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(71) Applicant: **TRAILHEAD BIOSYSTEMS INC.** [US/US]; 10000 Cedar Avenue, Cleveland, OH 44106 (US).

(72) Inventor: **AMINI, Nooshin**; 1600 Euclid Ave. Apt 1608, Cleveland, OH 44115 (US).

(74) Agent: **REMILLARD, Jane, E.** et al.; Nelson Mullins Riley & Scarborough LLP, One Financial Center, Suite 3500, Boston, MA 02111 (US).

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(54) Title: METHODS AND COMPOSITIONS FOR GENERATING OLIGODENDROCYTE PROGENITOR CELLS

(57) Abstract: Methods for generating pre-oligodendrocyte progenitor cells (pre-OPCs) and oligodendrocyte progenitor cells (OPCs) from human pluripotent stem cells are provided using chemically- defined culture media that allow for generation of pre-OPCs and OPCs in as little as three days. Culture media, isolated cell populations and kits are also provided.

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METHODS AND COMPOSITIONS FOR GENERATING OLIGODENDROCYTE PROGENITOR CELLS

Related Applications

This application claims priority to U.S. Provisional Application No. 63/168,065, filed March 30, 2021, the entire contents of which is hereby incorporated by reference.

Government Licensed Rights

This invention was made with government support under Grant Number: W911NF-17-3-0003 awarded by the U.S. ARMY ACC-AGP-RTP. The government has certain rights in the invention.

Background of the Invention

Oligodendrocytes (OLs) are a type of glial cells that synthesize the myelin sheath around axons. Thus, they are critical for nerve conduction in the central nervous system (CNS). A greater understanding of oligodendrocyte biology is likely to be very important in the development of therapies for the treatment of neurodegenerative disorders, including demyelinating diseases, such as multiple sclerosis and leukodystrophies, as well as amyotrophic lateral sclerosis (ALS), which can involve demyelination later in the course of the disease. Additionally, radiation therapy to the brain can be associated with the side-effect of oligodendrocyte depletion, leading to cognitive decline and/or impairment of motor coordination.

Since mature human oligodendrocytes are not readily isolatable from human subjects, human oligodendrocyte cell lines have been developed to allow study of the cells. However, immortalized cell lines may not mimic the full biology of the native cells and are not suitable for therapeutic uses. Thus, the ability to generate human oligodendrocytes *in vitro*, such as from stem cells, is highly desirable. Various protocols have been reported for differentiation of oligodendrocytes from human pluripotent stem cells. However, these protocols remain inefficient and variable in terms of oligodendrocyte yield and require very long differentiation times to generate myelin basic protein (MBP)-positive oligodendrocytes.

An early protocol used a four-step process (Hu et al. (2009) *Nature Protocols* 4:1614-1622; see also Wang et al. (2013) *Cell Stem Cell* 12:252-264). The protocol first involved induction of human embryonic stem cells (hESCs) to differentiate into neuroepithelial cells for

two weeks, forming neural-tube like rosettes, followed by a 10 day treatment with retinoic acid (RA) and sonic hedgehog (SHH), leading to OLIG2-expressing progenitors. Treatment with fibroblast growth factor (FGF2) for another 10 days led to conversion to OLIG2 and NKX2.2-expressing pre-OPCs. Finally, the pre-OPCs were cultured for an additional 8-9 weeks in the absence of FGF2 to differentiate into OPCs, expressing markers such as platelet-derived growth factor receptor alpha (PDGFRα), SOX10 and NG2. Thus, using this protocol, it required approximately 24 days to generate OLIG2-expressing progenitors and approximately 34 days to generate OLIG2 and NKX2.2-expressing pre-OPCs, with about 100 days needed to obtain mature Ols. A variant of this protocol was reported by Douvaras et al. (*Stem Cell Reports* (2014) 3:250-259), but still required about 20 days to obtain pre-OPCs and about 50 days to obtain OPCs, including culture with exogenously-added growth factors PDGF, IGF-1 and HGF.

Subsequently, alternative protocols have been reported, yet these protocols still utilized an approximately week-long neural induction and patterning phase (also referred to as neuralization), followed by induction of cells expressing pre-OPC and OPC markers using media that included exogenously-added growth factors, such as FGF2, PDGF, IGF-1 and/or HGF depending on the protocol (see e.g., Piao et al. (2015) *Cell Stem Cell* 16:198-210; Douvaras & Fossati (2015) *Nature Protocols* 10:1143-1154; Livesey et al. (2016) *Stem Cells* 34:1040-1053; and Yamashita et al. (2017) *PLOS One* 12: e0171947).

More recently, a protocol has been reported in which hESCs were first neurally induced to generate neural progenitor cells (NPCs), followed by overexpression of the SOX10 transcription factor in the NPCs (via viral transduction) and expansion in the presence of bFGF, leading to generation of MBP-positive oligodendrocytes in only about 20 days (Garcia-Leon et al. (2018) *Stem Cell Reports* 10:655-672). Furthermore, transient and partial inhibition of the SHH pathway transcription factor GLI1 in neural stem cells (generated by neuralization) by a small molecule inhibitor GANT61 was found to generate OPCs that were more migratory and could differentiate earlier toward myelin-producing oligodendrocytes (Namchaiw et al. (2019) *Stem Cell Res & Therapy* 10:272).

Accordingly, while some progress has been, there remains a need for efficient and robust methods and compositions for generating oligodendrocyte progenitor cells from human pluripotent stem cells.

Summary of the Invention

This disclosure provides methods of generating human pre-OPCs and OPCs from pluripotent stem cells using chemically-defined culture media that allows for generation of OLIG2 and NKX2.2-positive pre-OPCs and OPCs in as little as three days of culture. The culture media lacks serum or other exogenously-added growth factors and comprises small molecule agents that either agonize or antagonize particular signaling pathway activity in the pluripotent stem cells such that differentiation along the OPC lineage is promoted, leading to cellular maturation and expression of OPC-associated biomarkers. The methods of the disclosure have the advantage that they bypass the neural induction step of prior art protocols and allow for direct differentiation of pluripotent stem cells to pre-OPCs and OPCs, thereby significantly shortening the time needed to generate pre-OPCs and OPCs. Moreover, the use of small molecule agents in the culture media allows for precise control of the culture components.

Accordingly, in one aspect, the disclosure pertains to a method of generating human pre-oligodendrocyte progenitor cells (pre-OPCs) or oligodendrocyte progenitor cells (OPCs) comprising:

culturing human pluripotent stem cells in a culture media lacking exogenously-added growth factors and comprising a retinoic acid (RA) pathway agonist, an Akt pathway agonist and an mTOR pathway agonist, such that OLIG2-expressing pre-OPCs or OPCs are generated.

In one embodiment, the OLIG2-expressing pre-OPCs or OPCs are generated within 72 hours of starting culture of the human pluripotent stem cells in the culture media. In one embodiment, the pre-OPCs or OPCs also express NKX2-2.

In another embodiment, the culture media further comprises a WNT pathway antagonist. In another embodiment, the culture media further comprises an SHH pathway agonist. In another embodiment, the culture media further comprises a BMP pathway antagonist. In another embodiment, the culture media further comprises a PKC pathway antagonist. In various embodiments, the pre-OPCs or OPCs also express OTX2 and/or FEZF2.

Accordingly, in another aspect, the disclosure pertains to a method of generating human pre-oligodendrocyte progenitor cells (pre-OPCs) or oligodendrocyte progenitor cells (OPCs) comprising:

culturing human pluripotent stem cells in a culture media lacking exogenously-added growth factors and comprising a retinoic acid (RA) pathway agonist, an Akt pathway agonist, an

mTOR pathway agonist, a WNT pathway antagonist, an SHH pathway agonist, a BMP pathway antagonist and a PKC pathway antagonist such that OLIG2-expressing pre-OPCs or OPCs are generated. In other embodiments, the pre-OPCs or OPCs also express NKX2-2, OTX2 and/or FEZF2.

Non-limiting examples of suitable agonist and antagonist agents, and concentrations therefor, are described in further detail herein.

In one embodiment, the human pluripotent stem cells are induced pluripotent stem cells (iPSCs). In another embodiment, the human pluripotent stem cells are embryonic stem cells.

In on embodiment, the human pluripotent stem cells are attached to vitronectin-coated plates during culturing.

In another aspect, the disclosure pertains to a culture media for obtaining pre-oligodendrocyte progenitor cells (pre-OPCs) or oligodendrocyte progenitor cells (OPCs) comprising a retinoic acid (RA) pathway agonist, an Akt pathway agonist and an mTOR pathway agonist and lacking exogenously-added growth factors. In one embodiment, the culture media further comprises a WNT pathway antagonist In one embodiment, the culture media further comprises an SHH pathway agonist. In one embodiment, the culture media further comprises a BMP pathway antagonist. In one embodiment, the culture media further comprises a PKC pathway antagonist.

