. 6** and **Fig. 7**). However, not all TH positive cells are necessarily DA neurons. All catecholamines, including dopamine, noradrenaline, and adrenaline are synthesised from tyrosine. TH is the rate-limiting enzyme that produces L-DOPA from tyrosine (Sharples et al., 2014). To confirm that the TH^+ cells are indeed DA neurons, further markers such as DAT are to be considered. Importantly, midbrain organoids developed asymmetric polar structures, similar to brain development *in vivo*. To this end, the present inventors successfully generated midbrain organoids by using neuroepithelial stem cells as starting population for the generation said midbrain organoids by contacting them with differentiation medium under agitating conditions in three-dimensional cell culture comprising a matrix.

[0010] Accordingly, the present invention provides in one aspect a method of generating a midbrain organoid, comprising contacting neuroepithelial stem cells, which are cultured in a three-dimensional cell culture comprising a matrix, with differentiation medium, wherein the culturing is performed under agitating conditions, thereby obtaining a midbrain organoid.

[0011] The present invention also provides in another aspect midbrain organoid obtainable by the method of the present invention as well as in yet another aspect uses and methods applying the midbrain organoids of the present invention for testing compounds for their ability to elicit a cellular response on said midbrain organoid.

[0012] Furthermore, the present invention provides in a further aspect midbrain organoids as described herein for use in transplantation.

[0013] Having recognized that neuroepithelial stem cells can successfully be used for the generation of midbrain organoids as described herein, the present invention also provides in a yet further aspect the use of neuroepithelial stem cells, which are cultured in a three-dimensional cell culture comprising a matrix under agitating conditions, for generating a midbrain organoid unit.

[0014] The above aspects of present invention as well as preferred aspects thereof may also be summarized in the following items:

(1) A method of generating a midbrain organoid, comprising:
contacting neuroepithelial stem cells, which are cultured in a three-dimensional cell culture comprising a matrix, with differentiation medium, wherein the culturing is performed under agitating conditions, thereby obtaining a midbrain organoid.

(2) The method of item 1, wherein said neuroepithelial stem cells are human neuroepithelial stem cells.

(3) The method of item 2, wherein said neuroepithelial stem cells are hNESC-K7 or smNPCs.

(4) The method of any one of items 1-3, wherein said neuroepithelial stem cells are genetically modified or obtained from a patient suffering from a neurological disease.

(5) The method of item 4, wherein the genetic modification comprises a mutation, a knock-out or a knock-in.

(6) The method of item 4, wherein the neurological disease is a neurodegenerative disease such as Parkinson's disease, Multiple sclerosis, Batten's disease or Alzheimer's disease.

(7) The method of any one of items 1-6, wherein said neuroepithelial stem cells have been produced from induced pluripotent stem cells (iPSCs).

(8) The method of item 7, wherein the iPSCs are produced from fibroblasts or peripheral blood mononuclear cells (PBMCs), wherein the fibroblasts or PBMC have preferably been obtained from a patient.

(9) The method of any one of items 1-8, wherein the three-dimensional cell culture is performed in a gel, a bioreactor in ultra-low adhesion conditions or a microchip, preferably a hydrogel and/or a hydrogel droplet such as a Matrigel droplet.

(10) The method of any one of items 1-9, wherein the matrix is an extracellular matrix and/or wherein the matrix comprises one or more of natural molecules, synthetic polymers, biological-synthetic hybrids, metals, ceramics, bioactive glasses and/or carbon nanotubes.

(11) The method of any one of items 1-10, wherein the matrix comprises collagen, Matrigel, fibrin, hyaluronic acid, chitosan, alginate, silk fibrils, ethylene glycol such as PEG, poly(vinyl alcohol) and/or poly(2-hydroxy ethyl methacrylate), preferably the matrix comprises Matrigel.

(12) The method of any one of items 1-11, wherein said differentiation medium (differentiation medium I) comprises

(i) a SHH-pathway activator;

(ii) at least two different neurotrophins; and

(iii) an antioxidant.

(13) The method of any one of items 1-12, wherein said differentiation medium (differentiation medium II) comprises

(i) at least two different neurotrophins; and

(ii) an antioxidant.

(14) The method of item 12, wherein the SHH-pathway activator is selected from the group consisting of purmorphamine, SHH, smoothened agonist (SAG), Hh-Ag 1.5 and Gli-2, preferably the SHH-pathway activator is purmorphamine.

(15) The method of any one of items 12-14, wherein the at least two neurotrophins are selected form the group consisting of NGF, BDNF, NT-3, NT-4, CNTF and GDNF, preferably the at least two neurotrophins are GDNF and BDNF.

(16) The method of any one of items 12-15, wherein the antioxidant is selected from the group consisting of ascorbic acid, superoxide dismutase 1, superoxide dismutase 2, superoxide dismutase 3, glutathione, lipoic acid, epigallocatechin gallate, curcumine, melatonin, hydroxytyrosol, ubiquinone, catalase, vitamin E and uric acid, preferably the antioxidant is ascorbic acid.

(17) The method of any one of items 12-16, wherein the differentiation medium (differentiation medium I and/or II) further comprises an activator of activin/transforming growth factor-β (TGF-β) signaling pathway and/or wherein the differentiation medium II does not comprise a SHH-pathway activator.

(18) The method of item 17, wherein the activator of activin/TGF-β signaling pathway is selected from the group consisting of TGFβ1, TGFβ2, TGFβ3, activin A, activin B, activin AB and nodal, preferably the activator of activin/TGF-β signaling pathway is TGFβ3.

(19) The method of any one of items 12-18, wherein the differentiation medium (differentiation medium I and/or II) further comprises a cAMP analogue.

(20) The method of item 19, wherein the cAMP analogue is selected from the group consisting of forskolin, 8-(4-chloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate (8CPT-2Me-cAMP), 8-Chloro-cAMP (8-Cl-cAMP), Bucladesine, Rp-adenosine, 3', 5',-cyclic monophosphorothioate sodium salt (Rp-cAMPS), Sp-8-hydroxyadenosine, 3', 5',-cyclic monophosphorothioate sodium salt (Sp-8OH-cAMPS) and Rp8-hydroxyadenosine, 3', 5',-cyclic monophosphorothioate sodium salt (Rp-8OH-cAMPS) and dbcAMP, preferably the cAMP analogue is dbcAMP.

(21) The method of any one of items 12-20, wherein the differentiation medium (differentiation medium I and/or II) is a N2B27 medium.

(22) The method of item 21, wherein the N2B27 medium comprises equal amounts of Neurobasal medium and DMEM/F12 medium.

(23) The method of any one of items 12-22, wherein the differentiation medium (differentiation medium I and/or II) further comprises penicillin and streptomycin.

(24) The method of any one of items 12-23, wherein the differentiation medium (differentiation medium I and/or II) further comprises glutamine, preferably L-glutamine, more preferably L-glutamine at a concentration of 2mM.

(25) The method of any one of items 12-24, wherein the differentiation medium further comprises B27 supplement without vitamin A, preferably at a concentration of 1:100 (supplement:medium).

(26) The method of any one of items 12-25, wherein the differentiation medium ((differentiation medium I and/or II) further comprises N2 supplement, preferably at a concentration of 1:200 (supplement:medium).

(27) The method of any one of items 12-26, wherein the differentiation medium (differentiation medium I and/or differentiation medium II) does not comprise FGF8.

(28) The method of any one of items 12-27, wherein the cells are kept in the differentiation medium I for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11 or more days, preferably the cells are kept in the differentiation medium I for 6 days and/or wherein the cells are kept in differentiation medium II for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 24, 38, 40, 50 or more days, preferably the cells are kept in the differentiation medium II for 1, 24 or 38 days.

(29) The method of item 28, wherein differentiation medium I is replaced by differentiation medium II after 6 days of differentiation by omitting the SHH-pathway activator from the differentiation medium.

(30) The method of any one of items 1-29, wherein FGF8 is added to the differentiation medium (differentiation medium I and/or II) after 8 days of differentiation.

(31) The method of any one of items 1-30, wherein the neuroepithelial stem cells are contacted by a maintenance medium before they are contacted with the differentiation medium.

(32) The method of item 31, wherein the maintenance medium comprises

(i) a SHH-pathway activator;

(ii) canonical WNT-signaling activator; and

(iii) an antioxidant.

(33) The method of item 31 or 32, wherein the maintenance medium comprises N2B27 medium as defined in any one of items 21-27.

(34) The method of item 32 or 33, wherein the canonical WNT-signaling activator is selected from the group consisting of Norrin, R-spondin 2 or WNT protein.

(35) The method of any one of items 32-34, wherein canonical WNT-signaling activator

blocks Axin or APC e.g. via siRNA.

(36) The method of any one of items 32-34, wherein canonical WNT-signaling activator is a GSK-3 inhibitor.

(37) The method of item 36, wherein the GSK-3 inhibitor is selected from the group consisting of CHIR 99021, SB-216763, 6-bromoindirubin-3'-oxime, Tideglusib, GSK-3 inhibitor 1, AZD1080, TDZD-8, TWS119, CHIR-99021 HCl, CHIR-98014, SB 415286, SB 216763, LY2090314, AR-A014418 and IM-12, preferably the GSK-3 inhibitor is CHIR 99021.

(38) The method of item 31-37, wherein the maintenance of the neuroepithelial stem cells takes place in a two-dimensional and/or three-dimensional cell culture.

(39) The method of item 38, wherein the maintenance of the neuroepithelial stem cells is performed in a three-dimensional cell culture for 1, 2, 3, 4, 5, 6 or more days, preferably wherein the maintenance of the neuroepithelial stem cells is performed in a three-dimensional cell culture for 2 days.

(40) The method of any one of items 1-39, wherein the neuroepithelial stem cells are present in a colony.

(41) The method of item 40, wherein the colony is a cluster of cell clones.

(42) The method of item 40 or 41, wherein said colony of neuroepithelial stem cells is obtainable by culturing said neuroepithelial stem cells for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 or more days in the maintenance medium as defined in any one of items 31-39, preferably said neuroepithelial stem cells are cultured for 10 days in the maintenance medium as defined in any one of items 31-39.

(43) The method of item 42, wherein at least 50, 500, 1000, 2000, 3000, 4000, 5000, 6000, 7000, 8000, 9000, 10000, 11000, 12000, 13000, 14000, 15000, 16000 or more neuroepithelial stem cells are used as a starting cell population.

(44) The method of any one of items 40-43, wherein the colony of neuroepithelial stem cells is obtainable by culturing neuroepithelial cells in round bottom ultralow attachment 96-well plates and/or ultralow attachment 24-well-plates and/or in a three-dimensional cell culture.

(45) The method of any one of items 40-44, wherein the colony of neuroepithelial stem cells is cultured in a Matrigel droplet for at least 1 day.

(46) The method of any one of items 9-45, wherein said Matrigel droplet is cultured under agitating conditions after at least 1, 2, 3, 4, 5, 6, 7 or more days after initiation of differentiation, preferably the Matrigel droplet is cultured under agitating conditions after 2

days after initiation of differentiation. 92845

(47) The method of any one of items 1-46, wherein said agitating conditions comprise shaking, spinning, stirring, moving and/or mixing of the three-dimensional cell culture.

(48) The method of item 47, wherein the spinning is performed with a spinning bioreactor and/or the shaking is performed with an orbital shaker.

(49) The method of item 47 or 48, wherein said orbital shaker is shaking at least at 40 rpm, 50 rpm, 60 rpm, 70 rpm, 80 rpm, 90 rpm, 100 rpm, 110 rpm, or more, preferably said orbital shaker is shaking at 80 rpm.

(50) The method of any one of items 1-49, comprising:

(i) contacting neuroepithelial stem cells with the maintenance medium as defined in any one of items 32-39;

(ii) contacting neuroepithelial stem cells, which are cultured in a three-dimensional cell culture comprising a matrix, with the differentiation medium (I) as defined in any one of items 12, 14-30, wherein the culturing is performed under agitating conditions,
wherein the agitating is started after 0, 1, 2, 3, 4, 5, 6, 7, 8 or more days, preferably 2 days, after starting culturing of the neuroepithelial stem cells in the differentiation medium I;

(iii) contacting neuroepithelial stem cells with the differentiation medium (II) as defined in any one of items 13-30 under agitating conditions.
thereby obtaining a midbrain organoid.

(51) The method of any one of items 1-50, comprising:

(i) contacting neuroepithelial stem cells with the maintenance medium as defined in any one of items 32-39;

(ii) culturing the neuroepithelial stem cells for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or more days in the maintenance medium;

(iii) contacting neuroepithelial stem cells with the differentiation medium (I) as defined in any one of items 12, 14-30,

(iv) culturing the cells of step (iii) for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or more days in the differentiation medium I, wherein the culturing is performed under agitating conditions,
wherein the agitating is started after 0, 1, 2, 3, 4, 5, 6, 7, 8 or more days, preferably 2 days, after starting culturing of the neuroepithelial stem cells in the differentiation medium I;

(v) contacting the cells obtained in step (iv) with the differentiation medium II as defined in any one of items 13-30; and

(vi) culturing the cells of step (v) for 1, 2, 3, 4, 5, 6, 7, 8, 9, or more weeks in the differentiation medium II under agitating conditions;
thereby obtaining a midbrain organoid.

(52) The method of item 51, wherein the culturing of the neuroepithelial stem cells in step (ii) is performed for 9 or 10 days.

(53) The method of item 51 or 52, wherein the culturing of the cells of step (iv) is performed for 6 days.

(54) The method of any one of items 51-53, wherein the culturing the cells of step (vi) is performed for 1, 24 or 38 days.

(55) The method of any one of items 1-54, wherein the midbrain organoid is obtainable after 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or more weeks of differentiation, preferably the differentiation is performed for 6, 7, 16, 30 or 44 days.

(56) The method of any one of items 1-55, wherein said midbrain organoid is an early midbrain organoid or a late midbrain organoid.

(57) The method of item 56, wherein the early midbrain organoid is a midbrain organoid, which has been differentiated for 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 days, preferably the early midbrain organoid has been differentiated for 6, 7 or 16 days.

(58) The method of item 56, wherein the late midbrain organoid is a midbrain organoid, which has been differentiated for at least 25, 30, 35, 40, 50, 60 or more days, preferably the late midbrain organoid has been differentiated for 30 or 44 days.

(59) The method of any one of items 1-58, wherein said midbrain organoid comprises

(a) neural progenitor cells;

(b) young neurons;

(c) young dopaminergic neurons;

(d) mature neurons;

(e) mature dopaminergic neurons;

(f) an asymmetric organization of the midbrain organoid;

(g) oligodendrocytes;

(h) oligodendrocyte progenitors;

(i) astrocytes; and/or

(j) processes that expand from the midbrain organoid through the matrix.

(60) The method of any one of items 56, 57 or 59, wherein the early midbrain organoid comprises

(a) neural progenitor cells;

(b) young neurons; and/or

(c) young dopaminergic neurons.

(61) The method of any one of items 56, 58 or 59, wherein the late midbrain organoid comprises

(a) neural progenitor cells;

(b) young neurons;

(c) young dopaminergic neurons;

(d) mature neurons;

(e) mature dopaminergic neurons;

(f) an asymmetric organization of the midbrain organoid;

(g) oligodendrocytes;

(h) oligodendrocyte progenitors;

(i) astrocytes;

(j) clustering of dopaminergic neurons within the organoid; and/or

(k) processes that expand from the midbrain organoid through the matrix.

(62) The method of any one of items 59-61, wherein said neural progenitor cells are characterized by the expression of the markers SOX2 and/or nestin.

(63) The method of any one of items 59-62, wherein said young neurons are characterized by the expression of the marker TUJ1.

(64) The method of any one of items 59-63, wherein said young dopaminergic neurons are characterized by the expression of the markers TUJ1 and tyrosine hydroxylase (TH).

(65) The method of any one of items 59-64, wherein said mature neurons are characterized by the expression of the marker MAP2.

(66) The method of any one of items 59-65, wherein said mature dopaminergic neurons are characterized by the expression of the markers MAP2 and TH.

(67) The method of any one of items 59-66, wherein the asymmetric organization of the midbrain organoid is an asymmetric polar organization of dopaminergic neurons and/or an asymmetric organization of neuronal progenitor cells within the midbrain organoid.

(68) The method of any one of items 59-67, wherein said asymmetric polar organization of dopaminergic neurons within the midbrain organoid is characterized by the localization of

(a) mature dopaminergic neurons in the outermost part of the midbrain organoid;

(b) young dopaminergic neurons in the inner parts of the midbrain organoid.

(69) The method of item 68, wherein young dopaminergic neurons migrate towards the outermost part of the midbrain organoid upon maturation.

(70) The method of any one of items 59-69, wherein the asymmetric organization of neuronal progenitor cells within the midbrain organoid is characterized by the localization of

neuronal progenitors in a ring-like structure surrounding the inner core of the midbrain organoid.

(71) The method of any one of items 59-70, wherein said oligodendrocytes are characterized by the expression of the marker O4.

(72) The method of any one of items 59-71, wherein said oligodendrocyte progenitors are characterized by the expression of the marker NG2.

(73) The method of any one of items 59-72, wherein said astrocytes are characterized by the expression of the markers GFAP and/or S100b.

(74) The method of any one of items 59-73, wherein said clustering of dopaminergic neurons within the organoid is characterized by the accumulation of more than 2, 3, 4, 5, 6, 7, 8, 9, 10 or more dopaminergic neurons in a specific region of the midbrain organoid.

(75) The method of any one of items 59-74, wherein said processes that expand from the midbrain organoid through the matrix have a length of 0.1 mm, 0.2 mm, 0.3 mm, 0.4 mm, 0.5 mm, 0.6 mm, 0.7 mm, 0.8 mm, 0.9 mm, 1 mm, 1.1 mm, 1.2 mm or more.

(76) The method of any one of items 1-75, wherein the neuroepithelial stem cell is obtainable by a method comprising

a) optionally obtaining/providing induced pluripotent stem cells (iPSCs);

b) cultivating said iPSCs in a medium comprising
   (i) an activin/transforming growth factor-β (TGF-β) signaling inhibitor;
   (ii) a canonical WNT-signaling activator;
   (iii) a bone morphogenetic protein (BMP) signaling inhibitor; and
   (iv) a SHH-pathway activator; and

c) cultivating the cells obtained in b) in a medium comprising
   (i) an activin/TGF-β signaling inhibitor;
   (ii) a canonical WNT-signaling activator;
   (iii) a BMP signaling inhibitor; and
   (iv) a SHH-pathway activator; and

d) further cultivating the cells obtained in c) in a medium comprising
   (i) a canonical WNT-signaling activator;
   (ii) SHH-pathway activator; and
   (iii) an antioxidant; and

thereby obtaining a neuroepithelial stem cell.

(77) The method of item 76, wherein the method further comprises

e) maintaining the cells obtained in d) in a medium comprising

(i) a FGF signaling activator;
(ii) an EGF signaling activator; and
(iii) a LIF signaling activator.

(78) Midbrain organoid obtainable by the method of any one of items 1-77.

(79) Use of the midbrain organoid of item 78 for testing compounds for their ability to elicit a cellular response on said midbrain organoid.

(80) Use of item 79, wherein said compound is a drug, small molecule, hormone, growth factor, binding protein, nucleic acid molecule, peptide protein or (co-cultured) cell.

(81) Use of item 79 or 80, wherein said cellular response is the frequency or survival of a certain type of cell.

(82) Use of item 81, wherein said type of cell is a dopaminergic neuron.

(83) Method for testing a compound of interest for its ability to elicit a cellular response, comprising:
(a) contacting the midbrain organoid of item 78 with said compound of interest; and
(b) determining whether said compound of interest elicits a cellular response.

(84) The method of item 83, wherein said compound is a drug, small molecule, hormone, growth factor, binding protein, nucleic acid molecule, peptide protein or (co-cultured) cell.
(85) The method of item 83 or 84, wherein said cellular response is the frequency or survival of a certain type of cell.

(86) The method of item 85, wherein said type of cell is a dopaminergic neuron.

(87) The method for identifying molecules promoting or inhibiting dopaminergic neuronal differentiation and/or death of dopaminergic neurons in a midbrain organoid as defined in item 78, the method comprising contacting the midbrain organoid with a molecule of interest, wherein an increase of the differentiation into dopaminergic neurons compared to a control indicates that the molecule of interest promotes dopaminergic neuronal differentiation and/or inhibits death of dopaminergic neurons and wherein a decrease of the differentiation into dopaminergic neurons compared to a control indicates that the molecule of interest inhibits dopaminergic neuronal differentiation and/or induces death of dopaminergic neurons.

(88) The method of item 87, wherein the differentiation into dopaminergic neurons is measured by comparing neurite outgrowth.

(89) The method of item 87 or 88, wherein the differentiation into dopaminergic neurons is measured by comparing the expression of TH.

(90) The method of any one of items 87-89, wherein the control is a midbrain organoid which is not contacted with the molecule of interest.

(91) Composition comprising a midbrain organoid unit of item 78.

(92) The composition of item 91, which is a pharmaceutical composition.

(93) Use of neuroepithelial stem cells, which are cultured in a three-dimensional cell culture comprising a matrix under agitating conditions, for generating a midbrain organoid unit.

(94) Midbrain organoid of item 78 for use in transplantation.