In another aspect, the disclosure pertains to an isolated cell culture of pre-oligodendrocyte progenitor cells (pre-OPCs) or oligodendrocyte progenitor cells (OPCs), the culture comprising: OLIG2-expressing pre-OPCs or OPCs cultured in a culture media comprising a retinoic acid (RA) pathway agonist, an Akt pathway agonist and an mTOR pathway agonist and lacking exogenously-added growth factors. In one embodiment, the culture media further comprises a WNT pathway antagonist In one embodiment, the culture media further comprises an SHH pathway agonist. In one embodiment, the culture media further comprises a BMP pathway antagonist. In one embodiment, the culture media further comprises a PKC pathway antagonist. In other embodiments, the pre-OPCs or OPCs also express NKX2-2, OTX2 and/or FEZF2. In one embodiment of the cell culture, the pre-OPCs or OPCs are attached to vitronectin-coated plates.

In another aspect, the disclosure pertains to a pre-oligodendrocyte progenitor cells (pre-OPCs) or oligodendrocyte progenitor cell (OPC), such as generated by a method of the

disclosure. In one embodiment, the disclosure provides a composition comprising a non-native pre-oligodendrocyte progenitor cells (pre-OPCs) or oligodendrocyte progenitor cell (OPC), wherein the pre-OPC or OPC expresses OLIG2, NKX2-2, OTX2 and FEZF2 and lacks expression of NESTIN.

In yet another aspect, the disclosure pertains to an isolated cell population of pre-oligodendrocyte progenitor cells (pre-OPCs) or oligodendrocyte progenitor cells (OPCs) comprising at least 1 x 10^6 OLIG2-expressing pre-OPCs or OPCs, wherein the cell population lacks NESTIN-expressing neural stem cells. In other embodiments, the pre-OPCs or OPCs also express NKX2-2, OTX2 and/or FEZF2. In one embodiment of the isolated cell population, the pre-OPCs or OPCs are bound with at least one antibody that binds at least one cell surface marker expressed by the pre-OPCs or OPCs.

In yet another aspect, the disclosure pertains to a method of isolating pre-oligodendrocyte progenitor cells (pre-OPCs) or oligodendrocyte progenitor cells (OPCs), the method comprising:

contacting the OLIG2-expressing pre-OPCs or OPCs generated by a method of the disclosure, with at least one binding agent that binds to a cell surface marker expressed by the OLIG2-expressing pre-OPCs or OPCs; and

isolating cells that bind to the binding agent to thereby isolate OPCs.

In one embodiment, the binding agent is an antibody.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

Brief Description of the Drawings

FIG. 1 shows results from an HD-DoE model of an 8-factor experiment optimized for maximum expression of NKX2-2. The upper section of the model shows the prediction of expression level of pre-selected 53 genes when optimized for NKX2-2. The lower section of the model shows the effectors that were tested in this model and their contribution to maximum expression of NKX2-2. The value column refers to required concentration of each effector to mimic the model.

FIG. 2 shows the results from an HD-DoE model of an 8-factor experiment optimized for maximum expression of PDGFRA. Upper and lower sections are as described for FIG. 1. This

condition highlights the effector PD0325901 with factor contribution of 30.05 as an important input for high expression of PDGFRA.

FIG. 3 shows the dynamic profile of expression levels of NKX2-2, OLIG1, OLIG2 and PDGFRA genes relative to the concentration of 8 effectors tested. The positive impact of TTNPB, MHY1485 and PD0325901 on expression of PDGFRA and their factor contribution is shown by the slope of the plots for each effector. The dotted box highlights the opposite impact of PD0325901 on NKX2-2 and OLIG2 compared to PDGFRA.

FIG. 4 shows the results from an HD-DoE model of a 13-factor experiment optimized for maximum expression of OTX2. This model introduced MK2206, PD0325901, CHIR99021, LDN193189, Go6983 and PD173074 as positive effectors on expression of OTX2.

FIG. 5 shows the results from an HD-DoE model of a 13-factor experiment optimized for maximum expression of FEZF2. This model confirmed the positive effect of LDN193189, MK2206 and PD0325901 on patterning the cells and introduced three other factors including SC79, XAV939 and Purmorphamine-500nM.

FIG. 6 shows the dynamic profile of expression level of OTX2 and FEZF2 relative to the concentration of 13 factors tested in this model. XAV939 and Purmorphamine-500nM have a significant positive impact on the expression of FEZF2 and no significant negative impact on the expression of OTX2.

FIG. 7A-D shows the dynamic profile analysis of the elimination process in an 8-factor modeling experiment and its effect on expression of NKX2-2, OLIG2 and PDGFRA.

FIG. 8A-D shows the dynamic profile analysis of the elimination process in a 13-factor modeling experiment and its effect on expression of FEZF2 and OTX2.

FIG. 9 shows photographs of images of cells cultured in the optimized OPC differentiation media after three days. Cells were stained with oligodendrocyte and neural biomarkers. Cells express anterior neuroectoderm biomarkers including OTX2 and NKX2-2 along with OPC specific biomarker OLIG2. NESTIN and PDGFRa, which are neuronal and late OPC biomarkers respectively, are not present. Expression of KI67 shows the proliferative state of precursor cells.

FIG. 10A-B shows RNA-seq data of cells cultured in optimized OPC differentiation media after three days. Expression level of stem cell genes NANOG and POU5F1 were

decreased while genes involved in early development of brain regions and oligodendrocyte lineage were elevated.

Detailed Description of the Invention

Described herein are methodologies and compositions that allow for the generation of pre-OPCs and OPCs from human pluripotent stem cells under chemically-defined culture conditions using a small molecule based approach. The methods of the disclosure have the advantage that the starting pluripotent stem cells do not go through neural induction, which many prior art protocols use. This allows for generation of pre-OPCs and OPCs in as little as three days, which is significantly shorter than current protocols, which average 10 days to generate pre-OPCs.

As described in Example 1, a High-Dimensional Design of Experiments (HD-DoE) approach was used to simultaneously test multiple process inputs (e.g., small molecule agonists or antagonists) on output responses, such as gene expression. These experiments allowed for the identification of chemically-defined culture media, comprising agonists and/or antagonists of particular signaling pathways, that is sufficient to generate pre-OPCs or OPCs in a very short amount of time. The optimized culture media was further validated by a factor criticality analysis, which examined the effects of eliminating individual agonist or antagonist agents, as described in Example 2. Immunohistochemistry further confirmed the phenotype of the cells generated by the differentiation protocol, as described in Example 3.

Various aspects of the invention are described in further detail in the following subsections.

I. Cells

The starting cells used in the cultures of the disclosure are human pluripotent stem cells. As used herein, the term "human pluripotent stem cell" (abbreviated as hPSC) refers to a human stem cell that has the capacity to differentiate into a variety of different cell types. The term "pluripotent" as used herein refers to a cell with the capacity, under different conditions, to differentiate to cell types characteristic of all three germ cell layers (endoderm, mesoderm and ectoderm). Pluripotent cells are characterized primarily by their ability to differentiate to all three germ layers, for example, using a nude mouse and teratomas formation assay. Pluripotency

can also evidenced by the expression of embryonic stem (ES) cell markers, although the preferred test for pluripotency is the demonstration of the capacity to differentiate into cells of each of the three germ layers.

Human pluripotent stem cells include, for example, induced pluripotent stem cells (iPSC) and human embryonic stem cells, such as ES cell lines. Non-limiting examples of induced pluripotent stem cells (iPSC) include 19-11-1, 19-9-7 or 6-9-9 cells (e.g, as described in Yu, J. *et al.* (2009) *Science* 324:797-801). Non-limiting examples of human embryonic stem cell lines include ES03 cells (WiCell Research Institute) and H9 cells (Thomson, J.A. *et al.* (1998) *Science* 282:1145-1147). Human pluripotent stem cells (PSCs) express cellular markers that can be used to identify cells as being PSCs. Non-limiting examples of pluripotent stem cell markers include TRA-1-60, TRA-1-81, TRA-2-54, SSEA1, SSEA3, SSEA4, CD9, CD24, OCT3, OCT4, NANOG and/or SOX2. Since the methods of generating pre-OPCs and/or OPCs of the disclosure are used to differentiate (maturate) the starting pluripotent stem cell population, in various embodiments the pre-OPC and/or OPC cell populations generated by the methods of the disclosure lack expression of one or more stem cell markers selected from the group consisting of TRA-1-60, TRA-1-81, TRA-2-54, SSEA1, SSEA3, SSEA4, CD9, CD24, OCT3, OCT4, NANOG and/or SOX2

The pluripotent stem cells are subjected to culture conditions, as described herein, that induce cellular differentiation. As used herein, the term "differentiation" refers to the development of a cell from a more primitive stage towards a more mature (i.e. less primitive) cell, typically exhibiting phenotypic features of commitment to a particular cellular lineage. An early progenitor cell that can be derived from human PSCs by neural induction (neuralization) is a neural precursor cell (NPC). As used herein, a "neural precursor cell" or "NPC" refers to a stem cell-derived progenitor cell that expresses the type VI intermediate filament protein Nestin. Since the methods of generating pre-OPCs and/or OPCs of the disclosure avoid the use of neural induction, and thus do not generate NPCs, in various embodiments the cell populations generated by the methods of the disclosure lack Nestin-positive cells.