[0015] An "organoid" resembles a whole organ. Organoids exhibit an intrinsic potential to self-organise, forming the cellular organisation of an organ. Organoids hold great promise for diagnostic and therapeutic applications. The organoid of the present invention is preferably a midbrain organoid. Accordingly, a midbrain organoid of the present invention resembles the midbrain. The midbrain is the region of the brain, where the majority of the neurotransmitter dopamine (DA) is produced. A midbrain organoid of the present invention is preferably from a single colony of a NESC, preferably a hNESC. A midbrain organoid of the present invention has preferably the phenotype of a midbrain. A midbrain organoid of the present invention is either an early midbrain organoid or a late midbrain organoid. A midbrain organoid of the present invention has preferably the phenotype of a midbrain As such, it comprises typical cells/cell types of a midbrain. Accordingly, a midbrain organoid of the present invention comprises neural progenitor cells, young neurons, young dopaminergic neurons, mature neurons, mature dopaminergic neurons, an asymmetric organization, oligodendrocytes, oligodendrocyte progenitors, astrocytes, and/or processes that expand from the midbrain organoid through the matrix. These cell types are preferably characterized by the markers as described herein. Likewise, the asymmetric organization is preferably characterized as described herein and as well as the clustering of dopaminergic neurons and the processes that expand from the midbrain organoid through the matrix. Presence of the cells/cell types in a midbrain organoid of the present invention can be tested by means and methods known in the art and as described herein. Likewise, expression of the markers as described herein by said cells/cell types or the asymmetric organization can be tested as is known in the art and as described herein.

[0016] Organoids have the potential to model degenerative and developmental diseases and/or cancer, and represent a valuable tool to study genetic disorders and to identify subtle phenotypes. Somatic cells derived from patients can be reprogrammed into iPSCs and thereof-derived organoids can be used as a patient-specific model for drug tests or in

regenerative medicine for organ replacement therapies. Insertion and correction of mutations in hiPSC-derived organoids might help to understand disease mechanisms. This is advantageously envisioned by the present invention, i.e. midbrain organoids of the present invention may serve as models which can, for example, be used to study neurodevelopmental and/or neurodegenerative diseases, such as Parkinson's disease (PD), Multiple sclerosis, Batten's disease or Alzheimer's disease.

[0017] The methods of the present invention can be carried out in any cell culture, while, however, three-dimensional cell culture is preferred. Culture conditions may vary, but the artificial environment in which the cells are cultured invariably consists of a suitable vessel comprising one or more of the following: a substrate or medium that supplies the essential nutrients (amino acids, carbohydrates, vitamins, minerals), growth factors, hormones, gases (O_2, CO_2) and/or regulated physico-chemical environment (pH, osmotic pressure, temperature). Cell culture as described herein refers to the maintenance and growth of cells in a controlled laboratory environment. Such *in vitro* cell culture models are well-known in experimental cell biological research. For example, cells can be cultured while attached to a solid or semi-solid substrate (adherent or monolayer culture). Cells can also be grown floating in the culture medium (suspension culture). However, it is preferred that cells of the present invention are cultured under agitating conditions.

[0018] Medium for cell culture, such as maintenance medium or differentiation medium is described herein elsewhere, e.g. in the items above.

[0019] Differentiation media as applied in the methods of the present invention comprise at least two different neurotrophins. The term "neurotrophins", as used herein, relates to a family of proteins that regulate the survival, development, and function of neurons. Exemplary neurotrophins include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 (NT-4) as well as GDNF family of ligands and ciliary neurotrophic factor (CNTF). The GDNF family of ligands includes glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN), and persephin (PSPN).

[0020] Accordingly, the term "at least two different neurotrophins" refers to two or more of the recited molecules. Preferably, the at least two different neurotrophins are BDNF and GDNF (Gene Symbols: BDNF and GDNF, respectively). BDNF can e.g. be the human BDNF protein of Uniprot/Swissprot accession no. P23560 (version 1 as of October 31, 1991). GDNF can e.g. be the human GDNF protein of Uniprot/Swissprot accession no. P39905 (version 1 as of January 31, 1995).

[0021] BDNF and GDNF can both independently from each other be employed in a concentration of between about 0.0001 and about 50 ng/µl each, more preferably between about 0.001 and about 25 ng/µl each, and most preferably the amount is about 0.001 ng/µl each. BDNF and GDNF may for example be obtained from Peprotech.

[0022] The differentiation medium as applied in the methods of the present invention may further comprise an antioxidant. An antioxidant is a molecule that inhibits the oxidation of other molecules. The terms "oxidation" and "antioxidant" are well known in the art and have been described, for example, in Nordberg J, Arnér ES. (2001) "Reactive oxygen species, antioxidants, and the mammalian thioredoxin system." Free Radic Biol Med. 31(11):1287-312. In short, oxidation is a chemical reaction involving the loss of electrons or an increase in oxidation state. Oxidation reactions can produce free radicals. In turn, these radicals can start chain reactions. When the chain reaction occurs in a cell, it can cause damage or death to the cell. Antioxidants terminate these chain reactions by removing free radical intermediates, and inhibit other oxidation reactions. Accordingly, an antioxidant refers to an inhibitor of a molecule involved in cellular oxidative processes.

[0023] Exemplary antioxidants include ascorbic acid, superoxide dismutase 1, superoxide dismutase 2, superoxide dismutase 3, glutathione, lipoic acid, epigallocatechin gallate, curcumine, melatonin, hydroxytyrosol, ubiquinone, catalase, vitamin E or uric acid. Thus, the antioxidant can be ascorbic acid.

[0024] Any medium for cell culture as described herein may contain a ROCK inhibitor. A "ROCK inhibitor" as used herein is compound that acts as an inhibitor of Rho-associated protein kinase, i.e. reduces or even abolishes ROCK functionality. The capability of a compound to act as a ROCK inhibitor can be assessed by various means, e.g. by determining its ability to compete with ATP for binding to ROCK and/or by assessing its effects on cell morphology, G1-S Transition and cytokinesis as described in Ishizaki T Mol Pharmacol. 2000 May;57(5):976-83. The inhibitor may be either unspecific or specific for either of the ROCK isoforms ROCK1 and/or ROCK2. ROCK inhibitors known in the art have been reviewed in Liao et al. J Cardiovasc Pharmacol. 2007 Jul; 50(1): 17–24 and include Fasudil, Y-27632, Thiazovivin, Y39983, Wf-536, SLx-2119, Azabenzimidazole-aminofurazans, DE-104, Olefins, Isoquinolines, Indazoles, pyridinealkene derivatives, H-1152P, ROKα inhibitor, XD-4000, 4-(1-aminoalkyl)-N-(4-pyridyl)cyclohexane-carboxamides, HMN-1152, Rhostatin , BA-210, BA-207, BA-215, BA-285, BA-1037, Ki-23095, VAS-012, with Y-27632 or Thiazovivin being particularly envisaged for use in the method of the invention.

[0025] Differentiation medium as applied in the methods of the present invention can further comprise an activator of activin/transforming growth factor-β (TGF-β) signaling pathway. The activin/TGF-β signaling pathway is known in the art and for example described in Heldin, Miyazono and ten Dijke (1997) "TGF-bold beta signaling from cell membrane to nucleus through SMAD proteins." Nature 390, 465-471. In short, Receptor ligands, including, for example, TGFB1 , TGFB2, TGFB3, ACTIVIN A, ACTIVIN B, ACTIVIN AB, and/or NODAL, bind to a heterotetrameric receptor complex consisting of two type I receptor kinases, including, for example, TGFBR2, ACVR2A, and/or ACVR2B, and two type II receptor kinases, including, for example, TGFBR1 , ACVR1 B, and/or ACVR1C. This binding triggers phosphorylation and activation of a heteromeric complex consisting of an R-smad, including, for example, SMAD2, and/or SMAD3, and a Co-smad, including, for example, SMAD4. Accordingly, the term "activator of the activin/TGF-β signaling pathway" refers to an activator of any one of the above recited molecules that form part of this signaling pathway.

[0026] Exemplary activators of the activin/TGF-β signaling pathway include TGFβ1, TGFβ2, TGFβ3, activin A, activin B, activin AB or nodal. Thus, the activator of activin/TGF-β signaling pathway can be TGFβ3. The activator of the activin/TGF-β signaling pathway such as TGFβ3 can be utilized in an amount of 0.0001 ng/µl to 0.1 ng/µl such as e.g. in an amount of 0.001 ng/µl.

[0027] Differentiation medium as applied in the methods of the present invention can further comprise a cAMP analogue. Such cAMP analogs are compounds that have similar physical, chemical, biochemical, or pharmacological properties as the cyclic adenosine monophosphate (cAMP). cAMP is known to the skilled artesian and described in e.g. Fimia GM, Sassone-Corsi P. (2001) "Cyclic AMP signalling." J Cell Sci; 114(Pt 11):1971-2.

[0028] Differentiation medium as applied in the methods of the present invention can further be a N2B27 medium (into which the different compounds are diluted). This means that the medium comprises a N2 supplement and a B27 supplement. Both supplements are well known to the person skilled in the art and freely available. The B27 supplement can be a B27 supplement without vitamin A. This B27 can be used at a concentration of 1:10-1:1000, such as 1:100 (supplement:medium). The B27 supplement can for example be obtained from Life technologies. Likewise, also the N2 supplement can for example be obtained from Life technologies. The N2 supplement may be used at a concentration of 1:20 to 1:2000, such as 1:200 (supplement:medium).

[0029] Differentiation medium may also be a Neurobasal medium and/or a DMEM-F12 medium. Both media can for example be obtained from Life technologies. The N2B27

medium can for example comprise equal amounts of Neurobasal medium and DMEM/F12 medium.

[0030] Differentiation medium as applied in the methods of the present invention can further comprise an antibiotic.

[0031] Differentiation medium as applied in the methods of the present invention can further comprise glutamine.

[0032] Differentiation medium as applied in the methods of the present invention can comprise N2B27 medium comprising about 50 % DMEM-F12 (e.g. from Life technologies)/ about 50 % Neurobasal (e.g. from Life technologies), about 1:200 N2 supplement (e.g. from Life technologies), about 1:100 B27 supplement lacking vitamin A (e.g. from Life technologies n), 1 % Penicillin/Streptomycin (e.g. from Life technologies) and 2 mM L-glutamine (e.g. from Life technologies).

[0033] In addition, cells may be cultured in a two-dimensional cell culture. This type of cell culture is well-known to the person skilled in the art. In two-dimensional (2D) cell culture cells are grown on flat plastic dishes such as Petri dish, flasks and multi-well plates. However, biologically derived matrices (e.g. fibrin, collagen and as further described herein) and synthetic hydrogels (e.g. PAA, PEG and as further described herein) can be used to elicit specific cellular phenotypes that are not expressed on rigid surfaces.

[0034] The methods of the present invention are, however, preferably be carried out in a three dimensional cell culture. A "three-dimensional cell culture" or "3D cell culture" as used herein means that cells are grown in an artificially-created environment in which cells are permitted to grow or interact with its surroundings in all three dimensions. For example, in order to achieve the three dimensional property of the cell culture, cells are grown or differentiated in matrices or scaffolds. In principle, suitable matrices or scaffolds, which can be used in three dimensional cell cultures are known to the skilled artesian. Such matrices or scaffolds can therefore be any matrix or scaffold. For example, the matrix or scaffold can be an extracellular matrix comprising either natural molecules or synthetic polymers, a biological and synthetic hybrid, metals, ceramic and bioactive glass or carbon nanotubes.

[0035] Exemplary natural extracellular matrix molecules include collagen, basement membranes such as laminin or fibrin, alginates, chitosan, hyaluronic acid, silk fibroin, cellulose actetate, casein, chitin, fibrinogen, gelatine, elastin or poly-(hydroxyalkanoate). Synthetic extracellular matrix polymers include Hyaluronic acid (HA) modified forms, Poly-ethylen glycol (PEG) modified forms, Self-assembling protein hydrogels, Poly(lactic-co-glycolic acid) (PLGA), Polycaprolactone (PCL), Polyurethane or PGS. Biological and synthetic hybrids can for example include Polycaprolactone-chitosan, PLLA-Hydroxyapatite,

Hydroxyapatite-bioglass-ceramic, Poly-(hydroxylalkanoate)-bioglass, Hydroxyapatite-collagen, PCL-gelatin or PCL-collagen. Exemplary metals include Tantalam, Magnesium and its alloys, Titanium and its alloys or Nitinol (nickel and titanium alloys). Examples of Ceramics and bioactive glass matrices/scaffolds include Titanium and tri calcium phosphate, Hydroxyapatite and Tricalcium phosphate, Bioactive silicate glass(SiO_2–Na_2O–CaO–P_2O_5), Hydroxyapatite and bioglass, Calcium phosphate glass or Phosphate glass. Carbon nanotubes can be constructed using graphite ranging from 0.4 to 2 nm. Carbon nanotubes can comprise CNT-polycaprolactone, CNT-ceramic matrix, 45S5 bioglass-CNT, CNT studded with gelatine hydrogel, CNT-TiO_2, CNT-laminin, CNT grafted with polyacrylic acid or CNT-TGF-β.

[0036] The matrix or scaffold can also be a hydrogel such as Matrigel, fibrin gel or alginate gel. Matrigels can be a reconstituted basement membrane preparation extracted from Engelbreth-Holm-Swarm mouse sarcoma, a tumor rich in extracellular matrix proteins. Matrigel can be constituted of 60% laminin, 30% type IV collagen and 8% entactin. Optionally growth factors and other molecules can be added to the Matrigel. The Matrigel can also be BD MatrigelTM (obtainable from BD Biosciences).

[0037] Neuroepithelial stem cells (NESCs) when referred to herein can be derived from actual stem cells in several different stages of neural development. Neuroepithelial cells are a class of stem cell and have similar characteristics as stem cells. For example, these cells are able to self-renew. Self-renewal is the ability to go through numerous cell cycles of cell division while maintaining the undifferentiated state. In addition, neuroepithelial stem cell cells have the capacity to differentiate further into multiple types of cells, such as neurons, astrocytes and other glial cells. Thus, these cells are also multipotent. They are restricted to the neural lineage and can differentiate into neurons, astrocytes, and oligodendrocytes (Gage, 2000). Methods for testing if a cell has the capacity to self-renew and if a cell is multipotent are known to the skilled artesian. Self-renewal may be tested by passaging the cells over more than 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30 or more passages. Passaging includes splitting of the cells before re-plaiting them as a single cell suspension. Multipotency can be tested by differentiating said cells into different lineages such as astrocytes, oligodendroctyes and neurons.

[0038] Furthermore, a neuroepithelial stem cell can express markers such as PAX6, Notch 1, Nestin, PCNA, Hes5 and Sox1. In particular, the neuroepithelial stem cells used in the methods of the present invention can be mammalian neural plate border stem cells (NPBSC) as described in WO2013104752. Furthermore, the neuroepithelial stem cells used in the methods of the present invention can also be NPBSCs as described in WO2013104752, which are also obtained by the method as described in WO2013104752. These NPBSC can

be characterized by the expression of at least three markers selected from the group consisting of FORSE1, MSX1 , PHOX2B, PAX3, PAX6, SOX1 , SOX2, NESTIN, IRX3, HOXA2, HOXB2, HES5, DACH1 , PLZF, LM03, EVI1 and ASCL1. Furthermore, these cells can be characterized by a lack of expression of at least one of the markers OCT4, NANOG, AFP, T, SOX17, EOMES, GSH2, OLIG2, CK8, CK18, NKX2.2, NKX6.1, HOXB8, HOXA5, FOXA2 and VCAM-1.

[0039] The neuroepithelial stem cell can be a mammalian NESC. It is also encompassed by the present invention that the NESC is a human NESC (hNESC), such as hNESC-K7. A neuroepithelial stem cell may be obtained by different means and methods known to the skilled artesian. For example, a neuroepithelial stem cell may be derived or obtained from pluripotent cells. NESC of the present invention may be genetically modified or obtained from a patient suffering from a neurological disease, such as PD. Also, NESC may be produced from iPSCs, fibroblasts or PBMCs as described herein.

[0040] Totipotent stem cells (lat. "capable of everything") can give rise to all cell types of the body, including the germ line of the trophectoderm (Weissman, 2000).

[0041] A "pluripotent stem cell" when referred to herein relates to a cell type having the capacity for self-renewal, and the potential of differentiation into different cell types. Pluripotent stem cells can differentiate into nearly all cells, i.e. cells derived from any of the three primary germ layers: ectoderm, endoderm, and mesoderm. The term pluripotent stem cells also encompasses stem cells derived from the inner cell mass of an early stage embryo known as a blastocyst.

[0042] Multipotent stem cells are already restricted to a tissue or an organ and can give rise to all the tissue-specific cells.

[0043] Notably, recent advances in embryonic stem cell research have led to the possibility of creating new embryonic stem cell lines without destroying embryos, for example by using a blastomere biopsy-based technique, which does not interfere with the embryo's developmental potential (Klimanskaya (2006) "Embryonic stem cells from blastomeres maintaining embryo viability." Semin Reprod Med. 2013 Jan;31(1):49-55). Furthermore, a large number of established embryonic stem cell lines are available in the art. Thus it is possible to work with embryonic stem cells without the necessity to destroy an embryo. Takahashi and Yamanaka addressed these concerns and published a method to generate induced pluripotent stem cells (iPSCs) from somatic cells (Takahashi and Yamanaka, 2006). Skin fibroblasts were induced with four defined factors to reprogram the cells back to the pluripotency state. Those stem cells have the same essential characteristics as ESCs (**Fig. 1**) (Takahashi et al., 2007; Takahashi and Yamanaka, 2006; Yu et al., 2007). In one

embodiment, the pluripotent stem cells are embryonic stem cells, which have not been obtained via the destruction of a human embryo. Thus, the pluripotent stem cells are embryonic stem cells obtained from an embryo, without the destruction of the embryo.

[0044] A neuroepithelial stem cell can also be derived or obtained from another pluripotent cell, namely an induced pluripotent stem cell (iPSC). "Induced pluripotent stem cells", as used herein, refers to adult somatic cells that have been genetically reprogrammed to an embryonic stem cell–like state by being forced to express genes and factors important for maintaining the defining properties of embryonic stem cells. Thus, induced pluripotent stem cells derived from a non-pluripotent cell.

[0045] Induced pluripotent stem cells are an important advancement in stem cell research, as they allow obtaining pluripotent stem cells without the use of embryos. Mouse iPSCs were first reported in 2006 (Takahashi, K; Yamanaka, S (2006). "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors". Cell 126 (4): 663–76), and human iPSCs were first reported in 2007 (Takahashi et al. (2007) "Induction of pluripotent stem cells from adult human fibroblasts by defined factors." Cell; 131(5):861-72). Mouse iPSCs demonstrate important characteristics of pluripotent stem cells, including expression of stem cell markers, forming tumors containing cells from all three germ layers, and being able to contribute to many different tissues when injected into mouse embryos at a very early stage in development. Human iPSCs also express stem cell markers and are capable of generating cells characteristic of all three germ layers. Such stem cell markers can include Oct3/4, Sox2, Nanog, alkaline phosphatase (ALP) as well as stem cell-specific antigen 3 and 4 (SSEA3/4). Also the chromatin methylation patterns of iPSC are also similar to that of embryonic stem cells (Tanabe, Takahashi, Yamanaka (2014) "Induction of pluripotency by defined factors." Proc. Jpn. Acad., 2014, Ser. B 90).

[0046] In addition, iPSCs are able to self-renew in vitro and differentiate into all three germ layers. The pluripotency or the potential to differentiate into different cell types of iPSC can tested, e.g., by in vitro differentiation into neural or glia cells or the production of germline chimaeric animals through blastocyst injection.

[0047] Methods for the generation of human induced pluripotent stem cells are well known to the skilled person. Usually forced expression of Oct3/4, Sox2 and Klf4 (as well as OCT3/4, SOX2 and KLF4) is sufficient to generate an induced pluripotent stem cell out of an adult somatic cell, such as a fibroblast. However, also the combination of Oct3/4, Sox2, c-Myc and Klf4 (as well as OCT3/4, SOX2, C-MYC) and KLF4 is sufficient for the generation of a iPSC from an adult somatic cell. In addition also the combination of OCT3/4, SOX2, NANOG and LIN28 was efficient for reprogramming (Tanabe, Takahashi, Yamanaka (2014) "Induction of

pluripotency by defined factors." Proc. Jpn. Acad., 2014, Ser. B 90). For this, these genes are usually cloned into a retroviral vector and transgene-expressing viral particles or vectors, with which the somatic cell is co-transduced. However, also other techniques known to the skilled artesian can be used for that purpose. Human skin fibroblasts can also be co-transduced with all four vectors e.g. via protein transduction or naked DNA.

[0048] Further methods for obtaining iPSCs are also known to the skilled artesian and for example described in WO2009115295, WO2009144008 or EP2218778. Thus, the skilled artesian can obtain an iPSC by any method.

[0049] In principle, induced pluripotent stem cells may be obtained from any adult somatic cell (of a subject). Exemplary somatic cells include peripheral blood Mononuclear Cells (PBMCs) from blood or fibroblasts, such as for example fibroblasts obtained from skin tissue biopsies.

[0050] Therefore, it is envisioned by the present invention that the NESC is produced or derived or obtained from an induced pluripotent stem cell (iPSC). Different ways how to differentiate iPCSs into neuroepithelial stem cells are known to the skilled artesian and for example described in WO2013/104752. In addition, it is envisioned by the present invention that the iPSCs can be produced from somatic cells such as fibroblasts. Furthermore, the iPSC can be a human iPSC (hiPSC).