In one embodiment, the cells generated by the methods of the disclosure are pre-oligodendrocyte progenitor cells (pre-OPCs). As used herein, a "pre-oligodendrocyte progenitor cells" or "pre-OPC" refers to a stem cell-derived progenitor cell that expresses the cellular markers OLIG2 and NKX2.2. A pre-OPC may express additional markers, including but not

limited to: OTX2 (anterior neuroectoderm biomarker), FEZF2 (anterior ectoderm biomarker), and/or OLIG1.

In one embodiment, the cells generated by the methods of the disclosure are oligodendrocyte progenitor cells (OPCs), which are more differentiated (more mature) cells than pre-OPCs. As used herein, an "oligodendrocyte progenitor cells" or "OPC" refers to a stem cell-derived progenitor cell that expresses the cellular markers OLIG2 and NKX2.2, as well as PDGFRa. An OPC may express additional markers, non-limiting example of which include SOX10 (neural crest marker), OTX2 (anterior neuroectoderm biomarker), FEZF2 (anterior ectoderm biomarker), and/or OLIG1.

The pre-OPCs and OPCs generated by the methods of the disclosure can be further cultured in vitro to generate mature oligodendrocytes (OL). Markers of mature OLs include but are not limited to myelin basic protein (MBP) and O4.

II. Culture Media Components

The method of the disclosure for generating pre-OPCs or OPCs comprise culturing human pluripotent stem cells in a culture media lacking exogenously-added growth factors and comprising specific agonist and/or antagonists of cellular signaling pathways.

As described in Example 1, a culture media comprising a retinoic acid (RA) pathway agonist, an Akt pathway agonist and an mTOR pathway agonist was sufficient to generate OLIG2 and NKX2.2-expressing pre-OPCs in as little as three days. Inclusion of additional agents optimized for expression of other markers, including PDGFRa as a marker of OPC differentiation. In other embodiments, the culture media further comprises at least one additional agent selected from the group consisting of WNT pathway antagonists, SHH pathway agonists, BMP pathway antagonists and PKC pathway antagonists. In one embodiment, the culture media further comprises a WNT pathway antagonist. In one embodiment, the culture media further comprises an SHH pathway agonist. In one embodiment, the culture media further comprises a BMP pathway antagonist. In one embodiment, the culture media further comprises a PKC pathway antagonist. In one embodiment, the culture media further comprises a WNT pathway antagonist and an SHH pathway agonist, wherein the differentiated cells express OTX2 and FEZF2, in addition to OLIG2 and NKX2.2.

In one embodiment, the culture media comprises a retinoic acid (RA) pathway agonist, an Akt pathway agonist, an mTOR pathway agonist, a WNT pathway antagonist, an SHH pathway agonist, a BMP pathway antagonist and a PKC pathway antagonist. In one embodiment, the differentiated cells are OPCs expressing at least OLIG2, NKX2.2 and PDGFRa (and may express additional markers, such as OTX2, FEZF2 and/or OLIG1).

As used herein, an "agonist" of a cellular signaling pathway is intended to refer to an agent that stimulates (upregulates) the cellular signaling pathway. Stimulation of the cellular signaling pathway can be initiated extracellularly, for example by use of an agonist that activates a cell surface receptor involved in the signaling pathway (e.g., the agonist can be a receptor ligand). Additionally or alternatively, stimulation of cellular signaling can be initiated intracellularly, for example by use of a small molecule agonist that interacts intracellularly with a component(s) of the signaling pathway.

As used herein, an "antagonist" of a cellular signaling pathway is intended to refer to an agent that inhibits (downregulates) the cellular signaling pathway. Inhibition of the cellular signaling pathway can be initiated extracellularly, for example by use of an antagonist that blocks a cell surface receptor involved in the signaling pathway. Additionally or alternatively, inhibition of cellular signaling can be initiated intracellularly, for example by use of a small molecule antagonist that interacts intracellularly with a component(s) of the signaling pathway.

Retinoic acid (RA) pathway agonists, Akt pathway agonists, mTOR pathway agonists, WNT pathway antagonists, SHH pathway agonists, BMP pathway antagonists and PKC pathway antagonists are known in the art and commercially available. They are used in the culture media at a concentration effective to achieve the desired outcome, e.g., generation of pre-OPCs and/or OPCs expressing markers of interest. Non-limiting examples of suitable agonist and antagonists agents, and effective concentration ranges, are described further below.

Agonists of the RA pathway include agents, molecules, compounds, or substances capable of stimulation of a retinoic acid receptor (RAR) that is activated by both all-trans retinoic acid and 9-cis retinoic acid. There are three RARs: RAR-alpha, RAR-beta and RAR-gamma, which are encoded by the *RARA, RARB, RARG* genes, respectively. Different retinoic acid analogs have been synthesized that can activate the retinoic acid pathway. Non-limiting examples of such compounds include TTNPB (agonist of RAR-alpha, beta and gamma), AM 580 (RARalpha agonist), CD 1530 (potent and selective RARgamma agonist), CD 2314

(selective RARbeta agonist), Ch 55 (potent RAR agonist), BMS 753 (RARalpha-selective agonist), Tazarotene (receptor-selective retinoid; binds RAR-beta and -gamma), Isotretinoin (endogenous agonist for retinoic acid receptors; inducer of neuronal differentiation), and AC 261066 (RARβ2 agonist). In some embodiments, the RA signaling pathway agonist is selected from the group consisting of: i) a retinoid compound, ii) a retinoid X receptor (RXR) agonist, and iii) a 25 retinoic acid receptor (RARs) agonist. In particular embodiments, the RA pathway agonist is selected from the group consisting of: retinoic acid, Sr11237, adapalene, EC23, 9-cis retinoic acid, 13-cis retinoic acid, 4-oxo retinoic acid, and All-trans Retinoic Acid (ATRA).

Accordingly, in one embodiment, the RA pathway agonist is selected from the group consisting of TTNPB, AM 580, CD 1530, CD 2314, Ch 55, BMS 753, Tazarotene, Isotretinoin, AC 261066, retinoic acid (RA), Sr11237, adapalene, EC23, 9-cis retinoic acid, 13-cis retinoic acid, 4-oxo retinoic acid, and All-trans Retinoic Acid (ATRA), or combinations thereof. In one embodiment, the RA pathway agonist is present in the culture media at a concentration within a range of 5-500 mM, or 10-100 nM or 25-75 nM. In one embodiment, the RA pathway agonist is TTNPB. In one embodiment, the RA pathway agonist is TTNPB, which is present in the culture media at a concentration within a range of 5-500 nM, or 10-100 nM or 25-75 nM. In one embodiment, the RA pathway agonist is TTNPB, which is present in the culture media at a concentration of 50 nM.

Agonists of the Akt pathway include agents, molecules, compounds, or substances capable of stimulating (activating) the signaling pathway of one or more of the serine/threonine kinase Akt family members, which include Akt1 (also designated PKB or RacPK), Akt2 (also designated PKBβ or RacPK-β) and Akt 3 (also designated PKBγ or thyoma viral proto-oncogene 3). In one embodiment, the Akt pathway agonist is a pan-Akt activator. In one embodiment, the pan Akt activator is SC79. In one embodiment, the Akt pathway agonist is present in the culture media at a concentration within a range of 0.1-10 μM. In one embodiment, the Akt pathway agonist is SC79. In one embodiment, the Akt pathway agonist is SC79, which is present in the culture media at a concentration of 0.1-10 μM, or 0.5-5 μM, or 0.5-2.5 μM or 0.5-1.5 μM. In one embodiment, the Akt pathway agonist is SC79, which is present in the culture media at a concentration of 1 μM.

Agonists of the mTOR (mammalian target of rapamycin) pathway include agents, molecules, compounds, or substances capable of stimulating (activating) signaling through

mTOR, a PI3K-related kinase family member which is a core component of the mTORC1 and mTORC2 complexes. In one embodiment, the mTOR pathway agonist is selected from the group consisting of MHY1485, 3BDO, Salidroside, L-Leucine, NV-5138, and combinations thereof. In one embodiment, the mTOR pathway agonist is present in the culture media at a concentration within a range of 0.1-10 μM, or 0.5-5 μM, or 0.5-2.5 μM or 0.5-1.5 μM. In one embodiment, the mTOR pathway agonist is MHY1485. In one embodiment, the mTOR pathway agonist is MHY1485, which is present in the culture media at a concentration of 0.1-10 μM, or 0.5-5 μM, or 0.5-2.5 μM or 0.5-1.5 μM. In one embodiment, the mTOR pathway agonist is MHY1485, which is present in the culture media at a concentration of 1 μM.

Antagonists of the WNT pathway include agents, molecules, compounds, or substances capable of inhibiting (downregulating) the canonical Wnt/β-catenin signaling pathway, which biologically is activated by binding of a Wnt-protein ligand to a Frizzled family receptor. In one embodiment, the WNT pathway antagonist is selected from the group consisting of XAV939, ICG001, Capmatinib, endo-IWR-1, IWP-2, IWP-4, MSAB, CCT251545, KY02111, NCB-0846, FH535, LF3, WIKI4, Triptonide, KYA1797K, JW55, JW 67, JW74, Cardionogen 1, NLS-StAx-h, TAK715, PNU 74654, iCRT3, WIF-1, DKK1, and combinations thereof. In one embodiment, the WNT pathway antagonist is present in the culture media at a concentration within a range of 10-500 nM, 50-250 nM or 50-150 nM. In one embodiment, the WNT pathway antagonist is XAV939. In one embodiment, the WNT pathway antagonist is XAV939, which is present in the culture media at a concentration of 10-500 nM, 50-250 nM or 50-150 nM. In one embodiment, the WNT pathway antagonist is XAV939, which is present in the culture media at a concentration of 100 nM.

Agonists of the SHH (sonic hedgehog) pathway include agents, molecules, compounds, or substances capable of stimulating (activating) signaling through the SHH pathway, which biologically involves binding of SHH to the Patched-1 (PTCH1) receptor and transduction through the Smoothened (SMO) transmembrane protein. In one embodiment, the SHH pathway agonist is selected from the group consisting of Purmorphamine, GSA 10, SAG, and combinations thereof. In one embodiment, the SHH pathway agonist is present in the culture media at a concentration within a range of 100-1000 nM, or 250-750 nM or 400-600 nM. In one embodiment, the SHH pathway antagonist is Purmorphamine. In one embodiment, the SHH pathway antagonist is Purmorphamine, which is present in the culture media at a concentration

of 100-1000 nM, or 250-750 nM or 400-600 nM. In one embodiment, the SHH pathway antagonist is Purmorphamine, which is present in the culture media at a concentration of 500 nM.

Antagonists of the BMP (bone morphogenetic protein) pathway include agents, molecules, compounds, or substances capable of inhibiting (downregulating) the BMP signaling pathway, which biologically is activated by binding of BMP to a BMP receptor, which are activin receptor-like kinases (ALK) (e.g., type I BMP receptor, including but not limited to ALK2 and ALK3). In one embodiment, the BMP pathway antagonist is selected from the group consisting of LDN193189, DMH1, DMH2, Dorsopmorphin, K02288, LDN214117, LDN212854, folistatin, ML347, Noggin and combinations thereof. In one embodiment, the BMP pathway antagonist is present in the culture media at a concentration within a range of 100-1000 nM, 150-750 nM, 100-500 nM, or 150-350 nM. In one embodiment, the BMP pathway antagonist is LDN193189. In one embodiment, the BMP pathway antagonist is LDN193189, which is present in the culture media at a concentration of 100-1000 nM, 150-750 nM, 100-500 nM, or 150-350 nM. In one embodiment, the BMP pathway antagonist is LDN193189, which is present in the culture media at a concentration of 250 nM.

Antagonists of the PKC (protein kinase C) pathway include agents, molecules, compounds, or substances capable of inhibiting (downregulating) a PKC signaling pathway, which biologically is mediated by a PKC family member. The PKC family of serine/threonine kinases comprises fifteen isozymes, including the "classical" PKC subcategory, which contain the isoforms α, $\beta1$, $\beta2$ and γ. In one embodiment, the PKC pathway antagonist inhibits the activity of at least one (and in other embodiments, at least two or three) PKC enzyme selected from PKCα, PKC$\beta1$, PKC$\beta2$ and PKCγ. In one embodiment, the PKC pathway antagonist is selected from the group consisting of Go 6983, Sotrastaurin, Enzastaurin, Staurosporine, LY31615, Go 6976, GF 109203X, Ro 31-8220 Mesylate, and combinations thereof. In one embodiment, the PKC pathway antagonist is present in the culture media at a concentration within a range of 10-500 nM, 50-300 nM, 50-150 nM or 75-150 nM. In one embodiment, the PKC pathway antagonist is Go 6983. In one embodiment, the PKC pathway antagonist is Go 6983, which is present in the culture media at a concentration of 10-500 nM, 50-300 nM, 50-150 nM or 75-150 nM. In one embodiment, the PKC pathway antagonist is Go 6983, which is present in the culture media at a concentration of 110 nM.

III. Culture Conditions

In combination with the chemically-defined and optimized culture media described in subsection II above, the methods of generating pre-OPCs and OPCs of the disclosure utilize standard culture conditions established in the art for cell culture. For example, cells can be cultured at 37 °C and under 5% O_2 and 5% CO_2 conditions. Cells can be cultured in standard culture vessels or plates, such as 96-well plates. In certain embodiments, the starting pluripotent stem cells are adhered to plates, preferably coated with an extracellular matrix material such as vitronectin. In one embodiment, the stem cells are cultured on a vitronectin coated culture surface (e.g., vitronectin coated 96-well plates).

Pluripotent stem cells can be cultured in commercially-available media prior to differentiation. For example, stem cells can be cultured for at least one day in Essential 8 Flex media (Thermo Fisher # A2858501) prior to the start of the differentiation protocol. In a non-limiting exemplary embodiment, stem cells are passaged onto vitronectin (Thermo Fisher # A14700) coated 96-well plates at 150,000 cells/cm2 density and cultured for one day in Essential 8 Flex media prior to differentiation.

To begin the differentiation protocol, the media the stem cells are being cultured in is changed to a basal differentiation media that has been supplemented with signaling pathway agonists and/or antagonists as described above in subsection II. A basal differentiation media can include, for example, a commercially-available base supplemented with additional standard culture media components needed to maintain cell viability and growth, but lacking serum (the basal differentiation media is a serum-free media) or any other exogenously-added growth factors, such as FGF2, PDGF, IGF or HGF. In a non-limiting exemplary embodiment, a basal differentiation media contains 1x IMDM (Thermo Fisher #12440046), 1x F12 (Thermo Fisher #11765047), poly(vinyl alcohol) (Sigma #p8136) at 1 mg/ml, chemically defined lipid concentrate (Thermo Fisher #11905031) at 1%, 1-thioglycerol (Sigma #M6145) at 450 uM, Insulin (Sigma #11376497001) at 0.7 ug/ml and transferrin (Sigma #10652202001) at 15 ug/ml.

The culture media typically is changed regularly to fresh media. For example, in one embodiment, media is changed every 24 hours.

To generate pre-OPCs and/or OPCs, the stem cells are cultured in the optimized culture media for sufficient time for cellular differentiation and expression of pre-OPC- or OPC-

associated markers. As described in the Examples, it has been discovered that culture of the stem cells in the optimized culture media for as little as 72 hours (3 days) was sufficient for pre-OPC and OPC differentiation. Accordingly, in one embodiment, cells are cultured for at least 72 hours. In other embodiments, cell are cultured for at least 60, 64, 68, 72, 76, 80, 84, 88, 92 or 96 hours. In other embodiments, cells are cultured for at least 2.5, 3, 3.5, 4, 4.5 or 5 days.

IV. Uses

The methods and compositions of the disclosure for generating pre-OPCs and OPCs allow for efficient and robust availability of these cell populations for a variety of uses. For example, the methods and compositions can be used in the study of oligodendrocyte development and biology to assist in the understanding of oligodendrocyte-related diseases and disorders. For example, the pre-OPCs and/or OPCs generated using the methods of the disclosure can be further purified according to methods established in the art using agents that bind to surface markers expressed on the cells. Accordingly, in one embodiment, the disclosure provides a method of isolating pre-oligodendrocyte progenitor cells (pre-OPCs) or oligodendrocyte progenitor cells (OPCs), the method comprising:

contacting OLIG2-expressing pre-OPCs or OPCs generated by a method of the disclosure with at least one binding agent that binds to a cell surface marker expressed by the pre-OPCs or OPCs; and

isolating cells that bind to the binding agent to thereby isolate the pre-OPCs or OPCs.

In one embodiment, the binding agent is an antibody, e.g., a monoclonal antibody (mAb) that binds to the cell surface marker. Non-limiting examples of suitable OPC cell surface markers include PDGFRa, O4 and A2B5. Cells that bind the antibody can be isolated by methods known in the art, including but not limited to fluorescent activated cell-sorting (FACS) and magnetic activated cell sorting (MACS).

Progenitors of the oligodendrocyte lineage also are contemplated for use in the treatment of various oligodendrocyte-related diseases and disorders, through delivery of the cells to a subject having the disease or disorder. Examples of oligodendrocyte-related diseases and disorders include, but are not limited to, multiple sclerosis (MS), progressive multifocal

leukoencephalopathy, periventricular leukomalacia, certain leukodystrophies and amyotrophic lateral sclerosis (ALS).

V. Compositions

In other aspects, the disclosure provides compositions related to the methods of generating pre-OPCs and OPCs, including culture media and cell cultures, as well as isolated progenitor cells and cell populations.

In one aspect, the disclosure provides a culture media for obtaining pre-OPCs or OPCs lacking exogenously-added growth factors and comprising a retinoic acid (RA) pathway agonist, an Akt pathway agonist and an mTOR pathway agonist. In one embodiment, the culture media further comprises a WNT pathway antagonist. In one embodiment, the culture media further comprises an SHH pathway agonist. In one embodiment, the culture media further comprises a WNT pathway antagonist and an SHH pathway agonist. In one embodiment, the culture media further comprises a BMP pathway antagonist. In one embodiment, the culture media further comprises a PKC pathway antagonist. In one embodiment, the culture media further comprises a BMP pathway antagonist and a PKC pathway antagonist. In one embodiment, the culture media comprises a retinoic acid (RA) pathway agonist, an Akt pathway agonist, an mTOR pathway agonist, a WNT pathway antagonist, an SHH pathway agonist, a BMP pathway antagonist and a PKC pathway antagonist.