[0051] It is further encompassed by the present invention that the somatic cells such as fibroblasts have been obtained from a subject. The term "subject" can also mean human or an animal. The subject can also be a subject suffering from a neurodegenerative disease such as Parkinson's disease. In particular, the subject may be a subject comprising the LRRK2-G2019S mutation, which is associated with familial Parkinson's disease. The subject can also be a subject not suffering from a neurodegenerative disease such as Parkinson's disease. Also encompassed by the present invention is that the subject is a healthy subject. The subject can be a vertebrate, more preferably a mammal. Mammals include, but are not limited to, farm animals, sport animals, pets, primates, dogs, horses, mice and rats. A mammal can be a human, dog, cat, cow, pig, mouse, rat etc. Thus, in one embodiment, the subject is a vertebrate. The subject can also be a human subject.

[0052] "Agitation" or "agitating" when used herein encompasses any technique that keeps cells in motion, i.e. cells are essentially not allowed to adhere to surfaces. Agitation can be achieved in a number of ways, including shaking, spinning, stirring, moving and/or mixing. Spinning can, for example, be achieved by the use of spinner flasks containing a magnetic paddle or impeller. Alternatively, the methods of the invention can be carried out in a bioreactor. A number of types of bioreactor are available, including bioreactors in which

agitation of the medium is achieved using a paddle or impeller and rotary wall bioreactors. Rotary wall bioreactors can additionally be used to simulate conditions of reduced gravity (microgravity).It is also desirable to monitor and/or control the shear forces experienced by cells in operation of the methods of the present invention. For example, optimal conditions in cultures subjected to agitation require balancing the requirement for even distribution of oxygen and nutrients throughout the culture against the need to avoid cell damage due to excessive shear forces.

[0053] The term "activator", as used herein, is defined as a compound/molecule enhancing or achieving the activity of a target molecule or pathway. The activator may achieve this effect by enhancing or inducing the transcription of the gene encoding the protein to be activated and/or enhancing the translation of the mRNA encoding the protein to be activated. It can also be that the protein to be activated performs its biochemical function with enhanced efficiency in the presence of the activator or that the protein to be activated performs its cellular function with enhanced efficiency in the presence of the activator. Accordingly, the term "activator" encompasses both molecules/compounds that have a directly activating effect on the specific pathway but also molecules that are indirectly activating, e.g. by interacting for example with molecules that negatively regulate (e.g. suppress) said pathway. The activator can also be an agonist of the pathway to be activated. Methods for testing if a compound/molecule is capable to induce or enhance the activity of a target molecule or pathway are known to the skilled artesian. For example an activator of a SHH, WNT or other activator as described herein can be tested by performing Western Blot analysis of the amount of e.g. pathway effector proteins such as Gli proteins, LEF1 or TCF1 protein, respectively.

[0054] The compound/molecule that can be used as an activator can be any compound/molecule, which can activate the respective pathway or which inhibits a suppressor of the pathway to be activated. Exemplary activators can include suitable binding proteins directed e.g. against suppressors of a certain pathway.

[0055] An activator may enhance or increase the pathway to be activated by 10 %, 20 %, 30 %, 40 %, 50 %, 60 %, 70 %, 80 %, 90 %, 100 % or more when compared to the activity of the pathway without the addition of the activator.

[0056] The "Hedgehog signaling pathway" or "SHH pathway" is well known in the art and has been described, for example, in et al. (2014) "Sonic hedgehog signalling pathway: a complex network." Ann Neurosci. 21(1):28-31. Hedgehog ligands, including, for example, Sonic hedgehog, Indian hedgehog, and/or Desert hedgehog, bind to the receptor, including, for example, Patched or the patched-smoothened receptor complex, which induces a

downstream signaling cascade. Downstream target genes of SHH signaling include GLI1, GLI2 and/or GLI3. Accordingly, the term "activator of the Hedgehog signalling pathway" also refers to an activator of any one of the above recited molecules that form part of this signaling pathway.

[0057] Accordingly, the term "canonical WNT-signaling activator" as described herein refers to an activator of any one of the above recited molecules that form part of this signaling pathway.

[0058] Exemplary canonical WNT-signaling activators include Norrin, R-spondin 2 or WNT protein. However, the canonical WNT-signaling activator can also block Axin or APC. This can be achieved for example via siRNA or miRNA technology. It is also encompassed by the present invention that the canonical WNT-signaling activator is a GSK-3 inhibitor.

[0059] Unless otherwise indicated, all numbers expressing quantities of ingredients, properties such as molecular weight, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term "about." As used herein the terms "about" and "approximately" means within 10 to 15%, preferably within 5 to 10%. Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained by the present invention. At the very least, and not as an attempt to limit the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques. Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any numerical value, however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements.

[0060] The terms "a," "an," "the" and similar referents used in the context of describing the invention (especially in the context of the following claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein is

intended merely to better illuminate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

[0061] Groupings of alternative elements or embodiments of the invention disclosed herein are not to be construed as limitations. Each group member may be referred to and claimed individually or in any combination with other members of the group or other elements found herein. It is anticipated that one or more members of a group may be included in, or deleted from, a group for reasons of convenience and/or patentability. When any such inclusion or deletion occurs, the specification is deemed to contain the group as modified thus fulfilling the written description of all Markush groups used in the appended claims.

[0062] Certain embodiments of this invention are described herein, including the best mode known to the inventors for carrying out the invention. Of course, variations on these described embodiments will become apparent to those of ordinary skill in the art upon reading the foregoing description. The inventor expects skilled artisans to employ such variations as appropriate, and the inventors intend for the invention to be practiced otherwise than specifically described herein. Accordingly, this invention includes all modifications and equivalents of the subject matter recited in the claims appended hereto as permitted by applicable law. Moreover, any combination of the above-described elements in all possible variations thereof is encompassed by the invention unless otherwise indicated herein or otherwise clearly contradicted by context.

[0063] Specific embodiments disclosed herein may be further limited in the claims using consisting of or consisting essentially of language. When used in the claims, whether as filed or added per amendment, the transition term "consisting of" excludes any element, step, or ingredient not specified in the claims. The transition term "consisting essentially of" limits the scope of a claim to the specified materials or steps and those that do not materially affect the basic and novel characteristic(s). Embodiments of the invention so claimed are inherently or expressly described and enabled herein.

[0064] Furthermore, numerous references have been made to patents and printed publications throughout this specification. Each of the above-cited references and printed publications are individually incorporated herein by reference in their entirety.

[0065] In closing, it is to be understood that the embodiments of the invention disclosed herein are illustrative of the principles of the present invention. Other modifications that may be employed are within the scope of the invention. Thus, by way of example, but not of limitation, alternative configurations of the present invention may be utilized in accordance

with the teachings herein. Accordingly, the present invention is not limited to that precisely as shown and described. 92845

The **Figures** show:

Figure 1: Generation and application of hiPSCs. Somatic cells, taken from a patient, are cultured. By adding the four pluripotency factors OCT4, SOX2, C-MYC & KLF4, the somatic cells are reprogrammed into pluripotent stem cells. These hiPSCs could be used in patient-specific cell replacement therapies, drug screening tests or serve as human disease model. (Yamanaka and Blau, 2010).

Figure Fehler! Kein Text mit angegebener Formatvorlage im Dokument.: A Square of Parafilm containing MATRIGEL droplets. Neuroepithelial tissue was placed in the centre of a Parafilm dimple and covered with liquid MATRIGEL.

Figure Fehler! Kein Text mit angegebener Formatvorlage im Dokument.: Confocal image of hNESCs cultured under two-dimensional conditions. hNESCs expressed the neural progenitor markers SOX1, SOX2, and NESTIN. The ability of hNESCs to spontaneous differentiation is demonstrated by ICC with antibodies raised against neuronal markers TUJ1 and DCX. FOXA2 staining reveals a low expression level of ventral neural tube marker. Nuclei are counterstained with Hoechst. Scale bars, 20µm.

Figure 4: Progression of midbrain organoid development from human NESCs. **(a)** A colony of human NESCs seeded on round-bottom ultralow attachment plates after six days. Colonies are globular and have bright and smooth edges. **(b)** An organoid at day 9, after embedding in MATRIGEL, shows darker tissue in the centre and bright tissue at the edge. The arrow indicates MATRIGEL surrounding the organoid. **(c)** Image of an organoid at day 13, two days under differentiation conditions. Small processes start to develop across the whole surface. Scale bars, 500 µm. **(d)** 5x magnification of a midbrain organoid at day 35 showing long processes that expand from the tissue. **(e)** 10x magnification of the same organoid. Arrowheads indicate cell bodies outside the colony. **(f)** An organoid without MATRIGEL (left, arrow) and an organoid embedded in MATRIGEL (right, arrowhead), showing long processes that expand through the whole MATRIGEL matrix. Bright field images were acquired with an inverted microscope. Scale bar, 500 µm (a-d).

Figure 5: Immunofluorescence staining of early midbrain organoids. **(a)** Image of an early organoids at day 6 with markers for neural progenitor cells (SOX2) and young neurons (TUJ1). Arrowheads indicate early differentiated neurons. **(b)** An early organoid at day 7,

expressing the neural progenitor markers NESTIN and SOX2. Nuclei are counterstained with Hoechst. Scale bars, 50 µm. 92845

Figure 6: Dopaminergic neurons in midbrain organoids. **(a)** Immunofluorescence staining of an early organoid at day 16 with antibodies against DA neurons (TH/TUJ1, arrowheads). **(b)** Tile scan of an organoid at day 30 expressing DA neuron markers. **(c)** Higher magnification of the tile scan in b. Nuclei are counterstained with Hoechst. Scale bars, 50 µm (a,c), 500 µm (b).

Figure 7A and B: Young and mature dopaminergic neurons in midbrain organoids. **(a)** Tile scan of an organoid expressing the neural progenitor marker SOX2, as well as the young DA neuronal markers TUJ1/TH. **(b)** Higher magnification of the tile scan in a. Arrows indicate the assumed migration direction of maturing DA neurons basally away from the proliferative zone. **(c)** Tile scan of an organoid expressing mature DA neuronal markers MAP2/TH. The dashed line marks the apical surface containing mostly neural progenitor cells. Arrow: Assumed direction of DA neuron migration upon maturation **(d)** Higher magnification of the tile scan in c. Arrowheads mark mature DA neurons. Nuclei are counterstained with Hoechst. Scale bar, 500 µm (a,c), 500 µm (b,d).

Figure 8A and B: Presence of glial cells in midbrain organoids. **(a)** Tile scan of a midbrain organoid stained for markers of young neurons (TUJ1), oligodendrocytes (O4), and astrocytes (GFAP). **(b)** Higher magnification of a. Arrowheads mark O4 positive and TUJ1 negative cells, indicating oligodendrocytes. **(c)** 63x image of another organoid showing a staining of O4 positive and TUJ1 negative cells (arrowheads). **(d)** GFAP and O4 positive and TUJ1 negative cell reveals differentiation of glia. Nuclei are counterstained with Hoechst. Scale bars, 500 µm (a), 50 µm (b-d).

Examples

I. Material and Methods

1. Cell culture

Cell culture work was performed under sterile conditions using a laminar flow hood. All cells were cultured in an incubator at constant 37°C and 5% CO_2. For cells that were grown under 2D conditions, cell culture plates (NUNC™, THERMO SCIENTIFIC™) were coated with MATRIGEL® (CORNING) resuspended in cold Knockout DMEM (LIFE TECHNOLOGIES). 1.5 ml of diluted MATRIGEL was added to each well of a 6-well-plate. The plates were kept at room temperature (RT) overnight. Coated plates were stored at 4°C for up to one month.

2. Generation of cerebral organoids

Cerebral organoids were generated according to the established protocol by Lancaster et al. (Lancaster and Knoblich, 2014a; Lancaster et al., 2013) with slight modifications. Feeder-independent human iPS cells were used as a starting population instead of feeder-dependent iPSCs. As an initial step, embryoid bodies (EBs) were generated. These 3D aggregates undergo cell specification through differentiation into the three germ lineages endoderm, mesoderm, and ectoderm, similar to the embryonic development described in Section 1.1 of the Introduction. Next, the formation of neuroepithelial tissue was initiated, restricting the cell fate to the neural lineage. Once the neuroepithelial tissue developed, the 3D structures were transferred to droplets of MATRIGEL, which gives structural support and helps the tissue to maintain its 3D shape. This matrix, derived from the Engelbroth-Holm-Swarm mouse sarcoma, is composed of laminin, collagen IV, nidogen/enactin, and proteoglycan, thus resembling the extracellular matrix (ECM). The MATRIGEL-embedded tissue was cultured in medium that favoured neuronal differentiation by using Neurobasal medium supplemented with N2 and B27 containing vitamin A. Non-essential amino acids and GlutaMax™ were added in order to increase cell growth and viability. The reducing agent 2-Mercaptoethanol prevented the formation of toxic oxygen radical levels. The tissue was kept under dynamic conditions to prevent the tissue from attaching to the bottom. It further increases the nutrient exchange and controls waste product exchange. For this purpose, Lancaster et al. used a spinning bioreactor. In this project, the tissue was kept in a non-treated 10 cm cell culture petri dish (GREINER) on an orbital shaker (IKA®), spinning at around 80 rpm.

2.1 Maintenance of human induced pluripotent stem cells

A wild type human induced pluripotent stem cell line from CORIELL was seeded on a cell culture 6-well-plate and cultured in Essential8™-medium (LIFE TECHNOLOGIES). At 70-80% confluence and within one week after the last seeding, cells were passaged. To detach the cells from the surface, the medium was aspirated and 0.5 mM EDTA (INVITROGEN) was added. After an incubation time of 4 min at 37°C and 5% CO_2, EDTA was aspirated. Cells were washed with PBS, and resuspended in Essential8™-medium. Cells were seeded into a new coated *6-well-plate* in a ratio of 1:3-1:6. Medium was changed every day.

2.2 Immunocytochemical characterisation of hiPSCs

Human iPS cells were seeded on cover slips in a 24-well-plate and cultured under the conditions described in section 2.1. After three days, cells were fixed with 4% paraformaldehyde (PFA) for 40 min at 4°C and washed three times with PBS for 5 min. The cell membrane was permeabilised with 0.2% Triton-X-100 at RT for 10 min. After three

washing steps with 0.05% Triton-X-100 in PBS, cells were blocked with 2% NGS + 2% BSA in 0.05% Triton-X-100 in PBS at RT for 60 min. Primary antibodies were diluted in the blocking buffer (see **Table 1**). 35 µl drops of primary antibody solution were prepared on Parafilm in a wet chamber. Cover slips were placed upside down on the drops and incubated at 4°C overnight. 92845

Table 1: Primary antibodies used for hiPSC ICC

Antibody	Host	Company	Dilution
Anti-OCT4	Rabbit	ABCAM	1:400
Anti-SSEA-4	Mouse	MILLIPORE	1:75
Anti-NANOG	Rabbit	MILLIPORE	1:200
Anti-TRA-1-81	Mouse	COVANCE	1:50
Anti-SOX2	Rabbit	ABCAM	1:200
Anti-TRA-1-60	Mouse	MILLIPORE	1:50

The next day, the cover slips were washed with three changes of PBS for 5 min. Secondary antibodies raised against primary antibodies (**Table 2**) were diluted together with Hoechst dye (INVITROGEN, 1:10000) and drops on Parafilm were prepared as before. The cover slips were incubated for 1 h at RT and then washed three times with PBS, once with H_2O, and mounted on glass slides using fluorescence mounting medium. The stainings were analysed using a confocal laser scanning microscope (ZEISS LSM 710).

Table Fehler! Kein Text mit angegebener Formatvorlage im Dokument.: Secondary antibodies used for hiPSC/hNESC ICC

Antibody	Host	Company	Dilution
Anti-RABBIT-568	Goat	INVITROGEN	1:1000
Anti-MOUSE-488	Goat	INVITROGEN	1:1000
Anti-MOUSE-568	Goat	INVITROGEN	1:1000
Anti-RABBIT-488	Goat	INVITROGEN	1:1000

2.3 Generation of EBs

For the generation of EBs, colonies were washed once with PBS and detached using 0.5 mM EDTA (INVITROGEN). After an incubation period of 4 min at 37°C, EDTA was replaced by 1 ml pre-warmed StemPro® Accutase® (LIFE TECHNOLOGIES) and cells were incubated for another 4 min at 37°C. The colonies were detached from the dish using a 1 ml pipette tip with

1 ml of Essential8™- medium (LIFE TECHNOLOGIES). The cell suspension was transferred to a Falcon tube and triturated in order to create a single cell suspension. Two repetitions of 5 µl were collected for cell counting. While the cells were centrifuged at 270*g* for 5 min at RT, cells were counted using an automated cell counter (Countess II FL LIFE TECHNOLOGIES) with trypan blue. After centrifugation, the supernatant was aspirated and cells were resuspended in 1 ml low-FGF2 hESC medium containing ROCK inhibitor (**Table 3**). Next, low-FGF2 hESC medium was added in order to obtain 9000 live cells per 150 µl. Finally, 150 µl were plated to each well of a round-bottom ultralow attachment 96-well-plate (CORNING), which allows the cells to settle down and favours the formation of EBs. Cells were cultured in an incubator at 37°C and 5% CO_2. Medium was changed every other day by replacing half of the medium with fresh low-FGF2 hESC medium. ROCK inhibitor and low-FGF2 were added only for four days.

Table 2: low-FGF2 hESC medium

Substance	Concentration
DMEM-F12/Knockout-Serum replacement (LIFE TECHNOLOGIES)	4:1
ESC-quality FBS (INVITROGEN)	1:300
MEM-NEAA (LIFE TECHNOLOGIES)	1:100
GlutaMax (INVITROGEN)	20 mM
2-Mercaptoethanol (GIBCO)	3.85 µM
Y-27632 ROCK inhibitor (MERCK) – 4 days	50 µM
FGF2 (PEPROTECH) – 4 days	4 ng/ml

2.4 Neural induction

After six days, EBs with smooth edges that brightened at the border were transferred with a cut pipette tip to an ultralow attachment 24-well-plate (CORNING) containing neural induction medium (**Table 4**) and cultured for four days.

Table 3: Neural induction medium

DMEM-F12 (LIFE TECHNOLOGIES) supplemented with:	Concentration
N2 Supplement (LIFE TECHNOLOGIES)	1:100
MEM-NEAA (LIFE TECHNOLOGIES)	1:100
Heparin (SIGMA)	1 µg/ml

2.5 Transferring neuroepithelial tissue to MATRIGEL droplets

To prepare a substrate that helps to generate MATRIGEL droplets, a square of Parafilm was placed over an empty tip tray for 200 µl pipette tips and small dimples were created by pressing a gloved finger into the Parafilm over the holes. The dimpled Parafilm was sprayed once again with ethanol and dried in a petri dish under the laminar flow hood. Once the Parafilm was dry, Neuroepithelial tissue was transferred into each dimple and the medium was gently removed. Next, 30 µl of MATRIGEL was added carefully and the tissue was placed in the centre of the droplet (**Fig. 2.1**). To allow the MATRIGEL to polymerise, it was incubated at 37°C for 30 min. After the incubation time, the droplets were collected in 15 ml cerebral organoid differentiation medium without vitamin A (**Table 5**). To remove the MATRIGEL droplets from Parafilm, sterile forceps were used to hold the sheet while shaking the dish gently until the drops fell off. The dish was kept in an incubator under static conditions and the medium was changed after 48 h. After four days in static culture, the medium was changed again with fresh differentiation medium containing vitamin A. Finally, the dishes were placed on an orbital shaker installed in the incubator, shaking at approximately 80 rpm, and medium was changed every third or fourth day.

Table 4: Cerebral organoid differentiation medium

Substance	Concentration	
	Static	*Dynamic*
DMEM-F12/Neurobasal (LIFE TECHNOLOGIES)	1:1	1:1
N2 Supplement (LIFE TECHNOLOGIES)	1:200	1:200
Insulin solution (SIGMA)	1:4000	1:4000
MEM-NEAA (LIFE TECHNOLOGIES)	1:200	1:200
GlutaMax (INVITROGEN)	20 mM	20 mM
2-Mercaptoethanol (GIBCO)	192.5 nM	192.5 nM
Penicillin (LIFE TECHNOLOGIES)	100 U/ml	100 U/ml
Streptomycin (LIFE TECHNOLOGIES)	100 µg/ml	100 µg/ml
B27 supplement w/o vitamin A (LIFE TECHNOLOGIES)	1:100	-
B27 supplement with vitamin A (LIFE TECHNOLOGIES)	-	1:100

3. Generation of midbrain organoids

3.1 Human neuroepithelial stem cells

To generate midbrain organoids, human neuroepithelial stem cells (hNESCs) served as a starting population. These neural progenitor cells have properties of stem cells, as they are capable of a robust, immortal expansion and can differentiate into cells of the CNS, including

neurons, astrocytes, oligodendrocytes, and also into neural crest lineages. They solely require small molecules for self-renewal and expansion. Neural induction was initiated through the inhibition of non-neural BMP and dorsalising TGF-β signalling. To maintain the immortal self-renewal state, WNT and SHH signals were triggered. WNT signalling induces the formation of cells at the lateral border of the neural plate, while its antagonist SHH specifies ventral neural tube fates. CHIR99021 was used to stimulate the canonical WNT signalling pathway, and purmorphamine (PMA) was added to stimulate the SHH pathway. hNESCs can be efficiently differentiated into motor neurons and mDAs, designating them as a powerful tool to study early human development and neurodegenerative diseases.

3.2 Maintenance of human neuroepithelial stem cells

A wildtype human neuroepithelial stem cell line (hNESC-K7) that was derived from human induced pluripotent stem cells served as a starting population (Reinhardt et al., 2013). Cells were cultured in freshly supplemented N2B27 maintenance medium (**Table 6** and **Table 7**). Cells were passaged at 80-90% confluence and within one week after the last seeding. To detach the cells from the surface, the medium was replaced by 700 µl warm StemPro® Accutase® (LIFE TECHNOLOGIES) and cells were incubated at 37°C and 5% CO_2 for 4-6 min. Cells were resuspended in 5 ml warm DMEM/F12 (LIFE TECHNOLOGIES) and centrifuged at 200*g* for 3 min. After aspirating the supernatant, the pellet was resuspended in supplemented N2B27-maintenance medium and cells were seeded into a new plate in a ratio of 1:10-1:20, depending on the confluence. Medium was changed every other day.

Table 5: N2B27 medium composition

Substance	Concentration
DMEM-F12/Neurobasal (LIFE TECHNOLOGIES)	1:1
Penicillin (LIFE TECHNOLOGIES)	100 U/ml
Streptomycin (LIFE TECHNOLOGIES)	100 µg/ml
L-Glutamine (LIFE TECHNOLOGIES)	20mM
B27 supplement w/o vitamin A (LIFE TECHNOLOGIES)	1:100
N2 supplement (LIFE TECHNOLOGIES)	1:200

Table 6: N2B27 maintenance medium composition

N2B27 supplemented with:	Concentration
CHIR-99021 (AXON MEDCHEM)	3 µM
PMA (ENZO LIFE SCIENCE)	0.75 µM
AA (SIGMA)	150 µM

3.3 Immunocytochemical characterisation of hNESCs

hNESCs were seeded on cover slips in a 24-well-plate and cultured under the conditions described in Section 3.2. After 3 days, cells were fixed with 4% PFA for 40 min at 4°C and washed three times with PBS for 5 min. ICC was performed according to the protocol described in Section 2.2 with different primary antibodies (**Table 8**) and the according secondary antibodies (**Table 2**).

Table 7: Primary antibodies used for hNESC ICC

Antibody	Host	Company	Dilution
Anti-SOX2	Rabbit	ABCAM	1:200
Anti-FOXA2	Mouse	SANTA CRUZ	1:100
Anti-SOX1	Rabbit	CELL SIGNALLING	1:200
Anti-NESTIN	Mouse	BD	1:600
Anti-DCX	Rabbit	ABCAM	1:400
Anti-TUJ1	Mouse	COVANCE	1:600

3.4 Generation and expansion of three-dimensional hNESC colonies

In order to generate single 3D hNESC colonies, hNESCs were passaged as described in section 3.2. After centrifugation and resuspension in 1 ml N2B27 maintenance medium, cells were counted using an automated cell counter (Countess II FL LIFE TECHNOLOGIES) with trypan blue. N2B27 maintenance medium was added in order to obtain 9000 live cells per 150 µl. 150 µl were plated to each well of a round-bottom ultralow attachment 96-well-plate (CORNING), which allows the cells to form one single colony per well. Medium was changed every other day by replacing half of the medium with fresh N2B27 maintenance medium. After six days, the colonies were transferred to an ultralow attachment 24-well-plate (CORNING) in order to remove side colonies and to allow the tissue to expand. After two days, the colonies were embedded into MATRIGEL droplets in the same manner as described in Section 2.5. The MATRIGEL-embedded colonies were cultured for two more days under static maintenance conditions in 10 cm petri-dishes, before differentiation was initiated at day 10 with N2B27 differentiation medium containing PMA (**Table 9**). The differentiation protocol was adapted from Reinhardt et al. 2013 with a slight modification. FGF8 was not added for the first eight days, since this decreases the differentiation efficiency into mDA neurons. After two days under differentiation conditions, the dishes were placed on an orbital shaker and kept under dynamic conditions at approximately 80 rpm. Medium was changed every third or fourth day. PMA was only added the first six days of differentiation.

Table 8: N2B27 differentiation medium

N2B27 supplemented with:	Concentration

hBDNF (PEPROTECH)	10 ng/ml
hGDNF (PEPROTECH)	10 ng/ml
dbcAMP (PEPROTECH)	500 µM
AA (SIGMA)	200 µM
TGF-β3 (PEPROTECH)	1 ng/ml
PMA (ENZO LIFE SCIENCE)	1 µM

4. Immunohistochemical characterisation of human cerebral and midbrain organoids

During brain development, cells rearrange themselves and form different functional and interdependent regions. Simultaneously, neuroepithelial cells differentiate into the various neurons and supportive glial cells of the CNS. In order to analyse whether cerebral and midbrain organoids develop different cell types and brain regions characteristic of the developing human brain, immunohistochemical (IHC) stainings were performed. The presence of neural progenitor cells, young and mature neurons, astrocytes, oligodendrocytes, dopaminergic neurons was analysed. Moreover, immunofluorescence stainings were performed to verify whether cerebral and midbrain organoids established midbrain identity.

4.1 Sectioning

At day 6 (*n=3*), 7 (*n=3*), 16 (*n=3*), 30 (*n=3*), and 44 (*n=8*), cerebral and midbrain organoids were fixed in 4% PFA at RT on a shaker overnight. Bigger organoids with a diameter >900 µm were sectioned prior to IHC. The fixed tissue was washed three times in PBS for 15 min and embedded into warm 3% low-melting point agarose and allowed to cool until solid. The block of agarose was then trimmed and glued on a metal block holder, and sectioned using a vibratome (LEICA, VT100 S) to 150 µm thickness at a speed of 0.5 mm/s and a frequency of 70 Hz.

4.2 IHC

The free floating agarose sections were collected in 24-well-plates containing TBS+++ (1x Tris-buffered saline with 0.5% Triton-X-100, 0.1% sodium azide, 0.1% sodium citrate and 5% fetal bovine serum or normal goat serum) and blocked/permeabilised for at least 1h at RT on a shaker. Primary antibodies (see **Table 10**) were diluted in TBS+++ and 300 µl of the antibody solution were added to each well. The sections were incubated for 48 h at 4°C on a shaker and afterwards washed with three changes of TBS for 15 min. The sections were incubated in secondary antibodies (see **Table 11**) diluted in TBS+++ for 2 h at RT on a shaker, which was followed by three washing steps in TBS for 15 min. Finally, the sections

were rinsed with H_2O and mounted with mounting medium on a glass slide. The stainings were analysed using a confocal laser scanning microscope (ZEISS LSM 710). 92845

Table 9: Primary antibodies used for IHC

Antibody	Host	Company	Dilution
	Day 7:		
Anti-NESTIN	Mouse	BD	1:600
Anti-OCT4	Rabbit	ABCAM	1:400
Anti-SOX2	Rabbit	ABCAM	1:200
Anti-SSEA4	Mouse	MILLIPORE	1:75
Anti-TRA-1-60	Mouse	MILLIPORE	1:50
	Day 16, Day 30:		
Anti-PAX6	Rabbit	SANTA CRUZ	1:300
Anti-TH	Rabbit	ABCAM	1:1000
	Day6, Day 16, Day 30, Day 44:		
Anti-SOX2	Goat	R&D	1:200
Anti-TUJ1	Mouse	COVANCE	1:600
	Day 44:		
Anti-EN1	Goat	SANTA CRUZ	1:100
Anti-FOXA2	Mouse	SANTA CRUZ	1:100
Anti-GFAP	Chicken	MILLIPORE	1:1000
Anti-LMX1	Rabbit	MILLIPORE	1:200
Anti-MAP2	Mouse	MILLIPORE	1:200
Anti-O4	Mouse	SIGMA ALDRICH	1:400
Anti-TH	Rabbit	SANTA CRUZ	1:1000
Anti-TUJ1	Rabbit	COVANCE	1:600

Table 10: Secondary antibodies used for IHC

Antibody	Host	Company	Dilution
Anti-Mouse-488	Donkey	INVITROGEN	1:1000
Anti-Goat-568	Donkey	INVITROGEN	1:1000
Anti-Rabbit-647	Donkey	INVITROGEN	1:1000
Anti-Rabbit-488	Goat	INVITROGEN	1:1000
Anti-Rabbit-568	Goat	INVITROGEN	1:1000
Anti-Mouse-488	Goat	INVITROGEN	1:1000
Anti-Mouse-568	Goat	INVITROGEN	1:1000
Anti-Mouse-647	Goat	INVITROGEN	1:1000
Anti-Chicken-568	Goat	INVITROGEN	1:1000

II. Results

1. Generation of midbrain organoids

1.1 Immunocytochemical characterisation of hNESCs

ICC of human neuroepithelial stem cells revealed a stable expression of the neural progenitor markers SOX2 and NESTIN under maintenance conditions. Another neural progenitor marker, SOX1, was also expressed in hNESCs, though fluorescence intensities varied. FOXA2, a marker of the ventral neural tube, was sligthly expressed in hNESCs. Upon early differentiation, few hNESCs differentiate into neurons that express neuron-specific class III beta-tubulin (TUJ1) and doublecortin (DCX), which mark early neurons (**Fig. 3**).

1.2 Development of midbrain organoids

For the generation of midbrain organoids, the wild type hNESC line K7 was used as a starting population. Human NESCs seeded on round-bottom ultralow attachment plates formed dense globular colonies with only few dead cells around. In some wells, small side colonies developed, which then merged with the main colony after some days. The colonies began to brighten within the first three days and showed smooth edges under maintenance conditions, indicating healthy tissue. However, early organoids at day 8 developed a dark core in the centre, indicative of cell death. **(Fig. 4 a, b)**. After two days of differentiation, the organoids developed small processes along the surface of the tissue **(Fig. 4 c)**. Within three weeks, the processes expanded and reached a length of approximately 1 mm with only few cell bodies outside the colony. This was not observed in colonies without MATRIGEL support (**Fig. 4 d-f**).

1.3 Immunohistochemical characterisation of human midbrain organoids

Early midbrain organoids at day 6 and day 7 under maintenance conditions were stained without sectioning. The developing three dimensional human NESC colonies showed a stable expression of the neural progenitor markers SOX2 and NESTIN, as well as early differentiated young neurons (**Fig 5**). Throughout the colonies, small cavities enclosed by radially organised progenitor cells developed.

Midbrain organoids at later stages under differentiation conditions developed a dense neuronal network, which gradually increased in complexity, consistent with gross morphological changes. Immunofluorescence staining with markers of dopaminergic neurons (TUJ1/TH) revealed that already at day 16 some DA neurons developed, increasing in density with later stages. Notably, DA neurons were located in distinct areas, which is suggestive of an asymmetric polar organisation of the 3D structures, consistent with brain development *in vivo* (**Fig. 6, Fig. 7**). To further analyse DA neurons in midbrain organoids, staining for the mature neuronal marker MAP2 together with TH was performed. A staining of

different sections of the same organoid revealed that mature neurons were located at the outermost part of the organoid, while young TUJ1 positive neurons were more abundant in the inner core (**Fig. 7 a, c**). Interestingly, some of the mature neurons also expressed TH, revealing the presence of mature DA neurons in midbrain organoids. Additionally, staining for neural progenitor marker SOX2 showed that some neural progenitor cells surrounded the inner core of the organoid at later stages. TH positive DA neurons were located adjacent to the layer of neural precursor cells (**Fig. 7**).

To further examine the cellular organisation of midbrain organoids, stainings of glial cell markers including GFAP (astrocytes) and O4 (oligodendrocytes) were performed. In some regions of midbrain organoids, O4 positive and TUJ1 negative cells were identified, indicating the development of oligodendrocytes in late-stage midbrain organoids (**Fig. 8 a-c**). Only few astrocytes were found in midbrain organoids. However, some GFAP positive processes without associated cell bodies were observed across the tissue (**Fig. 8 c**), as well as few GFAP positive cells (**Fig. 8 d**)

Finally, midbrain organoids at later stages were stained for midbrain markers LMX1A, FOXA2, and EN1. Within the analysed sections and organoids, no cells expressing these transcription factors were found. Some cells showed evidence of LMX1A expression, though it was not located in the nucleus and diffuse, potentially revealing non-specific binding. Altogether the data indicate that midbrain organoids develop various neural cell types and exhibit an asymmetric polarisation of the tissue.

References

Abeliovich, A., and Hammond, R. (2007). Midbrain dopamine neuron differentiation: factors and fates. Developmental biology 304, 447-454.

Ang, S.L. (2009). Foxa1 and Foxa2 transcription factors regulate differentiation of midbrain dopaminergic neurons. Advances in experimental medicine and biology 651, 58-65.

Gage, F.H. (2000). Mammalian neural stem cells. Science (New York, NY) *287*, 1433-1438.

Gale, E., and Li, M. (2008). Midbrain dopaminergic neuron fate specification: Of mice and embryonic stem cells. Mol Brain *1*, 8.

Lancaster, M.A., and Knoblich, J.A. (2014a). Generation of cerebral organoids from human pluripotent stem cells. Nature protocols 9, 2329-2340.

Lancaster, M.A., and Knoblich, J.A. (2014b). Organogenesis in a dish: modeling development and disease using organoid technologies. Science 345, 1247125.

Lancaster, M.A., Renner, M., Martin, C.A., Wenzel, D., Bicknell, L.S., Hurles, M.E., Homfray, T., Penninger, J.M., Jackson, A.P., and Knoblich, J.A. (2013). Cerebral organoids model human brain development and microcephaly. Nature 501, 373-379.

Lewis, T.L., Jr., Courchet, J., and Polleux, F. (2013). Cell biology in neuroscience: Cellular and molecular mechanisms underlying axon formation, growth, and branching. The Journal of cell biology 202, 837-848.

Niwa, H. (2007). How is pluripotency determined and maintained? Development 134, 635-646.

Reinhardt, P., Glatza, M., Hemmer, K., Tsytsyura, Y., Thiel, C.S., Hoing, S., Moritz, S., Parga, J.A., Wagner, L., Bruder, J.M., et al. (2013). Derivation and expansion using only small molecules of human neural progenitors for neurodegenerative disease modeling. PloS one 8, e59252.

Sharples, S.A., Koblinger, K., Humphreys, J.M., and Whelan, P.J. (2014). Dopamine: a parallel pathway for the modulation of spinal locomotor networks. Frontiers in neural circuits 8, 55.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., and Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131, 861-872.

Takahashi, K., and Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126, 663-676.

Thomson, J.A., and Odorico, J.S. (2000). Human embryonic stem cell and embryonic germ cell lines. Trends in biotechnology 18, 53-57.

Weissman, I.L. (2000). Stem cells: units of development, units of regeneration, and units in evolution. Cell 100, 157-168.

Yamanaka, S., and Blau, H.M. (2010). Nuclear reprogramming to a pluripotent state by three approaches. Nature 465, 704-712.

Yu, J., Vodyanik, M.A., Smuga-Otto, K., Antosiewicz-Bourget, J., Frane, J.L., Tian, S., Nie, J., Jonsdottir, G.A., Ruotti, V., Stewart, R., et al. (2007). Induced pluripotent stem cell lines derived from human somatic cells. Science 318, 1917-1920.

Patentansprüche

1. Verfahren zum Herstellen eines Mittelhirn-Organoids, umfassend Kontaktieren neuroepithelialer Stammzellen, welche in einer dreidimensionalen Zellkultur, umfassend eine Matrix, gezüchtet werden, mit Differenzierungsmedium, wobei das Züchten unter agitierenden Bedingungen durchgeführt wird, wodurch Erhalten eines Mittelhirn-Organoids.

2. Verfahren nach Anspruch 1, wobei die neuroepithelialen Stammzellen menschliche neuroepitheliale Stammzellen sind.

3. Verfahren nach Anspruch 2, wobei die neuroepithelialen Stammzellen hNESC-K7 oder smNPCs sind.

4. Verfahren nach einem der Ansprüche 1-3, wobei die neuroepithelialen Stammzellen genetisch modifiziert oder von einem Patienten, der unter einer neurologischen Erkrankung leidet, erhalten wurden.

5. Verfahren nach Anspruch 4, wobei die genetische Modifikation eine Mutation, einen Knock-out oder einen Knock-in umfasst.

6. Verfahren nach Anspruch 4, wobei die neurologische Erkrankung eine neurodegenerative Erkrankung ist, wie Parkinson-Krankheit, Multiple Sklerose, Batten-Krankheit oder Alzheimer-Krankheit.

7. Verfahren nach einem der Ansprüche 1-6, wobei die neuroepithelialen Stammzellen aus induzierten pluripotenten Stammzellen (iPSCs) hergestellt wurden.

8. Verfahren nach Anspruch 7, wobei die iPSCs aus Fibroblasten oder peripheren mononukleären Blutzellen (PBMC) hergestellt wurden, wobei die Fibroblasten oder PBMC vorzugsweise von einem Patienten erhalten wurden.

9. Verfahren nach einem der Ansprüche 1-8, wobei die dreidimensionale Zellkultur in einem Gel, einem Bioreaktor, unter niedrigst-Adhäsions-Bedingungen oder einem Mikrochip durchgeführt wird, vorzugsweise einem Hydrogel und/oder einem Hydrogel-Tropfen, wie einem Matrigel-Tropfen.

10. Verfahren nach einem der Ansprüche 1-9, wobei die Matrix eine extrazelluläre Matrix ist und/oder wobei die Matrix ein oder mehrere natürliche Molekülen, synthetische Polymere, biologisch-synthetische Hybride, Metalle, Keramiken, bioaktive Gläser und/oder Kohlenstoffnanoröhrchen umfasst.

11. Verfahren nach einem der Ansprüche 1-10, wobei die Matrix Kollagen, vorzugsweise Kollagen IV, Laminin, Entaktin, Proteoglykane, Matrigel, Fibrin, Hyaluronsäure, Chitosan, Alginat, Seidenfibrillen, Ethylenglykol wie PEG, Polyvinylalkohol und/oder poly(2-Hydroxyethylmethacrylat) umfasst, vorzugsweise umfasst die Matrix Kollagen IV, Laminin, Entaktin, Proteoglykane oder Matrigel, gegebenenfalls umfasst die Matrix weiterhin Wachstumsfaktoren für das Überleben, die Proliferation und/oder Differenzierung von Stammzellen.

12. Verfahren nach einem der Ansprüche 1-11, wobei das Differenzierungsmedium (Differenzierungsmedium I) umfasst

    (i) einen SHH-Signalweg-Aktivator;

    (ii) mindestens zwei verschiedene Neurotrophine; und

    (iii) ein Antioxidans.

13. Verfahren nach einem der Ansprüche 1-11, wobei das Differenzierungsmedium (Differenzierungsmedium II) umfasst

    (i) mindestens zwei verschiedene Neurotrophine; und

    (ii) ein Antioxidans.

14. Verfahren nach Anspruch 12, wobei der SHH-Signalweg-Aktivator ausgewählt ist aus der Gruppe bestehend aus Purmorphamin, SHH, smoothened Agonist (SAG), Hh-Ag 1.5 und Gli-2, vorzugsweise ist der SHH-Signalweg-Aktivator Purmorphamin.

15. Verfahren nach einem der Ansprüche 12-14, wobei die die mindestens zwei verschiedenen Neurotrophine ausgewählt sind aus der Gruppe bestehend aus NGF, BDNF, NT-3, NT-4, CNTF und GDNF, vorzugsweise sind die mindestens zwei Neutrophine GDNF und BDNF.

16. Verfahren nach einem der Ansrpüche 12-15, wobei das Antioxidans ausgewählt ist aus der Gruppe bestehend aus Ascorbinsäure, Superoxid-Dismutase 1, Superoxid-Dismutase 2, Superoxid-Dismutase 3, Glutathion, Liponsäure, Epigallocatechingallat,

Curcumin, Melatonin, Hydroxytyrosol, Ubichinon, Katalase, Vitamin E und Harnsäure, vorzugsweise ist das Antioxidans Ascorbinsäure.

17. Verfahren nach einem der Ansprüche 12-16, wobei das Differenzierungsmedium (Differenzierungsmedium I und/oder II) weiterhin einen Aktivator des Aktivin/transformierenden Wachstumsfaktor-β (TGF-β)-Signalwegs umfasst und/oder wobei das Differenzierungsmedium II keinen SHH-Signalweg-Aktivator umfasst.

18. Verfahren nach einem der Ansprüche 12-17, wobei das Differenzierungsmedium (Differenzierungsmedium I und/oder II) weiterhin ein cAMP-Analogon umfasst.

19. Verfahren nach einem der Ansprüche 12-18, wobei FGF8 nach 8 Tagen der Differenzierung zu dem Differenzierungsmedium (Differenzierungsmedium I und/oder II) zugegeben wird.

20. Verfahren nach einem der Ansprüche 1-19, wobei die neuroepithelialen Stammzellen durch ein Erhaltungsmedium kontaktiert werden, bevor sie mit dem Differenzierungsmedium kontaktiert werden.

21. Verfahren nach Anspruch 20, wobei das Erhaltungsmedium umfasst

    (i) einen SHH-Signalweg-Aktivator;

    (ii) einen kanonischen WNT-Signal-Aktivator; und

    (iii) ein Antioxidans.

22. Verfahren nach Anspruch 21, wobei das Erhalten der neuroepithelialen Stammzellen in einer zweidimensionalen und/oder dreidimensionalen Zellkultur stattfindet.

23. Verfahren nach einem der Ansprüche 1-22, wobei die neuroepithelialen Stammzellen in einer Kolonie vorliegen.

24. Verfahren nach Anspruch 23, wobei die Kolonie eine Anhäufung von Zellklonen ist.

25. Verfahren nach einem der Ansprüche 1-24, wobei die agitierenden Bedingungen Schütteln, Drehen, Rühren, Bewegen und/oder Mischen der dreidimensionalen Zellkultur umfassen.