In one aspect, the disclosure provides an isolated cell culture of pre-OPCs or OPCs, the culture comprising: OLIG2-expressing pre-OPCs or OPCs cultured in a culture media comprising a retinoic acid (RA) pathway agonist, an Akt pathway agonist and an mTOR pathway agonist and lacking exogenously-added growth factors. In various embodiments, the culture media can also comprise a WNT pathway antagonist, an SHH pathway agonist, a BMP pathway antagonist and/or a PKC pathway antagonist. In one embodiment, the pre-OPCs or OPCs also express NKX2.2. In other embodiments, the pre-OPCs or OPCs also express OTX2 and FEZF2. In one embodiment, the pre-OPCs or OPCs are attached to vitronectin-coated plates.

In another aspect, the disclosure provides a pre-oligodendrocyte progenitor cell (pre-OPC) or an oligodendrocyte progenitor cell (OPC) generated by a differentiation method of the disclosure. In one embodiment, the disclosure provides a composition comprising a non-native

pre-OPC or OPC wherein the pre-OPC or OPC expresses OLIG2, NKX2-2, OTX2 and FEZF2 and lacks expression of NESTIN. In another embodiment, the disclosure provides an isolated cell population of pre-OPCs or OPCs comprising at least 1 x 10^6 OLIG2 and NKX2.2-expressing pre-OPCs or OPCs, wherein the cell population lacks NESTIN-expressing neural stem cells. In other embodiments, the cell population comprises at least 5 x 10^6, 1 x 10^7, 5 x 10^7, 1 x 10^8, 5 x 10^8 or 1 x 10^9 OLIG2 and NKX2.2-expressing pre-OPCs or OPCs. In on embodiment of the isolated cell population, the pre-OPCs or OPCs are bound with at least one antibody that binds at least one cell surface marker expressed by the pre-OPCs or OPCs. Non-limiting examples of suitable OPC cell surface markers include PDGFRa, O4 and A2B5.

The present invention is further illustrated by the following examples, which should not be construed as further limiting. The contents of figures and all references, patents and published patent applications cited throughout this application are expressly incorporated herein by reference.

EXAMPLES

Example 1: Culture Protocol Development for Generation of Stem Cell-Derived Oligodendrocyte Progenitors

In this example, a culture media recipe for generation of oligodendrocyte progenitors was developed that can guide human pluripotent stem cells to differentiate to oligodendrocyte progenitors expressing NKX2-2 and OLIG2 after 3 days in culture. These cells can be further differentiated to mature oligodendrocytes.

This example utilizes a method of High-Dimensional Design of Experiments (HD-DoE), as previously described in Bukys et al. (2020) *Iscience* 23:101346. The method employs computerized design geometries to simultaneously test multiple process inputs and offers mathematical modeling of a deep effector/response space. The method allows for finding combinatorial signaling inputs that control a complex process, such as during cell differentiation. It allows testing of multiple plausible critical process parameters, as such parameters impact output responses, such as gene expression. Because gene expression provides hallmark features of the phenotype of, for example, a human cell, the method can be applied to identify, and

understand, which signaling pathways control cell fate. In the current example, the HD-DOE method was applied with the intent to find conditions for induction of oligodendrocyte progenitor-expressed genes, directly from the pluripotent stem cell state.

Specifically, to develop a novel method to generate oligodendrocytes, the impact of agonists and antagonists of multiple signaling pathways (herein called effectors) were tested on expression of two sets of 53 pre-selected genes after a 3-day treatment. These effectors are small molecules that are commonly used during stepwise differentiation of stem cells to specific fates. Choice of the effectors were based on current literature on neural induction in anterior ectoderm and differentiation of stem cells to oligodendrocytes.

HD-DoE #1

To test the effectors, experiments with at least 8 factors were designed that can assess the response of cells to 48 or more different combinations of effectors in a range of concentrations. To analyze the models, we focused on expression of genes expressed during early development of anterior neuroectoderm and oligodendrocytes including NKX2-2, OLIG2, OLIG1, and PDGFRA. The impact of each effector on gene expression level is defined by a parameter called factor contribution that is calculated for each effector during the modeling.

As shown in the results summarized in **FIG. 1**, one model specifically showed promising results on upregulation of NKX2-2 and OLIG2 genes when optimized for maximum expression of NKX2-2 at 12480.6. This model tested 8 factors: PD0325901, MK2206, TTNPB, SC79, MHY1485, ZM336372, AGN193109 and AZD3147.

Out of the eight factors tested, three of them, namely TTNPB (agonist of retinoic acid pathway), SC79 (agonist of Akt signaling pathway) and MHY1485 (agonist of mTOR signaling pathway), had significant positive effect on expression of targeted genes, with TTNPB having the most impact with factor contribution of 31.3 and MHY1485 with factor contribution of 13.8 and SC79 at 1.47. These factors could bring up expression of NKX2-2 and OLIG2 significantly. OLIG1 and PDGFRA had average expression levels (129.9 and 346.45 respectively) which is compatible with the pattern of gene expression during oligodendrocyte differentiation.

As shown in the results summarized in **FIG. 2**, normalized expression of PDGFRA in this model could reach up to 832.9 which was the highest expression level out of all models and therefore, the model was also optimized for maximum expression of PDGFRA. This setting

showed that TTNPB (agonist of retinoic acid pathway) and MHY1485 (agonist of mTOR signaling pathway) also have a positive effect on upregulation of PGFRA with factor contribution of 49.01 and 13.4, respectively. It was also observed that PD0325901 can have a significant positive effect on this gene with factor contribution of 30.6. Because of low factor contribution of ZM336372 (<1), this factor was not included in the recipe. In this condition, OLIG1 had average expression level at 228.36 similar to conditions of optimization of NKX2-2. One difference in this condition was downregulation of OLIG2 gene from 1049.4 at previous condition to 241.9.

As shown in the results summarized in **FIG. 3**, out of effectors with positive contribution to expression levels of NKX2-2, OLIG2 or PDGFRA, two factors were negatively impacting NKX2-2 and OLIG2 expression levels. Thus, these two factors, PD0325901 and ZM336372, were eliminated from list of candidates for the recipe of oligodendrocyte differentiation.

Thus, this first HD-DoE screening identified a culture media lacking exogenously-added growth factors and comprising an agonist of retinoic acid pathway, an agonist of Akt signaling pathway and an agonist of mTOR signaling pathway as sufficient to lead to the generation of OLIG2-expressing OPCs from pluripotent stem cells after 3 days (72 hours) of culture.

HD-DoE #2

To further enhance the conditions for oligodendrocyte differentiation from pluripotency, we performed an additional HD-DoE experiment. We obtained additional gene regulatory models that were used for preparation of a differentiation protocol. The basis of this was a 13-factor HD-DoE experiment with focus on initiation of differentiation of cells toward anterior neuroectoderm. In this model, we focused on expression of FEZF2 and OTX2.

As shown in the results summarized in **FIG. 4**, the model was optimized for highest expression of OTX2 at 12755.9. According to the model of high expression of OTX2, seven effectors had positive contribution including MK2206, PD0325901, CHIR99021, LDN193189, Go6983, PD173074 and BLU9931 with highest factor contribution of 22.2 for MK2206 and lowest factor contribution of 1.7 for PD173074 and BLU9931.

As shown in the results summarized in **FIG. 5**, the model was optimized for highest expression of FEZF2 at 4466. When the model was optimized for maximum expression level of FEZF2, three effectors including LDN193189 with factor contribution of 19.5, PD0325901 with

factor contribution of 14.6 and MK2206 with factor contribution of 12.3 were common with previous condition and three new effectors including purmorphamine-500nM, XAV939 and SC79 were introduced.

As shown in the results summarized in **FIG. 6**, to fine-tune the recipe and find the optimum combination of factors for high expression of both OTX2 and FEZF2, dynamic profiling analysis was done. According to this analysis, XAV939 (inhibitor of WNT signaling pathway) and Purmorphamine (agonist of SHH signaling pathway and known for ventralizing the cells during development of regions of the brain) had significant positive effects on expression of FEZF2 and no negative impact on expression level of OTX2. Therefore, these two factors were added to the optimized culture recipe.

In addition to inclusion of factors that promoted expression of OPC-associated surface markers, certain factors that inhibited expression of such markers were eliminated from the optimized culture recipe. CHIR99021, which is the agonist of WNT signaling pathway, was eliminated. MK2206 and PD0325901 were also eliminated, since according to the 8-factor model, they had negative effect on expression of oligodendrocyte genes. PD173074 and BLU9931 were also eliminated because of low factor contribution of 1.7.

Summary

Considering both models, culture conditions that maximized differentiation of human induced pluripotent stem cells to cells having oligodendrocyte progenitor cell (OPC) identity, leading to elevated expression of OTX2, FEZF2, NKX2-2 and OLIG2, included the following effector inputs: TTNPB (RA pathway agonist), SC79 (Akt pathway agonist), MHY1485 (mTOR pathway agonist), Purmorphamine (SHH pathway agonist), XAV939 (WNT pathway antagonist), LDN193189 (BMP pathway antagonist) and Go6983 (PKC pathway antagonist).