26. Verfahren nach Anspruch 25, wobei das Rühren mit einem rührenden Bioreaktor und/oder das Schütteln mit einem Horizontalschüttler durchgeführt werden.

27. Verfahren nach Anspruch 26, wobei der Horizontalschüttler mit mindestens 40 U/min, 50 U/min, 60 U/min, 70 U/min, 80 U/min, 90 U/min, 100 U/min, 110 U/min oder mehr schüttelt, vorzugsweise schüttelt der Horizontalschüttler mit 80 U/min.

28. Verfahren nach einem der Ansprüche 1-27, umfassend:

(i) Kontaktieren neuroepithelialer Stammzellen mit dem Erhaltungsmedium, wie in Anspruch 21 definiert;

(ii) Kontaktieren neuroepithelialer Stammzellen, die in einer dreidimensionalen Zellkultur, umfassend eine Matrix, gezüchtet werden, mit dem Differenzierungsmedium (I), wie in einem der Ansprüche 12, 14-19 definiert,

wobei das Züchten unter agitierenden Bedingungen durchgeführt wird,

wobei das Agitieren nach 0, 1, 2, 3, 4, 5, 6, 7, 8 oder mehr Tagen, vorzugsweise 2 Tagen, gestartet wird, nach dem Starten des Züchtens der neuroepithelialen Stammzellen in Differenzierungsmedium I;

(iii) Kontaktieren neuroepithelialer Stammzellen mit dem Differenzierungsmedium (II), wie in einem der Ansprüche 13-27 definiert, unter agitierenden Bedingungen,

wodurch ein Mittelhirn-Organoids erhalten wird.

29. Verfahren nach einem der Ansprüche 1-28, wobei das Mittelhirn-Organoid nach 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 oder mehr Wochen der Differenzierung erhältlich ist.

30. Verfahren nach einem der Ansprüche 1-29, wobei das Mittelhirn-Organoid ein frühes Mittelhirn-Organoid oder ein spätes Mittelhirn-Organoid ist.

31. Verfahren nach Anspruch 30, wobei das frühe Mittelhirn-Organoid ein Mittelhirn-Organoid ist, welches für 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 oder 20 Tage differenziert wurde, vorzugsweise wurde das frühe Mittelhirn-Organoid für 6, 7 oder 16 Tage differenziert.

32. Verfahren nach Anspruch 30, wobei das späte Mittelhirn-Organoid ein Mittelhirn-Organoid ist, welches für mindestens 25, 30, 35, 40, 50, 60 oder mehr Tage differenziert wurde, vorzugsweise wurde das späte Mittelhirn-Organoid für 30 oder 44 Tage differenziert.

33. Verfahren nach einem der Ansprüche 1-32, wobei das Mittelhirn-Organoid umfasst

(a) neurale Vorläuferzellen;

(b) junge Neuronen;

(c) junge dopaminerge Neuronen;

(d) reife Neuronen;

(e) reife dopaminerge Neuronen;

(f) eine asymmetrischen Ordnung des Mittelhirn-Organoids;

(g) Oligodendrozyten;

(h) Oligodendrozytenvorläufer;

(i) Astrozyten; und/oder

(j) Fortsätze, die sich von dem Mittelhirn-Organoid durch die Matrix ausbreiten.

34. Verfahren nach einem der Ansprüche 30, 31 oder 33, wobei das frühe Mittelhirn-Organoid umfasst

(a) neurale Vorläuferzellen;

(b) junge Neuronen; und/oder

(c) junge dopaminerge Neuronen.

35. Verfahren nach einem der Ansprüche 30, 32 oder 33, wobei das späte Mittelhirn-Organoid umfasst

(a) neurale Vorläuferzellen;

(b) junge Neuronen;

(c) junge dopaminerge Neuronen;

(d) reife Neuronen;

(e) reife dopaminerge Neuronen;

(f) eine asymmetrische Ordnung des Mittelhirn-Organoids;

(g) Oligodendrozyten;

(h) Oligodendrozytenvorläufer;

(i) Astrozyten;

(j) Zusammenlagern von dopaminergen Neuronen innerhalb des Organoids; und/oder

(k) Fortsätze, die sich von dem Mittelhirn-Organoid durch die Matrix ausbreiten.

36. Verfahren nach einem der Ansprüche 33-35, wobei die neuralen Vorläuferzellen durch die Expression des Markers SOX2 und/oder Nestin charakterisiert sind,

wobei die jungen Neuronen durch die Expression des Markers TUJ1 charakterisiert sind,

wobei die jungen dopaminergen Neuronen durch die Expression der Marker TUJ1 und Tyrosinhydroxylase (TH) charakterisiert sind,

wobei die reifen Neuronen durch die Expression des Markers MAP2 charakterisiert sind,

wobei die reifen dopaminergen Neuronen durch die Expression der Marker MAP2 und TH charakterisiert sind,

wobei die Oligodendrozyten durch die Expression des Markers O4 charakterisiert sind,

wobei die Oligodendrozytenvorläufer durch die Expression des Markers NG2 charakterisiert sind, und/oder

wobei die Astrozyten durch die Expression der Marker GFAP und/oder S100b charakterisiert sind.

37. Verfahren nach einem der Ansprüche 33-36, wobei die asymmetrische Organisation des Mittelhirn-Organoids eine asymmetrische polare Organisation dopaminerger Neuronen und/oder eine asymmetrische Organisation neuraler Vorläuferzellen innerhalb des Mittelhirn-Organoids ist.

38. Verfahren nach Anspruch 37, wobei die asymmetrische polare Organisation von dopaminergen Neuronen innerhalb des Mittelhirn-Organoids durch die Lokalisierung von

(a) reifen dopaminergen Neuronen in dem äußersten Teil des Mittelhirn-Organoids; und/oder

(b) jungen dopaminergen Neuronen im inneren Teil des Mittelhirn-Organoids

charakterisiert ist.

39. Verfahren nach Anspruch 37 oder 38, wobei junge dopaminerge Neuronen bei der Reifung in Richtung des äußersten Teils des Mittelhirn-Organoids wandern.

40. Verfahren nach Anspruch 37, wobei die asymmetrische polare Organisation von neuralen Vorläuferzellen innerhalb des Mittelhirn-Organoids durch die Lokalisierung von neuralen Vorläuferzellen in einer ringähnlichen Struktur, die den inneren Kern des Mittelhirn-Organoids umschließt, charakterisiert ist.

41. Verfahren nach einem der Ansprüche 33-40, wobei das Zusammenlagern von dopaminergen Neuronen innerhalb des Mittelhirn-Organoids durch die Anhäufung von mehr als 2, 3, 4, 5, 6, 7, 8, 9, 10 oder mehr dopaminergen Neuronen, in einer bestimmten Region des Mittelhirn-Organoids, charakterisiert ist.

42. Verfahren nach einem der Ansprüche 33-41, wobei die Fortsätze, die sich von dem Mittelhirn-Organoid durch die Matrix ausbreiten, eine Länge von 0,1 mm, 0,2 mm, 0,3 mm, 0,4 mm, 0,5 mm, 0,6 mm, 0,7 mm, 0,8 mm, 0,9 mm, 1 mm, 1,1 mm, 1,2 mm oder mehr haben.

43. Mittelhirn-Organoid-Einheit, erhältlich durch das Verfahren nach einem der Ansprüche 1-42.

44. Verfahren zum Testen eines Stoffs von Interesse, auf seine Fähigkeit hin eine zelluläre Reaktion hervorzurufen, umfassend:

(a) Kontaktieren des Mittelhirn-Organoids nach Anspruch 43 mit dem Stoff von Interesse; und

(b) Feststellen, ob der Stoff von Interesse eine zelluläre Reaktion hervorruft.

45. Verfahren zum Auffinden von Molekülen, die dopaminerge neuronale Differenzierung und/oder den Tod von dopaminergen Neuronen in einem Mittelhirn-Organoid, wie in Anspruch 43 definiert, fördern oder hemmen, wobei das Verfahren umfasst Kontaktieren des Mittelhirn-Organoids mit einem Molekül von Interesse,

wobei eine Erhöhung der Differenzierung in dopaminerge Neuronen, verglichen mit einer Kontrolle, andeutet, dass das Molekül von Interesse dopaminerge neuronale Differenzierung fördert und/oder den Tod von dopaminergen Neuronen hemmt und wobei eine Erniedrigung der Differenzierung in dopaminerge Neuronen, verglichen mit einer Kontrolle, andeutet, dass das Molekül von Interesse dopaminerge neuronale Differenzierung hemmt und/oder den Tod von dopaminergen Neuronen hervorruft.

46. Verfahren nach Anspruch 44 oder 45, wobei der Stoff von Interesse ein Medikament, kleines Molekül, Hormon, Wachstumsfaktor, Bindeprotein, Nukleinsäuremolekül, Peptid, Protein oder eine (ko-gezüchtete) Zelle ist.

47. Verfahren nach Anspruch 44, wobei die Art der Zelle ein dopaminerges Neuron ist.

48. Verfahren nach Anspruch 45, wobei die Differenzierung in dopaminerge Neuronen durch das Vergleichen des Neuritenauswuchses gemessen wird.

49. Verfahren nach Anspruch 45 oder 48, wobei die Differenzierung in dopaminerge Neuronen durch das Vergleichen der Expression von TH gemessen wird.

50. Verfahren nach einem der Ansprüche 45, 48 oder 49, wobei die Kontrolle ein Mittelhirn-Organoid ist, welches nicht mit dem Molekül von Interesse kontaktiert wurde.

51. Zusammensetzung, umfassend ein Mittelhirn-Organoid-Einheit nach Anspruch 43.

52. Verwendung von neuroepithelialen Stammzellen, welche in einer dreidimensionalen Zellkultur, umfassend eine Matrix, unter agitierenden Bedingungen gezüchtet werden, zur Herstellung einer Mittelhirn-Organoid-Einheit.

53. Verfahren nach einem der Ansprüche 1-42, wobei die neuroepitheliale Stammzelle durch ein Verfahren erhalten wurde, umfassend

    a) gegebenenfalls Erhalten/Bereitstellen induzierter pluripotenter Stammzellen (iPSCs);

    b) Züchten der iPSCs in einem Medium, umfassend

        (i) einen Aktivin/transformierenden Wachstumsfaktor-β (TGF-β)-Signal-Inhibitor;

        (ii) einen kanonischen WNT-Signal-Aktivator;

(iii) einen knochenmorphogenetisches Protein (BMP)-Signal-Inhibitor; und 92845

(iv) einen SHH-Signalweg-Aktivator; und

c) Züchten der in b) erhaltenen Zellen in einem Medium, umfassend

(i) einen Aktivin/TGF-β-Signal-Inhibitor;

(ii) einen kanonischen WNT-Signal-Aktivator;

(iii) einen BMP-Signal-Inhibitor; und

(iv) einen SHH-Signalweg-Aktivator; und

d) weiteres Züchten der in c) erhaltenen Zellen in einem Medium, umfassend

(i) einen kanonischen WNT-Signal-Aktivator;

(ii) einen SHH-Signalweg-Aktivator; und

(iii) ein Antioxidans;

wodurch eine neuroepitheliale Stammzelle erhalten wird.

Figure 1

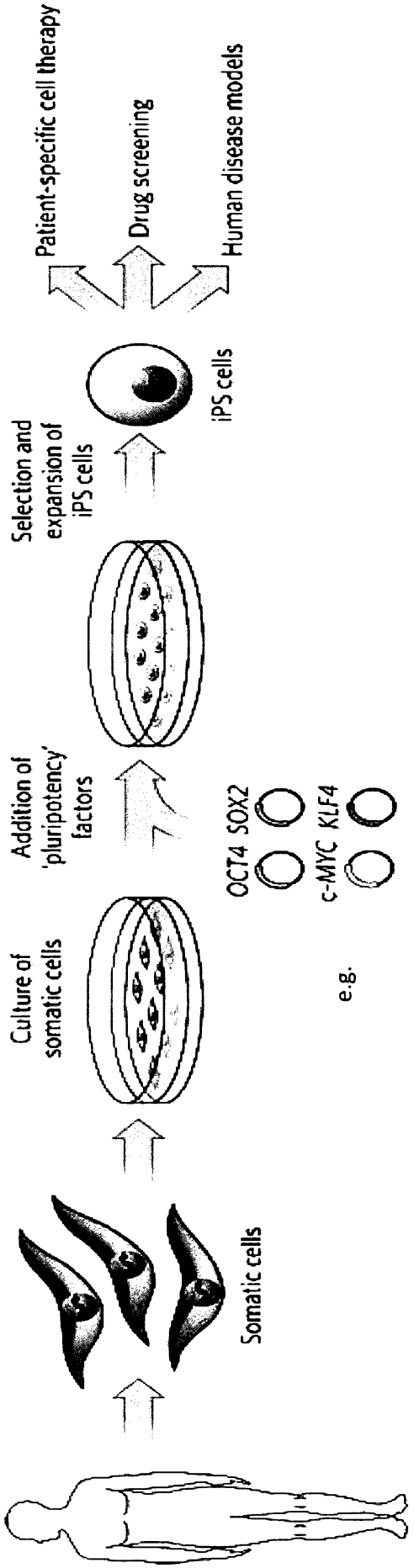

Patient-specific cell therapy
Drug screening
Human disease models
iPS cells
Selection and expansion of iPS cells
Addition of 'pluripotency' factors
OCT4 SOX2
c-MYC KLF4
e.g.
Culture of somatic cells
Somatic cells

Figure 2

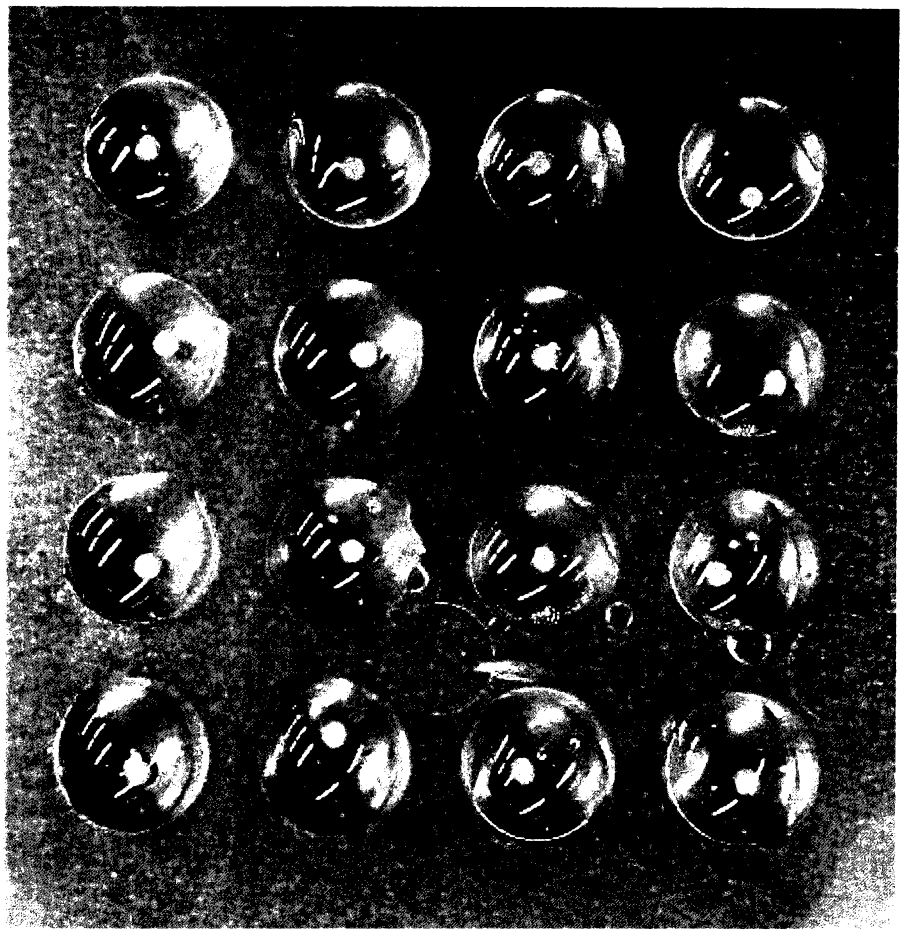


Figure 3

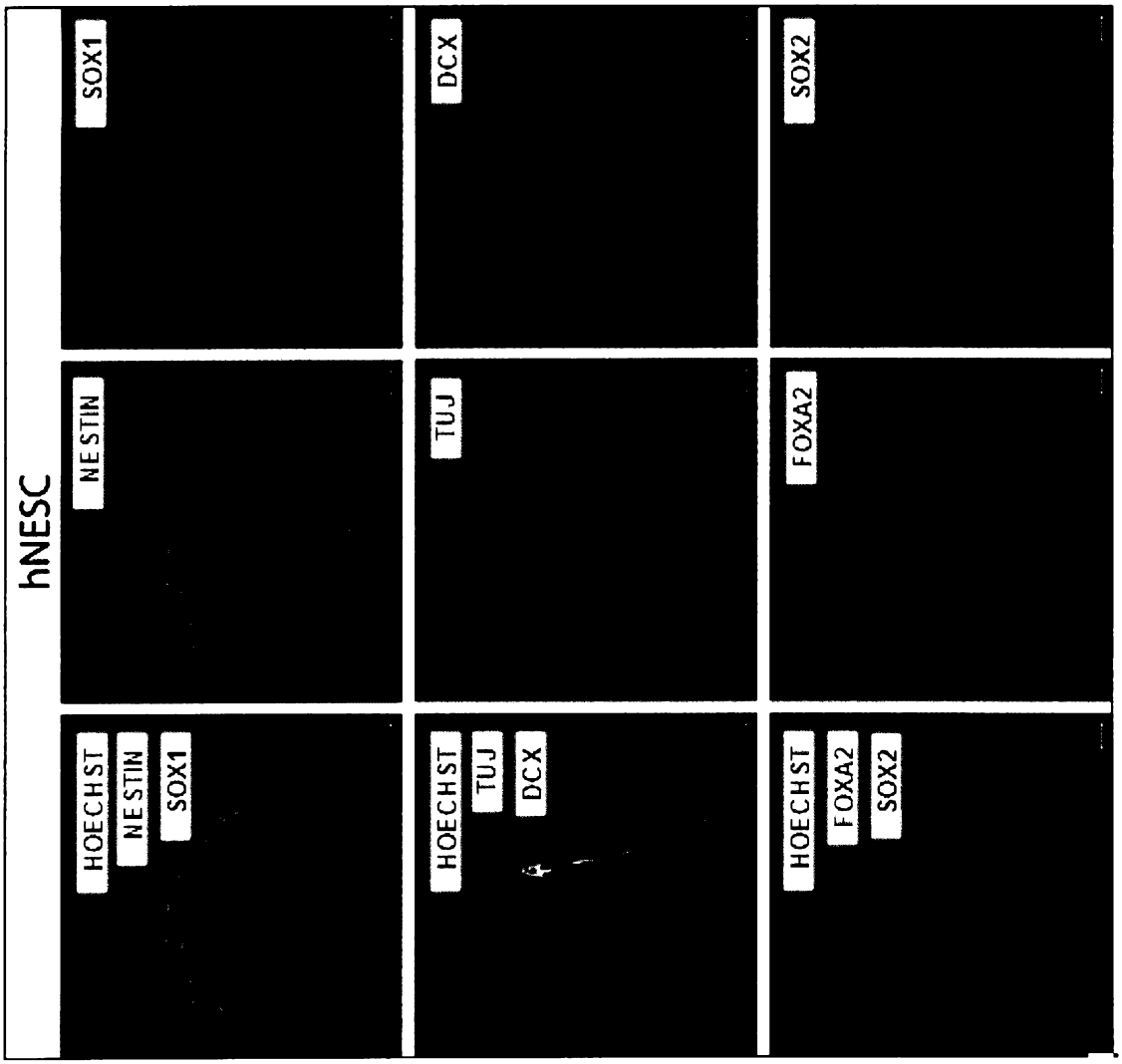

hNESC
SOX1
NESTIN
HOECHST
NESTIN
SOX1
TUJ
DCX
HOECHST
TUJ
DCX
FOXA2
SOX2
HOECHST
FOXA2
SOX2

Figure 4

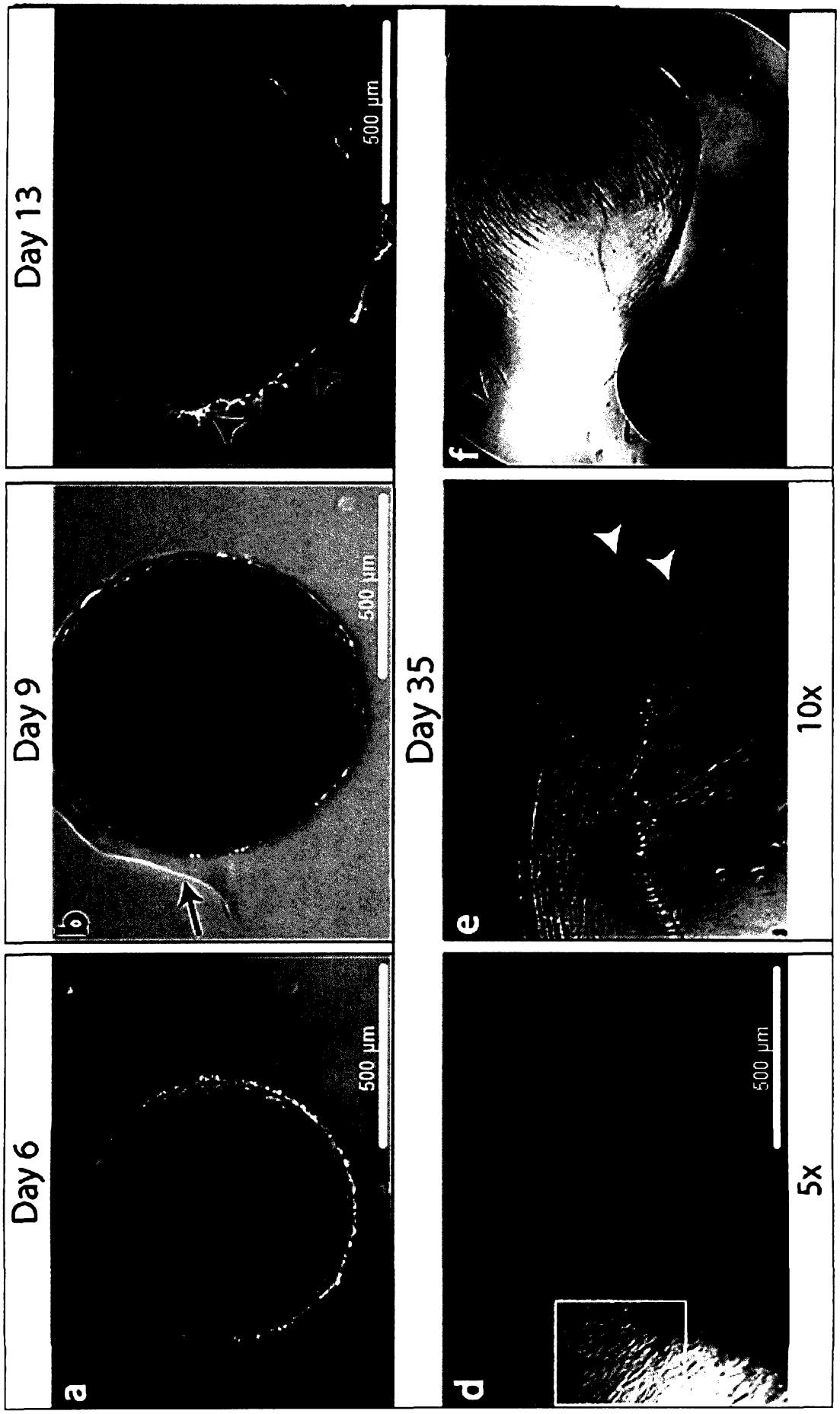


Figure 5

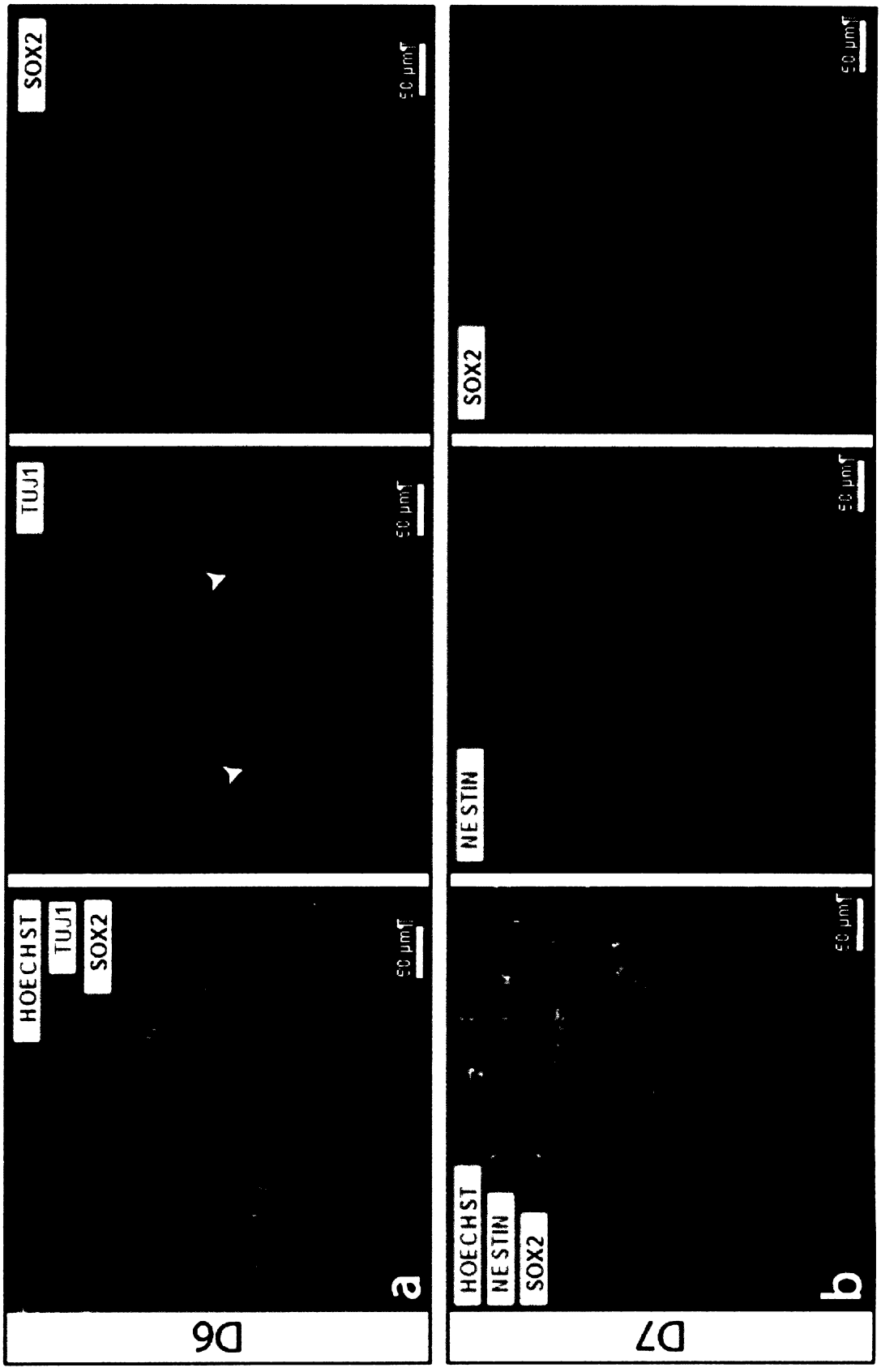

D6
a
HOECHST
TUJ1
SOX2
TUJ1
SOX2
50 µm
D7
b
HOECHST
NESTIN
SOX2
NESTIN
SOX2
50 µm

Figure 6

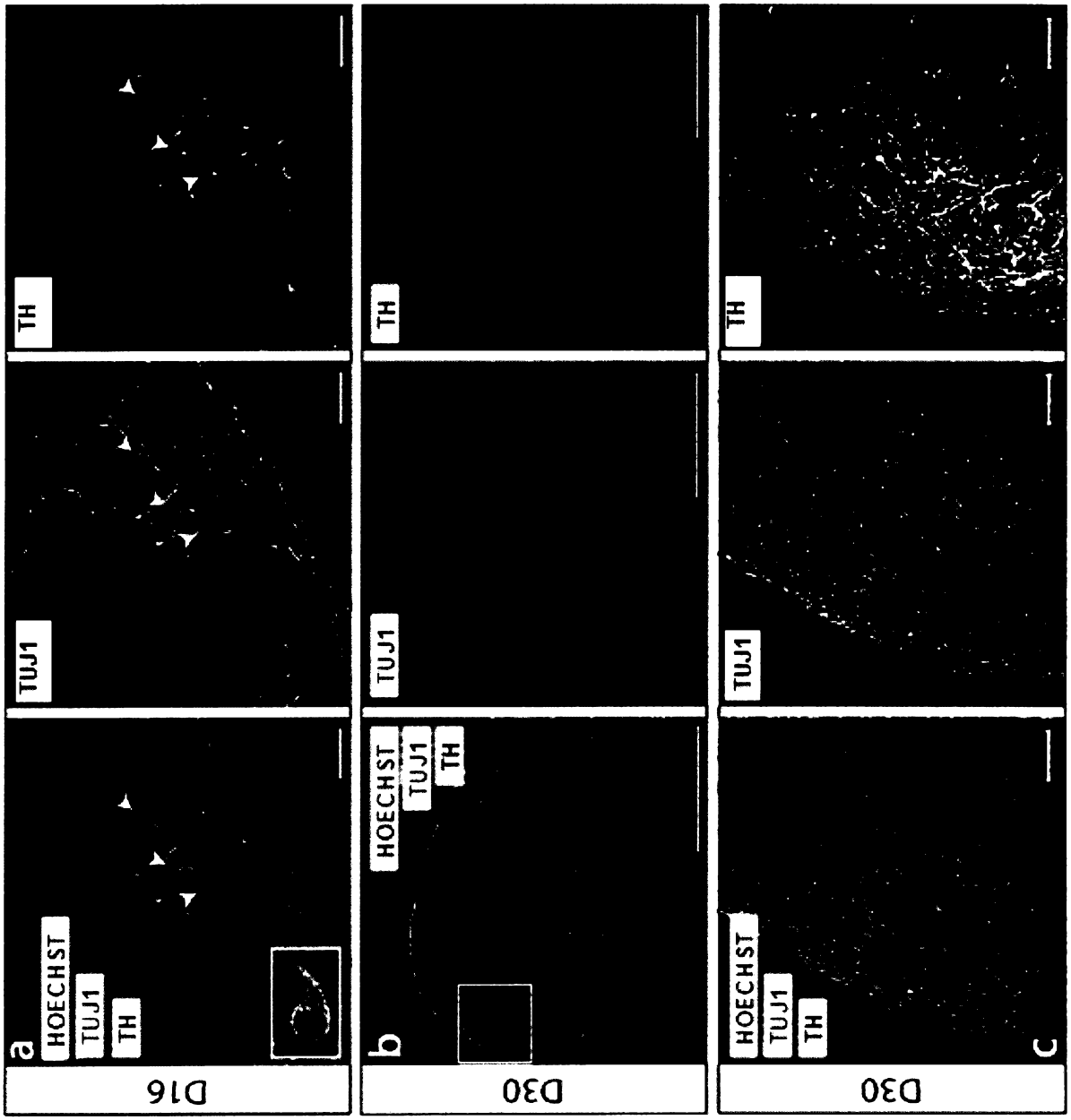

a
HOECHST
TUJ1
TH
TUJ1
TH
D16
b
HOECHST
TUJ1
TH
TUJ1
TH
D30
c
HOECHST
TUJ1
TH
TUJ1
TH
D30

Figure 7A

92845

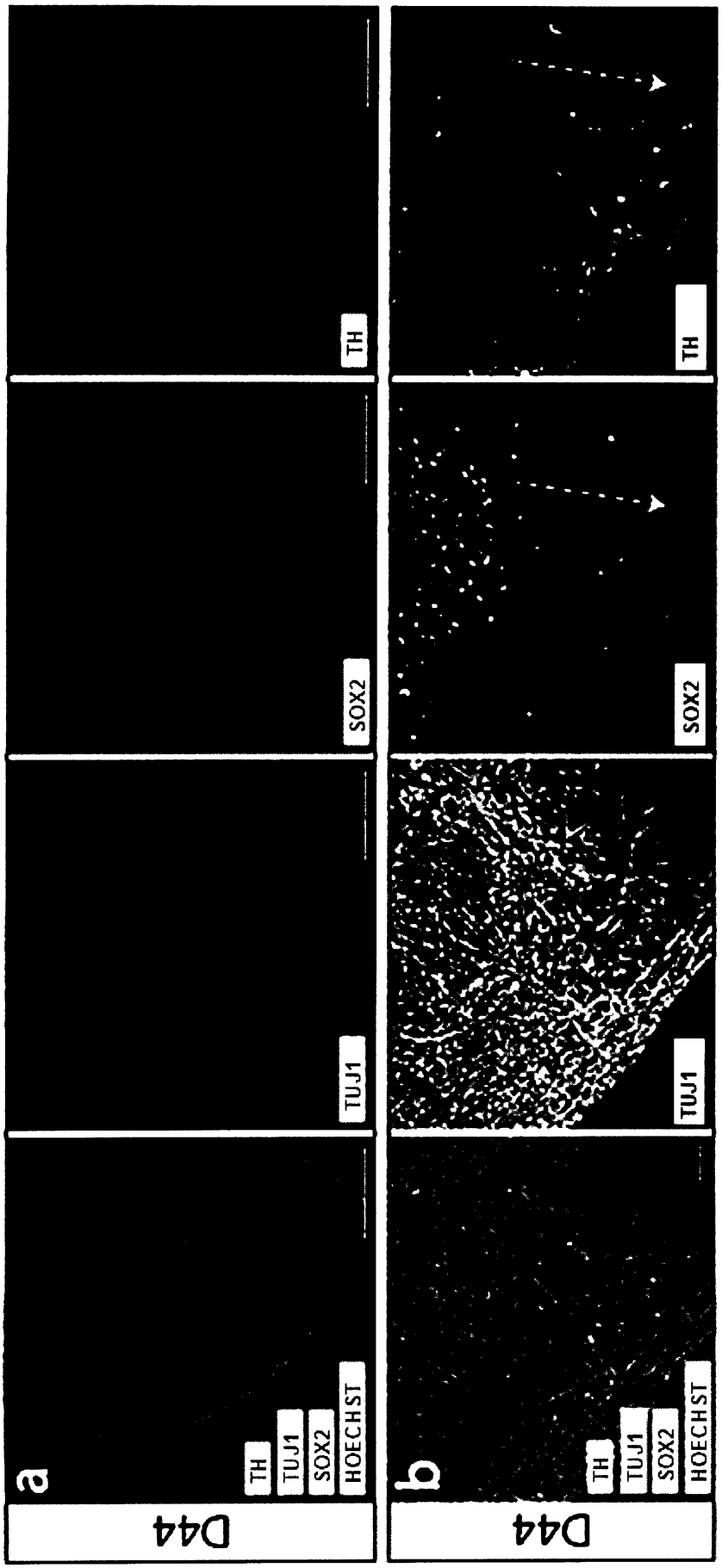


Figure 7B

92845

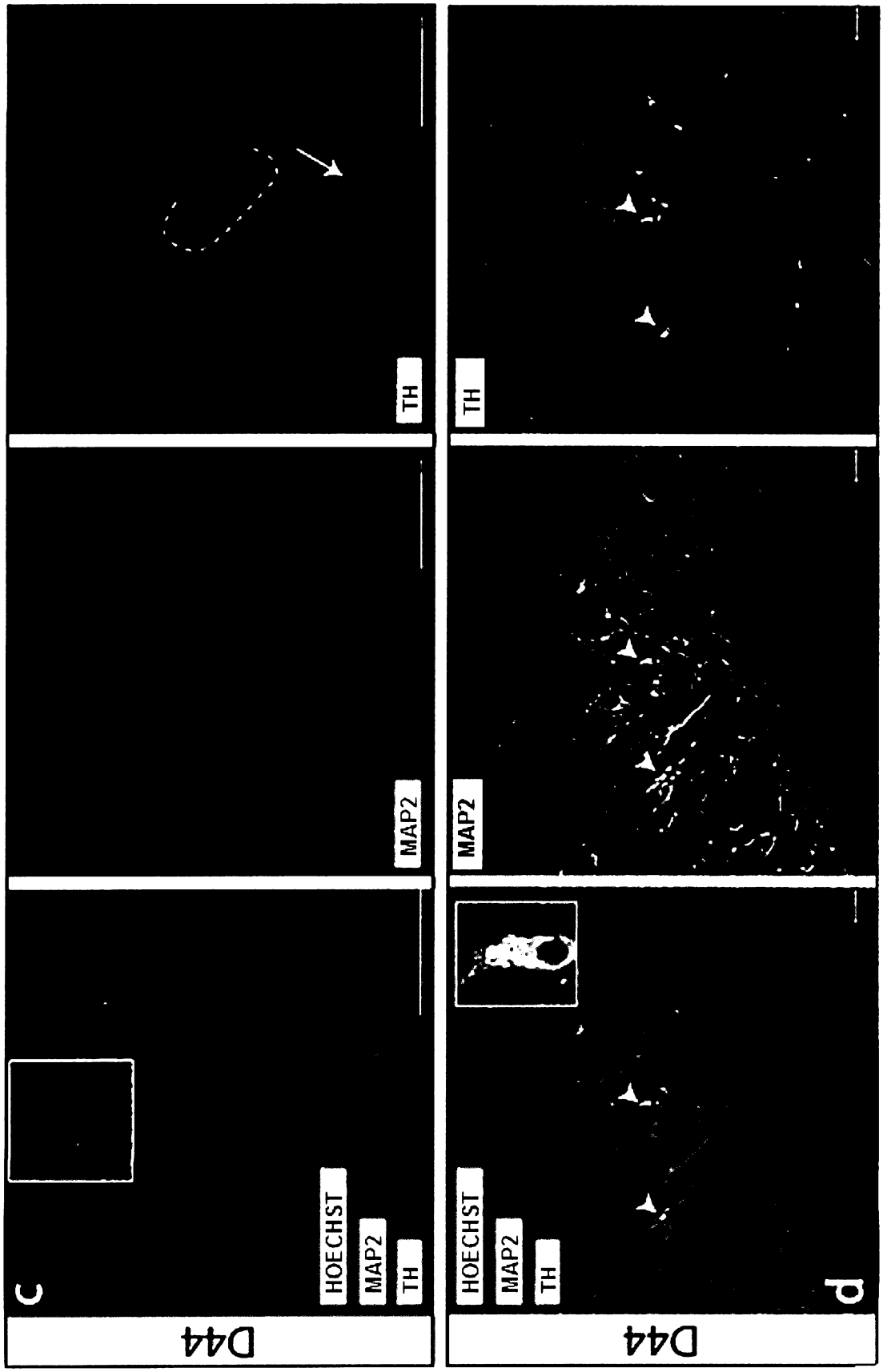


Figure 8A

92845

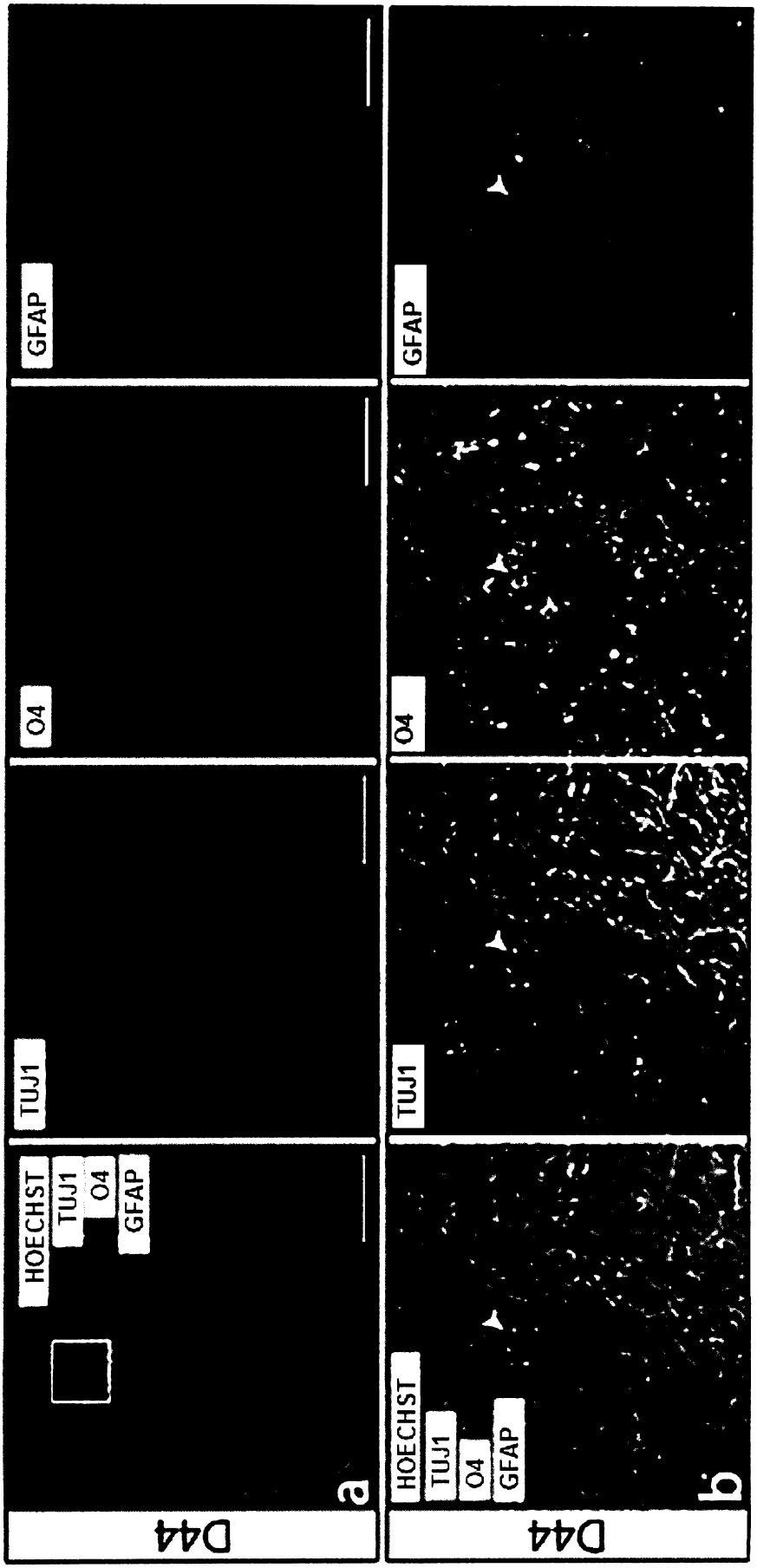
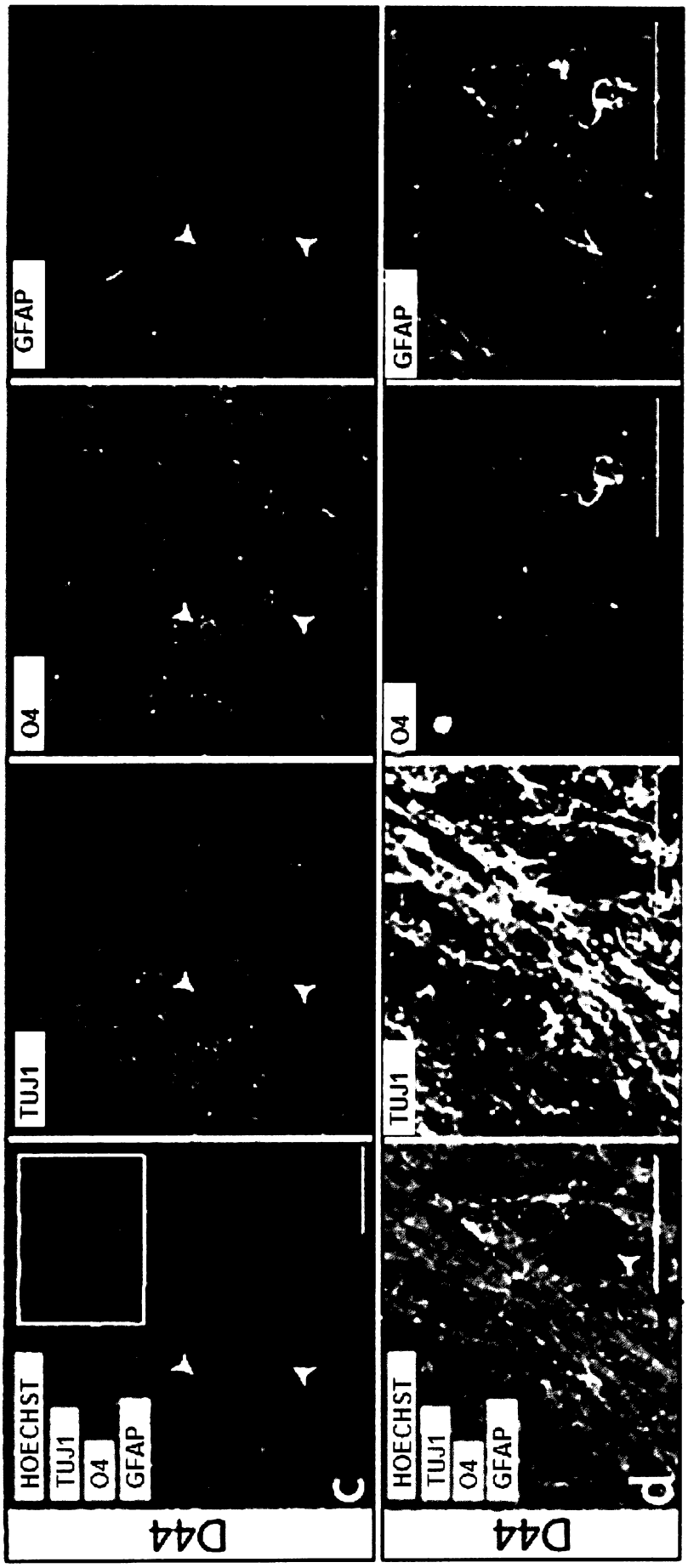


Figure 8B


D44
HOECHST
TUJ1
O4
GFAP
c
TUJ1
O4
GFAP
D44
HOECHST
TUJ1
O4
GFAP
d

Abstract

The present invention provides means and methods for the generation of midbrain organoids which are useful for studying neurodevelopmental and neurodegenerative diseases. Neuroepithelial stem cells serve as a starting population for the generation of midbrain organoids by contacting them with differentiation medium under agitating conditions in three-dimensional cell culture comprising a matrix.

LE GOUVERNEMENT
DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère de l'Économie
et du Commerce extérieur

National Application Number

SEARCH REPORT

in accordance with Article 35.1 a)
of the Luxembourg law on patents
dated 20 July 1992

LO 1209
LU 92845

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
Y,D	MADELINE A LANCASTER ET AL: "Generation of cerebral organoids from human pluripotent stem cells", NATURE PROTOCOLS, vol. 9, no. 10, 4 September 2014 (2014-09-04), pages 2329-2340, XP055234395, GB ISSN: 1754-2189, DOI: 10.1038/nprot.2014.158 * the whole document *	1-53	INV. C12N5/0797 C12N5/0793 C12N5/079 ADD. G01N33/50
Y,D	MADELINE A. LANCASTER ET AL: "Cerebral organoids model human brain development and microcephaly", NATURE, vol. 501, no. 7467, 28 August 2013 (2013-08-28), pages 373-379, XP055166627, ISSN: 0028-0836, DOI: 10.1038/nature12517	1-25,43, 51-53	
A	* page 374; figure 1 * * page 377 - page 378 *	26-42, 44-50	TECHNICAL FIELDS SEARCHED (IPC) C12N G01N
Y	EP 2 614 829 A1 (MAX PLANCK GESELLSCHAFT [DE]) 17 July 2013 (2013-07-17) * column 42 - column 43; example 1 * * column 51 - column 52; claims 6, 9 *	1-53	
Y	US 2015/087541 A1 (GONZALEZ RODOLFO [US] ET AL) 26 March 2015 (2015-03-26) * page 9 - page 10; examples 1-4 *	44-50	

-/--

The present search report has been drawn up for all claims

	Date of completion of the search	Examiner
	10 December 2015	Heiduschat, Carola

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

2

EPO FORM 1503 03.82 (P04C55)

LE GOUVERNEMENT
DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère de l'Économie
et du Commerce extérieur

SEARCH REPORT

in accordance with Article 35.1 a)
of the Luxembourg law on patents
dated 20 July 1992

National Application Number

LO 1209
LU 92845

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
Y,D	PETER REINHARDT ET AL: "Derivation and Expansion Using Only Small Molecules of Human Neural Progenitors for Neurodegenerative Disease Modeling", PLOS ONE, vol. 8, no. 3, 22 March 2013 (2013-03-22), page e59252, XP055234383, DOI: 10.1371/journal.pone.0059252	3	
A	* page 2 * * page 10 * * page 14 - page 15 *	1,2,4-53	

Y,D	GALE EMILY ET AL: "Midbrain dopaminergic neuron fate specification: Of mice and embryonic stem cells", MOLECULAR BRAIN, BIOMED CENTRAL LTD, LONDON UK, vol. 1, no. 1, 30 September 2008 (2008-09-30), page 8, XP021045971, ISSN: 1756-6606, DOI: 10.1186/1756-6606-1-8	4,6	
A	* page 6 - page 10 *	1-3,5, 7-53	TECHNICAL FIELDS SEARCHED (IPC)

Y	MARIUS ADER ET AL: "Modeling human development in 3D culture", CURRENT OPINION IN CELL BIOLOGY, vol. 31, 1 December 2014 (2014-12-01), pages 23-28, XP055173769, ISSN: 0955-0674, DOI: 10.1016/j.ceb.2014.06.013	5	
A	* page 24 - page 25; figure 1 *	1-4,6-53	

	-/--		

The present search report has been drawn up for all claims

	Date of completion of the search	Examiner
	10 December 2015	Heiduschat, Carola

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

2

EPO FORM 1503 03.82 (P04C55)

LE GOUVERNEMENT
DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère de l'Économie
et du Commerce extérieur

National Application Number

LO 1209
LU 92845

SEARCH REPORT

in accordance with Article 35.1 a)
of the Luxembourg law on patents
dated 20 July 1992

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
Y	NANCY STANSLOWSKY ET AL: "Functional differentiation of midbrain neurons from human cord blood-derived induced pluripotent stem cells", STEM CELL RESEARCH & THERAPY, BIOMED CENTRAL LTD, LONDON, UK, vol. 5, no. 2, 17 March 2014 (2014-03-17), page 35, XP021184185, ISSN: 1757-6512, DOI: 10.1186/SCRT423 * page 2 - page 3 *	7,8	
A	MOTOTSUGU EIRAKU ET AL: "Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals", CELL STEM CELL, ELSEVIER, CELL PRESS, AMSTERDAM, NL, vol. 3, no. 5, 6 November 2008 (2008-11-06), pages 519-532, XP008141935, ISSN: 1934-5909, DOI: 10.1016/J.STEM.2008.09.002 [retrieved on 2008-11-05] * the whole document *	1-53	TECHNICAL FIELDS SEARCHED (IPC)
A	STUART M CHAMBERS ET AL: "Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling", NATURE BIOTECHNOLOGY, vol. 27, no. 3, 1 March 2009 (2009-03-01), pages 275-280, XP055007827, ISSN: 1087-0156, DOI: 10.1038/nbt.1529 * page 278 - page 279; figure 4 *	1-53	

-/--

The present search report has been drawn up for all claims

Date of completion of the search	Examiner
10 December 2015	Heiduschat, Carola

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

2

EPO FORM 1503 03.82 (P04C55)

LE GOUVERNEMENT
DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère de l'Économie
et du Commerce extérieur

National Application Number

LO 1209
LU 92845

SEARCH REPORT

in accordance with Article 35.1 a)
of the Luxembourg law on patents
dated 20 July 1992

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A,D	M. A. LANCASTER ET AL: "Organogenesis in a dish: Modeling development and disease using organoid technologies", SCIENCE, vol. 345, no. 6194, 17 July 2014 (2014-07-17), pages 1247125-1247125, XP055130123, ISSN: 0036-8075, DOI: 10.1126/science.1247125 * the whole document *	1-53	
A	MASATO NAKAGAWA ET AL: "A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells", SCIENTIFIC REPORTS, vol. 4, 8 January 2014 (2014-01-08), XP055132358, DOI: 10.1038/srep03594 * page 6 *	1-53	
A	Taisuke Kadoshimaa ET AL: "Self-organization of axial polarity, inside-out layer pattern, and species-specific progenitor dynamics in human ES cell-derived neocortex", Proceedings of the National Academy of Sciences, 10 December 2013 (2013-12-10), pages 20284-20289, XP055234150, Retrieved from the Internet: URL:http://www.pnas.org/content/110/50/20284.full.pdf [retrieved on 2015-12-07] * the whole document *	1-53	TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

Date of completion of the search	Examiner
10 December 2015	Heiduschat, Carola

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

2

EPO FORM 1503 03.82 (P04C55)

ANNEX TO THE SEARCH REPORT ON LUXEMBOURG PATENT APPLICATION NO. LO 1209 LU 92845

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

10-12-2015

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 2614829 A1	17-07-2013	EP 2614829 A1	17-07-2013
		EP 2802332 A1	19-11-2014
		US 2015010515 A1	08-01-2015
		WO 2013104752 A1	18-07-2013
US 2015087541 A1	26-03-2015	AU 2013251749 A1	13-11-2014
		CA 2871337 A1	31-10-2013
		CN 104379732 A	25-02-2015
		EP 2841566 A1	04-03-2015
		HK 1201873 A1	11-09-2015
		JP 2015514437 A	21-05-2015
		KR 20150004834 A	13-01-2015
		US 2015087541 A1	26-03-2015
		WO 2013163228 A1	31-10-2013

LE GOUVERNEMENT
DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère de l'Économie
et du Commerce extérieur

National Application Number

SEARCH REPORT

in accordance with Article 35.1 a)
of the Luxembourg law on patents
dated 20 July 1992

LO 1209
LU 92845

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
Y,D	MADELINE A LANCASTER ET AL: "Generation of cerebral organoids from human pluripotent stem cells", NATURE PROTOCOLS, vol. 9, no. 10, 4 September 2014 (2014-09-04), pages 2329-2340, XP055234395, GB ISSN: 1754-2189, DOI: 10.1038/nprot.2014.158 * the whole document *	1-53	INV. C12N5/0797 C12N5/0793 C12N5/079 ADD. G01N33/50