Example 2: Factor Criticality Analysis of OPC-Inducing Culture Conditions

To assess the factor criticality of each component in the optimized culture media described in Example 1, we performed in-silico prediction analysis of the outcome under conditions in which individual effectors was eliminated, while keeping others present. To do this, we used dynamic profile analysis at setpoint, while comparing the expression level of genes of interest in absence of each factor. Since expression of genes of interest reveal whether the

desired outcome is reachable, this factor criticality analysis revealed the extent of importance of each input effector.

FIG. 7A-D summarize the results for the factor criticality analysis of the effectors TTNPB (RA pathway agonist), SC79 (Akt pathway agonist) and MHY1485 (mTOR pathway agonist). **FIG. 7A** shows the expression level of the OPC genes of interest in presence of TTNPB, MHY1485 and SC79 when the model is otherwise optimized for maximum expression of NKX2-2. As shown in **FIG. 7B**, upon removal of TTNPB, the predicted expression levels of NKX2-2, OLIG2 and PDGFRA dropped significantly from more than 12000 to 4500 for NKX2-2, 1000 to 400 for OLIG2 and 350 to less than 100 for PDGFRA. This outcome signifies a significant deleterious effect on expression of all the desired markers when the RA pathway agonist is removed. As shown in **FIG. 7C**, when MHY1485 was removed, again expression levels decreased, however, not as drastically as previous condition. As shown in **FIG. 7D**, when SC79 was removed, only a small shift in the plots was observed, which suggests this factor is less critical than TTNPB and MHY1485 for attaining maximal OPC marker induction.

FIG. 8A-D summarize the results for the factor criticality analysis of the effectors Purmorphamine (SHH pathway agonist), XAV939 (WNT pathway antagonist), LDN193189 (BMP pathway antagonist) and Go6983 (PKC pathway antagonist). To attain the desired patterning of the oligodendrocyte population to anterior region of the brain, these additional factor inputs were queried, such as FEZF2 and OTX2. Expression levels of FEZF2 and OTX2 were examined in absence of either LDN193189, XAV, Purmorphamine or Go6983, when the model was optimized for maximum expression of FEZF2. The most significant change was observed in absence of LDN193189 which led to almost 50% reduction in expression of FEZF2 (from 4500 to 2500). Expression of OTX2 was also reduced from 9000 to 7000, which was the lowest in all four elimination processes. When XAV939 and Purmorphamine were removed, expression level of FEZF2 decreased to 3000 and 3500 respectively, while expression of OTX2 was only slightly higher in both cases. When Go6983 was removed, we did not observe any significant changes in the expression level of genes of interest, therefore suggesting G06983 being optional related to control of FEZF2 and OTX2.

Example 3: Immunocytochemistry Validation of Stem Cell-Derived OPCs

To further validate the optimized culture media described in Example 1, cells were cultured in the optimized media for 3 days and immunocytochemistry was used to assess expression of biomarkers of anterior neuroectoderm and oligodendrocyte progenitors. Biomarkers included OTX2 and oligodendrocyte precursor biomarkers including NKX2-2, OLIG2 and PDGF. Nestin, an early neuronal marker was used to distinguish between neural stem cells and oligodendrocyte progenitors. Ki67 was also used to confirm the proliferation of cells after induction. Representative immunohistochemistry results are shown in **FIG. 9**. These immunocytochemistry images confirmed that most of the cells expressed OTX2. However, there was no trace of the neuronal biomarker Nestin detected, confirming that the differentiated OPC population lacks neural stem cells. Expression of OLIG2 and NKX2-2 was also observed in more than 90% of the cells, thereby confirming the oligodendrocyte lineage of the cells. None of the cells expressed PDGFR which was expected since this gene is expressed at later stages of differentiation of oligodendrocytes.

Example 4: RNA-seq Validation of Stem Cell-Derived Pre-OPC

RNA sequencing was used to obtain a gene profile of cells cultured in the differentiation media detailed in Examples 1 and 2. hiPSCs were cultured for 3 days in the media and RNA from the generated cells was sequenced by standard RNA-seq analysis. The results in **FIG. 10A** show normalized expression levels of selected genes representative of various regions of the brain, early oligodendrocyte identity (NKX2-2, OLIG2, PDGFRa) and stem cell state (NANOG, POU5F1) in three replicates at day 0 and day 3. The results demonstrated that the level of stem cell genes decreased in pre-OPC cells while the level of oligodendrocyte genes increased, which validated the differentiation of hiPSCs to oligodendrocyte lineage using the differentiation media. The results of **FIG. 10B** show differential expression and fold change of the selected genes, with HOXA1 at highest level (15) and OLIG2, NKX2-2 and PDGFRa at 5. This data demonstrates the ability of developed recipe as a stage 1 media in directing the cells toward oligodendrocyte identity.

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents of the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims

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CLAIMS

1. A method of generating human pre-oligodendrocyte progenitor cells (pre-OPCs) or oligodendrocyte progenitor cells (OPCs) comprising:

culturing human pluripotent stem cells in a culture media lacking exogenously-added growth factors and comprising a retinoic acid (RA) pathway agonist, an Akt pathway agonist and an mTOR pathway agonist such that OLIG2-expressing pre-OPCs or OPCs are generated.

2. The method of claim 1, wherein the human pluripotent stem cells are induced pluripotent stem cells (iPSCs).

3. The method of claim 1, wherein the human pluripotent stem cells are embryonic stem cells.

4. The method of any one of claims 1-3, wherein OLIG2-expressing pre-OPCs or OPCs are generated within 72 hours of starting culture of the human pluripotent stem cells in the culture media.

5. The method of any one of claims 1-4, wherein the pre-OPCs or OPCs also express NKX2-2.

6. The method of any one of claims 1-5, wherein the human pluripotent stem cells are attached to vitronectin-coated plates during culturing.

7. The method of any one of claims 1-6, wherein the RA pathway agonist is selected from the group consisting of TTNPB, AM 580, CD 1530, CD 2314, Ch 55, BMS 753, Tazarotene, Isotretinoin, AC 261066, retinoic acid (RA), Sr11237, adapalene, EC23, 9-cis retinoic acid, 13-cis retinoic acid, 4-oxo retinoic acid, All-trans Retinoic Acid (ATRA), and combinations thereof.

8. The method of claim 7, wherein the RA pathway agonist is present in the culture media at a concentration within a range of 10-100 nM.

9. The method of claim 8, wherein the RA pathway agonist is TTNPB, which is present in the culture media at a concentration of 50 nM.

10. The method of any one of claims 1-6, wherein the Akt pathway agonist is SC79

11. The method of claim 10, wherein SC79 is present in the culture media at a concentration within a range of 0.1-10 μM.

12. The method of claim 10, wherein SC79 is present in the culture media at a concentration of 1 μM.

13. The method of any one of claims 1-6, wherein the mTOR pathway agonist is selected from the group consisting of MHY1485, 3BDO, Salidroside, L-Leucine, NV-5138, and combinations thereof.

14. The method of claim 13, wherein the mTOR pathway agonist is present in the culture media at a concentration within a range of 0.1-10 μM.

15. The method of claim 14, wherein the mTOR pathway agonist is MHY1485, which is present in the culture media at a concentration of 1 μM.

16. The method of any one of claims 1-15, wherein the culture media further comprises a WNT pathway antagonist and an SHH pathway agonist.

17. The method of claim 16, wherein the WNT pathway antagonist is selected from the group consisting of XAV939, ICG001, Capmatinib, endo-IWR-1, IWP-2, IWP-4, MSAB, CCT251545, KY02111, NCB-0846, FH535, LF3, WIKI4, Triptonide, KYA1797K, JW55, JW 67, JW74, Cardionogen 1, NLS-StAx-h, TAK715, PNU 74654, iCRT3, WIF-1, DKK1, and combinations thereof.

18. The method of claim 17, wherein the WNT pathway antagonist is present in the culture media at a concentration within a range of 50-150 nM.

19. The method of claim 18, wherein the WNT pathway antagonist is XAV939, which is present in the culture media at a concentration of 100 nM.

20. The method of claim 16, wherein the SHH pathway agonist is selected from the group consisting of Purmorphamine, GSA 10, SAG, and combinations thereof.

21. The method of claim 20, wherein the SHH pathway agonist is present in the culture media at a concentration within a range of 250-750 nM.

22. The method of claim 21, wherein the SHH pathway antagonist is Purmorphamine, which is present in the culture media at a concentration of 500 nM.

23. The method of any one of claims 1-22, wherein the culture media further comprises a BMP pathway antagonist.

24. The method of claim 23, wherein the BMP pathway antagonist is selected from the group consisting of LDN193189, DMH1, DMH2, Dorsopmorphin, K02288, LDN214117, LDN212854, folistatin, ML347, Noggin, and combinations thereof.

25. The method of claim 24, wherein the BMP pathway antagonist is present in the culture media at a concentration within a range of 100-500 nM.

26. The method of claim 25, wherein the BMP pathway antagonist is LDN193189, which is present in the culture media at a concentration of 250 nM.