Y,D	MADELINE A. LANCASTER ET AL: "Cerebral organoids model human brain development and microcephaly", NATURE, vol. 501, no. 7467, 28 August 2013 (2013-08-28), pages 373-379, XP055166627, ISSN: 0028-0836, DOI: 10.1038/nature12517	1-25,43, 51-53	
A	* page 374; figure 1 * * page 377 - page 378 *	26-42, 44-50	TECHNICAL FIELDS SEARCHED (IPC) C12N G01N
Y	EP 2 614 829 A1 (MAX PLANCK GESELLSCHAFT [DE]) 17 July 2013 (2013-07-17) * column 42 - column 43; example 1 * * column 51 - column 52; claims 6, 9 *	1-53	
Y	US 2015/087541 A1 (GONZALEZ RODOLFO [US] ET AL) 26 March 2015 (2015-03-26) * page 9 - page 10; examples 1-4 *	44-50	

-/--

The present search report has been drawn up for all claims

	Date of completion of the search	Examiner
	10 December 2015	Heiduschat, Carola

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

2

EPO FORM 1503 03.82 (P04C55)

LE GOUVERNEMENT
DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère de l'Économie
et du Commerce extérieur

SEARCH REPORT

in accordance with Article 35.1 a)
of the Luxembourg law on patents
dated 20 July 1992

National Application Number

LO 1209
LU 92845

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
Y,D	PETER REINHARDT ET AL: "Derivation and Expansion Using Only Small Molecules of Human Neural Progenitors for Neurodegenerative Disease Modeling", PLOS ONE, vol. 8, no. 3, 22 March 2013 (2013-03-22), page e59252, XP055234383, DOI: 10.1371/journal.pone.0059252	3	
A	* page 2 * * page 10 * * page 14 - page 15 *	1,2,4-53	

Y,D	GALE EMILY ET AL: "Midbrain dopaminergic neuron fate specification: Of mice and embryonic stem cells", MOLECULAR BRAIN, BIOMED CENTRAL LTD, LONDON UK, vol. 1, no. 1, 30 September 2008 (2008-09-30), page 8, XP021045971, ISSN: 1756-6606, DOI: 10.1186/1756-6606-1-8	4,6	
A	* page 6 - page 10 *	1-3,5, 7-53	TECHNICAL FIELDS SEARCHED (IPC)

Y	MARIUS ADER ET AL: "Modeling human development in 3D culture", CURRENT OPINION IN CELL BIOLOGY, vol. 31, 1 December 2014 (2014-12-01), pages 23-28, XP055173769, ISSN: 0955-0674, DOI: 10.1016/j.ceb.2014.06.013	5	
A	* page 24 - page 25; figure 1 *	1-4,6-53	

	-/--		

The present search report has been drawn up for all claims

	Date of completion of the search	Examiner
	10 December 2015	Heiduschat, Carola

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

2

EPO FORM 1503 03.82 (P04C55)

LE GOUVERNEMENT
DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère de l'Économie
et du Commerce extérieur

National Application Number

SEARCH REPORT

in accordance with Article 35.1 a)
of the Luxembourg law on patents
dated 20 July 1992

LO 1209
LU 92845

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
Y	NANCY STANSLOWSKY ET AL: "Functional differentiation of midbrain neurons from human cord blood-derived induced pluripotent stem cells", STEM CELL RESEARCH & THERAPY, BIOMED CENTRAL LTD, LONDON, UK, vol. 5, no. 2, 17 March 2014 (2014-03-17), page 35, XP021184185, ISSN: 1757-6512, DOI: 10.1186/SCRT423 * page 2 - page 3 *	7,8	
A	MOTOTSUGU EIRAKU ET AL: "Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals", CELL STEM CELL, ELSEVIER, CELL PRESS, AMSTERDAM, NL, vol. 3, no. 5, 6 November 2008 (2008-11-06), pages 519-532, XP008141935, ISSN: 1934-5909, DOI: 10.1016/J.STEM.2008.09.002 [retrieved on 2008-11-05] * the whole document *	1-53	TECHNICAL FIELDS SEARCHED (IPC)
A	STUART M CHAMBERS ET AL: "Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling", NATURE BIOTECHNOLOGY, vol. 27, no. 3, 1 March 2009 (2009-03-01), pages 275-280, XP055007827, ISSN: 1087-0156, DOI: 10.1038/nbt.1529 * page 278 - page 279; figure 4 *	1-53	

-/--

The present search report has been drawn up for all claims

Date of completion of the search	Examiner
10 December 2015	Heiduschat, Carola

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

2

EPO FORM 1503 03.82 (P04C55)

LE GOUVERNEMENT
DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère de l'Économie
et du Commerce extérieur

National Application Number

LO 1209
LU 92845

SEARCH REPORT

in accordance with Article 35.1 a)
of the Luxembourg law on patents
dated 20 July 1992

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (IPC)
A,D	M. A. LANCASTER ET AL: "Organogenesis in a dish: Modeling development and disease using organoid technologies", SCIENCE, vol. 345, no. 6194, 17 July 2014 (2014-07-17), pages 1247125-1247125, XP055130123, ISSN: 0036-8075, DOI: 10.1126/science.1247125 * the whole document *	1-53	
A	MASATO NAKAGAWA ET AL: "A novel efficient feeder-free culture system for the derivation of human induced pluripotent stem cells", SCIENTIFIC REPORTS, vol. 4, 8 January 2014 (2014-01-08), XP055132358, DOI: 10.1038/srep03594 * page 6 *	1-53	
A	Taisuke Kadoshimaa ET AL: "Self-organization of axial polarity, inside-out layer pattern, and species-specific progenitor dynamics in human ES cell-derived neocortex", Proceedings of the National Academy of Sciences, 10 December 2013 (2013-12-10), pages 20284-20289, XP055234150, Retrieved from the Internet: URL:http://www.pnas.org/content/110/50/20284.full.pdf [retrieved on 2015-12-07] * the whole document *	1-53	TECHNICAL FIELDS SEARCHED (IPC)

The present search report has been drawn up for all claims

	Date of completion of the search	Examiner
	10 December 2015	Heiduschat, Carola

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

2

EPO FORM 1503 03.82 (P04C55)

ANNEX TO THE SEARCH REPORT ON LUXEMBOURG PATENT APPLICATION NO. LO 1209 LU 92845

This annex lists the patent family members relating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

10-12-2015

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 2614829 A1	17-07-2013	EP 2614829 A1	17-07-2013
		EP 2802332 A1	19-11-2014
		US 2015010515 A1	08-01-2015
		WO 2013104752 A1	18-07-2013
US 2015087541 A1	26-03-2015	AU 2013251749 A1	13-11-2014
		CA 2871337 A1	31-10-2013
		CN 104379732 A	25-02-2015
		EP 2841566 A1	04-03-2015
		HK 1201873 A1	11-09-2015
		JP 2015514437 A	21-05-2015
		KR 20150004834 A	13-01-2015
		US 2015087541 A1	26-03-2015
		WO 2013163228 A1	31-10-2013

LE GOUVERNEMENT
DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère de l'Économie
et du Commerce extérieur

WRITTEN OPINION

File No.	Filing date *(day/month/year)*	Priority date *(day/month/year)*	Application No.
LO1209	08.10.2015		LU92845

International Patent Classification (IPC)
INV. C12N5/0797 C12N5/0793 C12N5/079 ADD. G01N33/50

Applicant
Université du Luxembourg

This report contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the application
- ☒ Box No. VIII Certain observations on the application

	Examiner
Form LU237A (Cover Sheet) (January 2007)	Heiduschat, Carola

LE GOUVERNEMENT
DU GRAND-DUCHÉ DE LUXEMBOURG
Ministère de l'Économie
et du Commerce extérieur

WRITTEN OPINION

File No.	Filing date *(day/month/year)*	Priority date *(day/month/year)*	Application No.
LO1209	08.10.2015		LU92845

International Patent Classification (IPC)
INV. C12N5/0797 C12N5/0793 C12N5/079 ADD. G01N33/50

Applicant
Université du Luxembourg

This report contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the application
- ☒ Box No. VIII Certain observations on the application

	Examiner
Form LU237A (Cover Sheet) (January 2007)	Heiduschat, Carola

Application No.
LU92845

WRITTEN OPINION

Box No. I Basis of the opinion

1. This opinion has been established on the basis of the latest set of claims filed before the start of the search.

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

   ☐ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☐ on paper

   ☐ in electronic form

   c. time of filing/furnishing:

   ☐ contained in the application as filed.

   ☐ filed together with the application in electronic form.

   ☐ furnished subsequently.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Box No. V Reasoned statement with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty	Yes:	Claims	1-53
	No:	Claims	
Inventive step	Yes:	Claims	
	No:	Claims	1-53
Industrial applicability	Yes:	Claims	1-53
	No:	Claims	

2. Citations and explanations

see separate sheet

WRITTEN OPINION

Box No. VIII Certain observations on the application

see separate sheet

Application No.
LU92845

WRITTEN OPINION

Box No. I Basis of the opinion

1. This opinion has been established on the basis of the latest set of claims filed before the start of the search.

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

   ☐ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☐ on paper

   ☐ in electronic form

   c. time of filing/furnishing:

   ☐ contained in the application as filed.