27. The method of any one of claims 1-26, wherein the culture media further comprises a PKC pathway antagonist.

28. The method of claim 27, wherein the PKC pathway antagonist is selected from the group consisting of Go 6983, Sotrastaurin, Enzastaurin, Staurosporine, LY31615, Go 6976, GF 109203X, Ro 31-8220 Mesylate, and combinations thereof.

29. The method of claim 28, wherein the PKC pathway antagonist is present in the culture media at a concentration within a range of 50-150 nM.

30. The method of claim 29, wherein the PKC pathway antagonist is Go6983, which is present in the culture media at a concentration of 110 nM.

31. A method of generating human pre-oligodendrocyte progenitor cells (pre-OPCs) or oligodendrocyte progenitor cells (OPCs) comprising:

culturing human pluripotent stem cells in a culture media lacking exogenously-added growth factors and comprising a retinoic acid (RA) pathway agonist, an Akt pathway agonist, an mTOR pathway agonist, a WNT pathway antagonist, an SHH pathway agonist, a BMP pathway antagonist and a PKC pathway antagonist such that OLIG2-expressing pre-OPCs or OPCs are generated.

32. A culture media for obtaining pre-oligodendrocyte progenitor cells (pre-OPCs) or oligodendrocyte progenitor cells (OPCs) comprising a retinoic acid (RA) pathway agonist, an Akt pathway agonist and an mTOR pathway agonist and lacking exogenously-added growth factors.

33. The culture media of claim 32, which further comprises a WNT pathway antagonist and an SHH pathway agonist.

34. The culture media of claim 33, which further comprises a BMP pathway antagonist and a PKC pathway antagonist.

35. An isolated cell culture of pre-oligodendrocyte progenitor cells (pre-OPCs) or oligodendrocyte progenitor cells (OPCs), the culture comprising: OLIG2-expressing pre-OPCs

or OPCs cultured in a culture media comprising a retinoic acid (RA) pathway agonist, an Akt pathway agonist and an mTOR pathway agonist and lacking exogenously-added growth factors.

36. The isolated cell culture of claim 35, wherein the culture media further comprises a WNT pathway antagonist and an SHH pathway agonist and wherein the pre-OPCs or OPCs also express OTX2 and FEZF2.

37. The isolated cell culture of claim 35 or 36, wherein the culture media further comprises a BMP pathway antagonist and a PKC pathway antagonist.

38. The isolated cell culture of any one of claims 35-37, wherein the pre-OPCs or OPCs are attached to vitronectin-coated plates.

39. A pre-oligodendrocyte progenitor cell (pre-OPC) or oligodendrocyte progenitor cell (OPC) generated by the method of any one of claims 1-32.

40. A composition comprising a non-native pre-oligodendrocyte progenitor cell (pre-OPC) or oligodendrocyte progenitor cell (OPC), wherein the pre-OPC or OPC expresses OLIG2, NKX2-2, OTX2 and FEZF2 and lacks expression of NESTIN.

41. An isolated cell population of pre-oligodendrocyte progenitor cells (pre-OPCs) or oligodendrocyte progenitor cells (OPCs) comprising at least 1 x 10^6 OLIG2-expressing pre-OPCs or OPCs, wherein the cell population lacks NESTIN-expressing neural stem cells.

42. The isolated cell population of claim 41, wherein the pre-OPCs or OPCs also express NKX2-2, OTX2 and FEZF2.

43. The isolated cell population of claim 41 or 42, wherein the pre-OPCs or OPCs are bound with at least one antibody that binds at least one marker expressed by the pre-OPCs or OPCs.

Objective | Setpoint (#19) | Alternative setpoints

	Response	Criterion	Value	Graph	log(D)	Prob. of failure	Cpk
24	MAFB	Predicted	262.762				
25	MAF	Predicted	1513.6				
26	MEF2C	Predicted	32.2549				
27	MKI67	Predicted	4271.5				
28	NETO1	Predicted	84.0921				
29	NETO2	Predicted	5207.9				
30	NEUROD6	Predicted	61.0956				
31	NKX2-1	Predicted	3594.47				
32	NKX2-2	Maximize	12480.6		-1.34983	19%	0.29305
33	NPAS1	Predicted	6.28653				
34	NPY	Predicted	24.7324				
35	NR2F1	Predicted	3138.48				
36	NR2F2	Predicted	2196.53				
37	NKXPH1	Predicted	-27.3136				
38	OLIG1	Predicted	129.939				
39	OLIG2	Predicted	1049.37				
40	PAX6	Predicted	1248.23				
41	PDGFRA	Predicted	346.458				
42	POU3F2	Predicted	402.112				
43	PROX1	Predicted	613.532				
44	PVALB	Predicted	158.934				
45	RELN	Predicted	1271.24				
46	RUNX1T1	Predicted	261.165				
47	SATB1	Predicted	3604.89				
48	SOX10	Predicted	-6.22496				
49	SOX2	Predicted	67561.9				
50	SP8	Predicted	254.048				
51	SST	Predicted	3239.19				
52	TUBB3	Predicted	19466.2				
53	VIM	Predicted	72202.5				
54	ZIC1	Predicted	1526.61				

	Factor	Role	Value	Graph	Factor contribution
1	PD0325901	Free	0.11493		17.3982
2	ZM336372	Free	0.000339067		16.8484
3	MK2206	Free	1.2371		8.96926
4	SC79	Free	0.999546		1.47518
5	AGN193109	Free	0.00080431		1.7969
6	TTNBP	Free	49.9982		31.2673
7	AZD3147	Free	8.51318e-05		8.46043
8	MHY1485	Free	1.99996		13.7844

FIG. 1

Objective | Setpoint (#19) | Alternative setpoints

	Response	Criterion	Value	Graph	log(D)	Prob. of failure	Cpk
24	MAFB	Predicted	502.928				
25	MAF	Predicted	1041.46				
26	MEF2C	Predicted	292.265				
27	MKI67	Predicted	6304.16				
28	NETO1	Predicted	177.072				
29	NETO2	Predicted	4452.29				
30	NEUROD6	Predicted	122.783				
31	NKX2-1	Predicted	2232.01				
32	NKX2-2	Predicted	3335.38				
33	NPAS1	Predicted	1.41842				
34	NPY	Predicted	38.0745				
35	NR2F1	Predicted	4260.34				
36	NR2F2	Predicted	4398.83				
37	NKXPH1	Predicted	-5.47688				
38	OLIG1	Predicted	228.358				
39	OLIG2	Predicted	241.971				
40	PAX6	Predicted	7874.96				
41	PDGFRA	Maximize	832.98		-0.60206	22%	0.247347
42	POU3F2	Predicted	239.865				
43	PROX1	Predicted	777.062				
44	PVALB	Predicted	63.8672				
45	RELN	Predicted	920.133				
46	RUNX1T1	Predicted	565.708				
47	SATB1	Predicted	3654.04				
48	SOX10	Predicted	-4.01141				
49	SOX2	Predicted	56097.1				
50	SP8	Predicted	434.992				
51	SST	Predicted	1861.62				
52	TUBB3	Predicted	13831				
53	VIM	Predicted	26669.6				
54	ZIC1	Predicted	404.143				

	Factor	Role	Value	Graph	Factor contribution
1	PD0325901	Free	100		30.0553
2	ZM336372	Free	1		0.427593
3	MK2206	Free	0		1.1434
4	SC79	Free	0		0.835633
5	AGN193109	Free	0		2.5985
6	TTNBP	Free	50		49.0142
7	AZD3147	Free	0		2.46744
8	MHY1485	Free	2		13.4579