   ☐ filed together with the application in electronic form.

   ☐ furnished subsequently.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

Box No. V Reasoned statement with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty	Yes:	Claims	1-53
	No:	Claims	
Inventive step	Yes:	Claims	
	No:	Claims	1-53
Industrial applicability	Yes:	Claims	1-53
	No:	Claims	

2. Citations and explanations

see separate sheet

Application No.

WRITTEN OPINION

LU92845

Box No. VIII Certain observations on the application

see separate sheet

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1 **Prior art**

Reference is made to the following documents:

D1 MADELINE A LANCASTER ET AL: "Generation of cerebral organoids from human pluripotent stem cells",
NATURE PROTOCOLS,
vol. 9, no. 10, 4 September 2014 (2014-09-04), pages 2329-2340, XP055234395,
GB
ISSN: 1754-2189, DOI: 10.1038/nprot.2014.158

D2 MADELINE A. LANCASTER ET AL: "Cerebral organoids model human brain development and microcephaly",
NATURE,
vol. 501, no. 7467, 28 August 2013 (2013-08-28), pages 373-379, XP055166627,
ISSN: 0028-0836, DOI: 10.1038/nature12517

D3 EP 2 614 829 A1 (MAX PLANCK GESELLSCHAFT [DE]) 17 July 2013 (2013-07-17)

D4 US 2015/087541 A1 (GONZALEZ RODOLFO [US] ET AL) 26 March 2015 (2015-03-26)

D5 PETER REINHARDT ET AL: "Derivation and Expansion Using Only Small Molecules of Human Neural Progenitors for Neurodegenerative Disease Modeling",
PLOS ONE,
vol. 8, no. 3, 22 March 2013 (2013-03-22), page e59252, XP055234383,
DOI: 10.1371/journal.pone.0059252

D6 GALE EMILY ET AL: "Midbrain dopaminergic neuron fate specification: Of mice and embryonic stem cells",
MOLECULAR BRAIN, BIOMED CENTRAL LTD, LONDON UK,
vol. 1, no. 1, 30 September 2008 (2008-09-30), page 8, XP021045971,
ISSN: 1756-6606, DOI: 10.1186/1756-6606-1-8

D7 MARIUS ADER ET AL: "Modeling human development in 3D culture", CURRENT OPINION IN CELL BIOLOGY,
vol. 31, 1 December 2014 (2014-12-01), pages 23-28, XP055173769,
ISSN: 0955-0674, DOI: 10.1016/j.ceb.2014.06.013

D8 NANCY STANSLOWSKY ET AL: "Functional differentiation of midbrain neurons from human cord blood-derived induced pluripotent stem cells", STEM CELL RESEARCH & THERAPY, BIOMED CENTRAL LTD, LONDON, UK,
vol. 5, no. 2, 17 March 2014 (2014-03-17), page 35, XP021184185,
ISSN: 1757-6512, DOI: 10.1186/SCRT423

D9 MOTOTSUGU EIRAKU ET AL: "Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals",
CELL STEM CELL, ELSEVIER, CELL PRESS, AMSTERDAM, NL,
vol. 3, no. 5, 6 November 2008 (2008-11-06), pages 519-532, XP008141935,
ISSN: 1934-5909, DOI: 10.1016/J.STEM.2008.09.002
[retrieved on 2008-11-05]

D10 STUART M CHAMBERS ET AL: "Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling",
NATURE BIOTECHNOLOGY,
vol. 27, no. 3, 1 March 2009 (2009-03-01), pages 275-280, XP055007827,
ISSN: 1087-0156, DOI: 10.1038/nbt.1529

D11 M. A. LANCASTER ET AL: "Organogenesis in a dish: Modeling development and disease using organoid technologies",
SCIENCE,
vol. 345, no. 6194, 17 July 2014 (2014-07-17), pages 1247125-1247125, XP055130123,
ISSN: 0036-8075, DOI: 10.1126/science.1247125

2 **Novelty**

The subject-matter of claims 1 to 53 is considered novel over the prior art.

3 **Lack of Inventive Step**

The present application does not meet the criteria of patentability, because the subject-matter of claims 1 to 53 does not involve an inventive step.

3.1 Claim 1 is directed to a method of generating a midbrain organoid, comprising contacting neuroepithelial stem cells, which are cultured in a three-dimensional cell culture comprising a matrix, with differentiation medium, wherein the culturing is performed under agitating conditions, thereby obtaining a midbrain organoid.

3.1.1 Methods for generating cerebral organoids are well known in the art (D1, D2. D11). D7 and D9 describe in addition the generation of cortical organoids.

3.1.2 Either D1 or D2 may be considered as the closest prior art. both disclose a method of generating cerebral organoids. Thus, the subject-matter of claim 1 differs in the more specified organ type of midbrain.

In the light of the prior art the technical problem to be solved can be seen as the provision of a method of generating a midbrain organoid.

The solution of this problem provided by the Applicant is the modification of the methods known in the art , e.g. D1, by using a differentiation medium suitable to generate midbrain structures.

This feature solves the problem of specific differentiation. However, the technical features of such a differentiation medium is only defined in claims 12 to 21.

Therefore, the above mentioned technical problem is only stated in claim 1 but not actually solved. Claim 1 therefore lacks essential features (see also Item VIII).

3.1.3 D3 discloses a method of generating midbrain neural cells such as dopaminergic neurons. A differentiation medium comprising the necessary factors is used.

Even when considering the technical feature of the differentiation medium, the subject-matter of claim 1 cannot be considered inventive over D1 or D2 disclosing the generation of cerebral organoids and D3 disclosing a method for the differentiation of midbrain cells, such as dopaminergic neurons (DA). Same applies to the organoids obtained by such a method, a composition comprising it (claims 43 and 51) and to the use of neuroepithelial cells for generating a midbrain organoid unit (claim 52).

3.1.4 Dependent claims 2 to 11 define the neuroepithelial stem cells (human, hNESC-K7, smNPC, genetically engineered, patient derived, from iPSCs, from UBC) and the 3D culture (type of conditions, type of matrix). These are routine design options known from the prior art (D1 to D3, D5 or D8), which do not provide any inventive step.

3.2 Further dependent claims additionally define the media used in the method:

3.2.1 Claims 12 to 19 are directed to a method of any of 1-11 wherein differentiation media I and II are defined by comprising

I: (i) a SHH-pathway activator

(ii) at least two different neurotrophins; and

(iii) an antioxidant

II: (i) at least two different neurotrophins; and

(ii) an antioxidant

3.2.2 Claims 20 and 21 define an additional step of contacting the cells to a maintenance medium before differentiation, comprising

(i) a SHH-pathway activator

(ii) a canonical WNT signalling activator; and

(iii) an antioxidant

The subject-matter of claims 12 to 21 is considered obvious over D1 combined with D3.

3.3 Claim 53 is directed to a method of claims 1 to 42, wherein the neuroepithelial stem cells are obtained by cultivation of iPSCs in a medium comprising steps

(b)

(i) an inhibitor of the activin/TGF-β signalling pathway;

(ii) a canonical WNT signalling activator;

(ii) a BMP signalling inhibitor ;

(iii) and a SHH-pathway activator

(c) culturing the cells obtained in step (b) in the same medium and

(d) further cultivating the cells obtained in step (c) in a medium comprising

(i) a canonical WNT signalling activator;

(ii) a SHH-pathway activator, and

(iii) an antioxidant

It is well known in the art which factors influence the differentiation to the neural lineage and to midbrain structures. Factors influencing anterior-posterior and dorso-ventral identity are retinoic acid, FGF-8 and SHH (D10).

Methods aiming at differentiating cells into cells of midbrain, such as dopaminergic neurons, use first FGF-8 and SHH and subsequently BDNF, GDNF, ascorbic acid, TGFbeta3 and cAMP (D10).

D3 describes a method using a medium comprising FGF8, a Hedgehog activator (PMA) and an antioxidant (Ascorbic acid (AA)) followed by a medium comprising at least two neurotrophins (BDNF, GDNF), an antioxidant (AA); TGFb3, cAMP and the for first two days also PMA (subsequently without PMA; see Example 1, paragraphs [0199], [0200] and [0202]). Thus, withdrawal of SHH during differentiation as refected in claim 12 was known.

Thus, D3 describes a method including th e cited sequence of media also including an inhibitor of the activin/TGF-β signalling pathway (SB321542) and a BMP signalling inhibitor (dorsomorphin).

Therefore, the subject-matter of claims 1, 2, 12, 20 and 53 cannot be considered inventive over D1 or D2 combined with D3.

Same applies to dependent claims 14 to 19 and 21 to 25.

3.4 Claims 26 to 32 define the method further by the agitation and the time of incubation. In the prior art D1 the agitation (85 rpm) starts 4 days after the addition of the differentiation medium. After 12 to 20 days of culture expanding neuroepithelium dominates while occasional neurons are visible. Neuronal differentiation is pronounced after approximately 4 weeks of culture. Thus, the features and ranges cited in said claims appear obvious over the prior art and do not add to inventive step.

3.5 Claims 33 to 35 further define the cell types to be present in the obtained organoids. Only claim 36 defines markers for these cell types, which are, however, known in the art and to be expected in this context.

It appears obvious that the organoid comprises cells several types of neural cells (as expected in D1) in several developmental stages.

Claims 37 to 42 further define the polarity within the organoids to be obtained. Such structure also appears obvious from the prior art (structure in cerebral organoids, D1) and seems to be an implicit feature resulting from the applied method.

3.6 Claim 44 is directed to a method of testing cellular response, in particular dopaminergic differentiation (claim 45-50).

D4 discloses a screening assay in order to identify compounds influencing the differentiation of neuralized stem cells (after treatment with FGF8 and PMA) into dopaminergic neurons. The effect on the cells is measured by analysing neurite extensions, DA maker expression (e.g. TH).

The technical problem to be solved may thus be considered as the provision of an alternative method of screening for compounds affecting differentiation of dopaminergic neurons.

Cerebral organoids were already known for disease modeling and developmental studies (D1). It appears obvious that midbrain organoids may also be useful in the screening of compounds involved in development and differentiation of DA neurons.

Thus, the subject-matter of Claim 44 cannot be considered inventive over D4 with D1. Same applies to dependent claims 45 to 50.

4 **Industrial Applicability**

The subject-matter of claims 1 to 53 is industrially applicable.

5 **Re Item VIII**

Certain observations on the application

5.1 Claims 33 to 42 attempts to define the subject-matter in terms of the result to be achieved (obtaining an organoid with a certain cellular structure), which merely amounts to a statement of the underlying problem, without providing the technical features necessary for achieving this result.

5.2 The term "hNESC-K7" used in claim 3 is vague and unclear and leaves the reader in doubt as to the meaning of the technical feature to which it refers, thereby rendering the definition of the subject-matter of said claim unclear.

5.3 It is clear from the description on pages 27 to 35 that features defining the differentiation medium as cited in claims 12 to 19 are essential to the definition of the invention.

Since independent claim 1 does not contain this feature it does not meet the requirement of clarity that any independent claim must contain all the technical features essential to the definition of the invention.

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1 **Prior art**

Reference is made to the following documents:

D1 MADELINE A LANCASTER ET AL: "Generation of cerebral organoids from human pluripotent stem cells",
NATURE PROTOCOLS,
vol. 9, no. 10, 4 September 2014 (2014-09-04), pages 2329-2340, XP055234395,
GB
ISSN: 1754-2189, DOI: 10.1038/nprot.2014.158

D2 MADELINE A. LANCASTER ET AL: "Cerebral organoids model human brain development and microcephaly",
NATURE,
vol. 501, no. 7467, 28 August 2013 (2013-08-28), pages 373-379, XP055166627,
ISSN: 0028-0836, DOI: 10.1038/nature12517

D3 EP 2 614 829 A1 (MAX PLANCK GESELLSCHAFT [DE]) 17 July 2013 (2013-07-17)

D4 US 2015/087541 A1 (GONZALEZ RODOLFO [US] ET AL) 26 March 2015 (2015-03-26)

D5 PETER REINHARDT ET AL: "Derivation and Expansion Using Only Small Molecules of Human Neural Progenitors for Neurodegenerative Disease Modeling",
PLOS ONE,
vol. 8, no. 3, 22 March 2013 (2013-03-22), page e59252, XP055234383,
DOI: 10.1371/journal.pone.0059252

D6 GALE EMILY ET AL: "Midbrain dopaminergic neuron fate specification: Of mice and embryonic stem cells",
MOLECULAR BRAIN, BIOMED CENTRAL LTD, LONDON UK,
vol. 1, no. 1, 30 September 2008 (2008-09-30), page 8, XP021045971,
ISSN: 1756-6606, DOI: 10.1186/1756-6606-1-8

D7 MARIUS ADER ET AL: "Modeling human development in 3D culture", CURRENT OPINION IN CELL BIOLOGY,
vol. 31, 1 December 2014 (2014-12-01), pages 23-28, XP055173769,
ISSN: 0955-0674, DOI: 10.1016/j.ceb.2014.06.013

D8 NANCY STANSLOWSKY ET AL: "Functional differentiation of midbrain neurons from human cord blood-derived induced pluripotent stem cells", STEM CELL RESEARCH & THERAPY, BIOMED CENTRAL LTD, LONDON, UK,
vol. 5, no. 2, 17 March 2014 (2014-03-17), page 35, XP021184185,
ISSN: 1757-6512, DOI: 10.1186/SCRT423

D9 MOTOTSUGU EIRAKU ET AL: "Self-organized formation of polarized cortical tissues from ESCs and its active manipulation by extrinsic signals",
CELL STEM CELL, ELSEVIER, CELL PRESS, AMSTERDAM, NL,
vol. 3, no. 5, 6 November 2008 (2008-11-06), pages 519-532, XP008141935,
ISSN: 1934-5909, DOI: 10.1016/J.STEM.2008.09.002
[retrieved on 2008-11-05]

D10 STUART M CHAMBERS ET AL: "Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling",
NATURE BIOTECHNOLOGY,
vol. 27, no. 3, 1 March 2009 (2009-03-01), pages 275-280, XP055007827,
ISSN: 1087-0156, DOI: 10.1038/nbt.1529

D11 M. A. LANCASTER ET AL: "Organogenesis in a dish: Modeling development and disease using organoid technologies",
SCIENCE,
vol. 345, no. 6194, 17 July 2014 (2014-07-17), pages 1247125-1247125, XP055130123,
ISSN: 0036-8075, DOI: 10.1126/science.1247125

2 **Novelty**

The subject-matter of claims 1 to 53 is considered novel over the prior art.

3 **Lack of Inventive Step**

The present application does not meet the criteria of patentability, because the subject-matter of claims 1 to 53 does not involve an inventive step.

3.1 Claim 1 is directed to a method of generating a midbrain organoid, comprising contacting neuroepithelial stem cells, which are cultured in a three-dimensional cell culture comprising a matrix, with differentiation medium, wherein the culturing is performed under agitating conditions, thereby obtaining a midbrain organoid.

3.1.1 Methods for generating cerebral organoids are well known in the art (D1, D2. D11). D7 and D9 describe in addition the generation of cortical organoids.

3.1.2 Either D1 or D2 may be considered as the closest prior art. both disclose a method of generating cerebral organoids. Thus, the subject-matter of claim 1 differs in the more specified organ type of midbrain.

In the light of the prior art the technical problem to be solved can be seen as the provision of a method of generating a midbrain organoid.

The solution of this problem provided by the Applicant is the modification of the methods known in the art , e.g. D1, by using a differentiation medium suitable to generate midbrain structures.

This feature solves the problem of specific differentiation. However, the technical features of such a differentiation medium is only defined in claims 12 to 21.

Therefore, the above mentioned technical problem is only stated in claim 1 but not actually solved. Claim 1 therefore lacks essential features (see also Item VIII).

3.1.3 D3 discloses a method of generating midbrain neural cells such as dopaminergic neurons. A differentiation medium comprising the necessary factors is used.

Even when considering the technical feature of the differentiation medium, the subject-matter of claim 1 cannot be considered inventive over D1 or D2 disclosing the generation of cerebral organoids and D3 disclosing a method for the differentiation of midbrain cells, such as dopaminergic neurons (DA). Same applies to the organoids obtained by such a method, a composition comprising it (claims 43 and 51) and to the use of neuroepithelial cells for generating a midbrain organoid unit (claim 52).

3.1.4 Dependent claims 2 to 11 define the neuroepithelial stem cells (human, hNESC-K7, smNPC, genetically engineered, patient derived, from iPSCs, from UBC) and the 3D culture (type of conditions, type of matrix). These are routine design options known from the prior art (D1 to D3, D5 or D8), which do not provide any inventive step.

3.2 Further dependent claims additionally define the media used in the method:

3.2.1 Claims 12 to 19 are directed to a method of any of 1-11 wherein differentiation media I and II are defined by comprising

I: (i) a SHH-pathway activator

(ii) at least two different neurotrophins; and

(iii) an antioxidant

II: (i) at least two different neurotrophins; and

(ii) an antioxidant

3.2.2 Claims 20 and 21 define an additional step of contacting the cells to a maintenance medium before differentiation, comprising

(i) a SHH-pathway activator

(ii) a canonical WNT signalling activator; and

(iii) an antioxidant

The subject-matter of claims 12 to 21 is considered obvious over D1 combined with D3.

3.3 Claim 53 is directed to a method of claims 1 to 42, wherein the neuroepithelial stem cells are obtained by cultivation of iPSCs in a medium comprising steps

(b)

(i) an inhibitor of the activin/TGF-β signalling pathway;

(ii) a canonical WNT signalling activator;

(ii) a BMP signalling inhibitor ;

(iii) and a SHH-pathway activator

(c) culturing the cells obtained in step (b) in the same medium and

(d) further cultivating the cells obtained in step (c) in a medium comprising

(i) a canonical WNT signalling activator;

(ii) a SHH-pathway activator, and

(iii) an antioxidant

It is well known in the art which factors influence the differentiation to the neural lineage and to midbrain structures. Factors influencing anterior-posterior and dorso-ventral identity are retinoic acid, FGF-8 and SHH (D10).

Methods aiming at differentiating cells into cells of midbrain, such as dopaminergic neurons, use first FGF-8 and SHH and subsequently BDNF, GDNF, ascorbic acid, TGFbeta3 and cAMP (D10).

D3 describes a method using a medium comprising FGF8, a Hedgehog activator (PMA) and an antioxidant (Ascorbic acid (AA)) followed by a medium comprising at least two neurotrophins (BDNF, GDNF), an antioxidant (AA); TGFb3, cAMP and the for first two days also PMA (subsequently without PMA; see Example 1, paragraphs [0199], [0200] and [0202]). Thus, withdrawal of SHH during differentiation as refected in claim 12 was known.

Thus, D3 describes a method including th e cited sequence of media also including an inhibitor of the activin/TGF-β signalling pathway (SB321542) and a BMP signalling inhibitor (dorsomorphin).

Therefore, the subject-matter of claims 1, 2, 12, 20 and 53 cannot be considered inventive over D1 or D2 combined with D3.

Same applies to dependent claims 14 to 19 and 21 to 25.

3.4 Claims 26 to 32 define the method further by the agitation and the time of incubation. In the prior art D1 the agitation (85 rpm) starts 4 days after the addition of the differentiation medium. After 12 to 20 days of culture expanding neuroepithelium dominates while occasional neurons are visible. Neuronal differentiation is pronounced after approximately 4 weeks of culture. Thus, the features and ranges cited in said claims appear obvious over the prior art and do not add to inventive step.

3.5 Claims 33 to 35 further define the cell types to be present in the obtained organoids. Only claim 36 defines markers for these cell types, which are, however, known in the art and to be expected in this context.

It appears obvious that the organoid comprises cells several types of neural cells (as expected in D1) in several developmental stages.

Claims 37 to 42 further define the polarity within the organoids to be obtained. Such structure also appears obvious from the prior art (structure in cerebral organoids, D1) and seems to be an implicit feature resulting from the applied method.

3.6 Claim 44 is directed to a method of testing cellular response, in particular dopaminergic differentiation (claim 45-50).

D4 discloses a screening assay in order to identify compounds influencing the differentiation of neuralized stem cells (after treatment with FGF8 and PMA) into dopaminergic neurons. The effect on the cells is measured by analysing neurite extensions, DA maker expression (e.g. TH).

The technical problem to be solved may thus be considered as the provision of an alternative method of screening for compounds affecting differentiation of dopaminergic neurons.

Cerebral organoids were already known for disease modeling and developmental studies (D1). It appears obvious that midbrain organoids may also be useful in the screening of compounds involved in development and differentiation of DA neurons.

Thus, the subject-matter of Claim 44 cannot be considered inventive over D4 with D1. Same applies to dependent claims 45 to 50.

4 **Industrial Applicability**

The subject-matter of claims 1 to 53 is industrially applicable.

5 **Re Item VIII**

Certain observations on the application

5.1 Claims 33 to 42 attempts to define the subject-matter in terms of the result to be achieved (obtaining an organoid with a certain cellular structure), which merely amounts to a statement of the underlying problem, without providing the technical features necessary for achieving this result.

5.2 The term "hNESC-K7" used in claim 3 is vague and unclear and leaves the reader in doubt as to the meaning of the technical feature to which it refers, thereby rendering the definition of the subject-matter of said claim unclear.

5.3 It is clear from the description on pages 27 to 35 that features defining the differentiation medium as cited in claims 12 to 19 are essential to the definition of the invention.

Since independent claim 1 does not contain this feature it does not meet the requirement of clarity that any independent claim must contain all the technical features essential to the definition of the invention.