FIG. 2

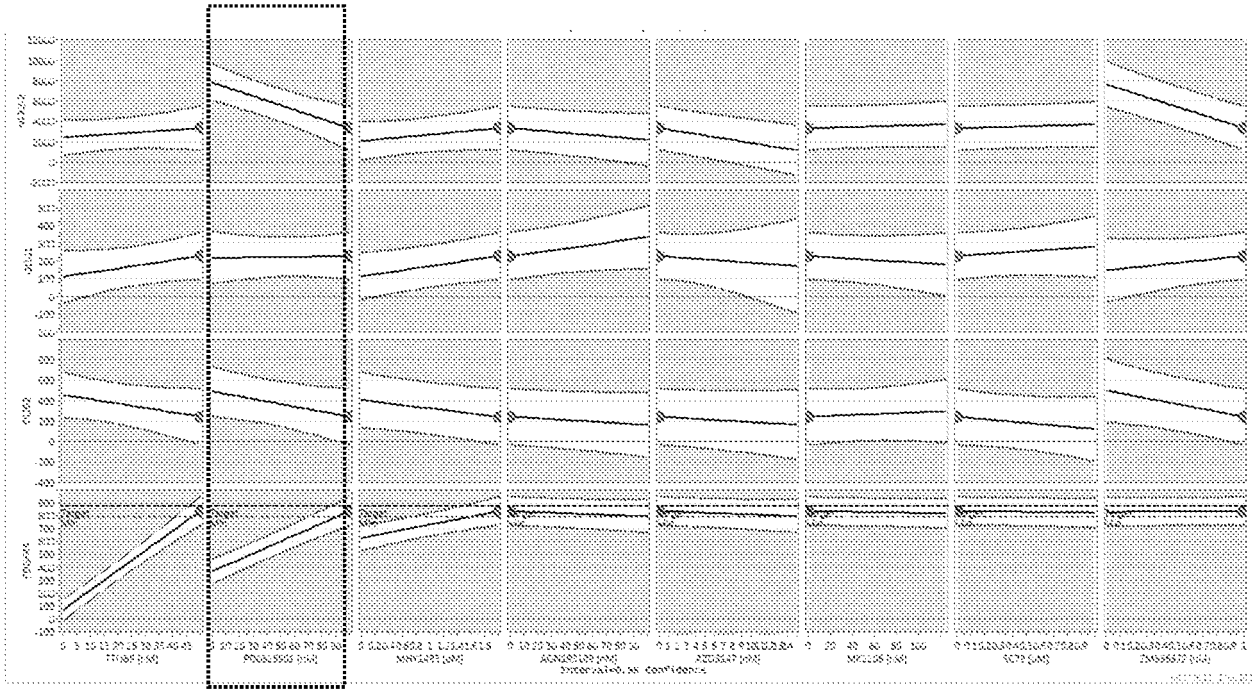


FIG. 3

Objective | Setpoint (#19) | Alternative setpoints

	Response	Criterion	Value	Graph	log(D)	Prob. of failure	Cpk
19	HES1	Predicted	330.554				
20	HMGB2	Predicted	14870.5				
21	HMX2	Predicted	-6.64705				
22	HOXB1	Predicted	-0.378681				
23	IRX3	Predicted	696.214				
24	ISL1	Predicted	-26.1116				
25	ISL2	Predicted	-17.4699				
26	LHX4	Predicted	68.0646				
27	LMO1	Predicted	905.208				
28	LMO3	Predicted	305.228				
29	LMX1A	Predicted	604.987				
30	LMX1B	Predicted	-112.196				
31	MKI67	Predicted	1461.79				
32	NEUROD1	Predicted	0.550277				
33	NEUDOD6	Predicted	22.9791				
34	NEUROG1	Predicted	48.9105				
35	NEUROG2	Predicted	57.2419				
36	NFE2L3	Predicted	202.664				
37	NKX6-2	Predicted	33.9382				
38	NR2F6	Predicted	2901.11				
39	NR4A2	Predicted	2.57416				
40	OLIG2	Predicted	39.718				
41	OTX2	Maximize	12755.9		-10	0.88%	0.777651
42	PITX2	Predicted	-5.0266				
43	PITX3	Predicted	5.75851				
44	POU4F1	Predicted	14.1262				
45	SIX3	Predicted	-2385.95				
46	TCF3	Predicted	7666.44				
47	TERF2	Predicted	1103.74				
48	TH	Predicted	-0.189617				
49	VIM	Predicted	10365.4				
50	WNT1	Predicted	447.584				
51	WNT8B	Predicted	157.511				
52	LHX3	Predicted	-0.494643				

	Factor	Role	Value	Graph	Factor contribution
1	LDN193189	Free	248.736		11.9807
2	PD173074+BLU9931	Free	49.994		1.79948
3	Purmorphamine 500	Free	18.6918		2.03588
4	Purmorphamine 200	Free	9.2549		0.38661
5	SC79	Free	0.988981		5.75937
6	MK2206	Free	124.722		22.2839
7	ZM336372	Free	0.415081		3.84831
8	PD0325901	Free	99.9991		18.0824
9	CHIR99021	Free	0.99711		13.5337
10	XAV939	Free	0.134963		1.98982
11	UCLA_GP130_2	Free	0.0207014		3.64899
12	Tofacitinib	Free	0.2591		12.0135
13	GO6983	Free	95.5821		2.63741

FIG. 4

Objective | Setpoint (#8) | Alternative setpoints

	Response	Criterion	Value	Graph	log(D)	Prob. of failure	Cpk
1	RNA	Predicted	240.061				
2	ALDH1A1	Predicted	0.50181				
3	ASCL1	Predicted	1.73552				
4	BARHL1	Predicted	339.575				
5	DBX2	Predicted	0.750382				
6	DDC	Predicted	-0.676181				
7	DMBX1	Predicted	613.121				
8	EN1	Predicted	0.439595				
9	EN2	Predicted	-0.0220256				
10	ETV4	Predicted	50.9694				
11	FERD3L	Predicted	51.6771				
12	FEV	Predicted	-0.0817658				
13	FEZF2	Maximize	4466		-10	0.02%	1.25582
14	FOXA1	Predicted	142.511				
15	FOXA2	Predicted	1111.45				
16	FOXD2	Predicted	24.5731				
17	GATA3	Predicted	253.497				
18	GBX2	Predicted	1.25974				
19	HES1	Predicted	996.596				
20	HMGB2	Predicted	17929.3				
21	HMX2	Predicted	19.6778				
22	HOXB1	Predicted	0.384939				
23	IRX3	Predicted	181.478				
24	ISL1	Predicted	26.9557				
25	ISL2	Predicted	118.897				
26	LHX4	Predicted	541.817				
27	LMO1	Predicted	1135.94				
28	LMO3	Predicted	138.926				
29	LMX1A	Predicted	240.188				
30	LMX1B	Predicted	5.61555				
31	MKI67	Predicted	2490.26				
32	NEUROD1	Predicted	1.62807				
33	NEUROD6	Predicted	44.8253				
34	NEUROG1	Predicted	57.8583				

	Factor	Role	Value	Graph	Factor contribution
1	LDN193189	Free	244.52		19.4571
2	PD173074+BLU9931	Free	38.0259		5.11884
3	Purmorphamine 500	Free	488.02		9.76177
4	Purmorphamine 200	Free	47.0196		3.29792
5	SC79	Free	0.999847		6.31136
6	MK2206	Free	124.995		12.2943
7	ZM336372	Free	0.0372426		9.80767
8	PD0325901	Free	98.3146		14.5858
9	CHIR99021	Free	0.809146		5.31386
10	XAV939	Free	99.9993		12.5423
11	UCLA_GP130_2	Free	0.564196		0.385926
12	Tofacitinib	Free	72.4413		0
13	GO6983	Free	34.9768		1.12322

FIG. 5

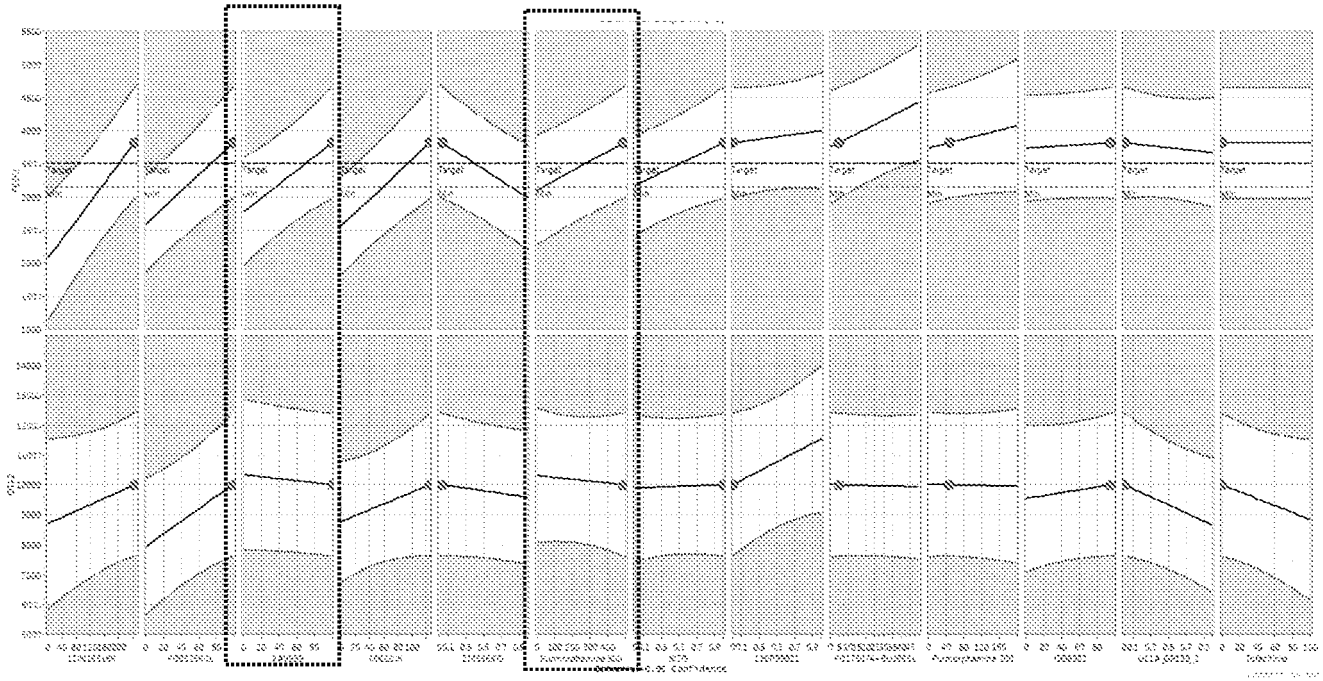


FIG. 6

FIG. 7A

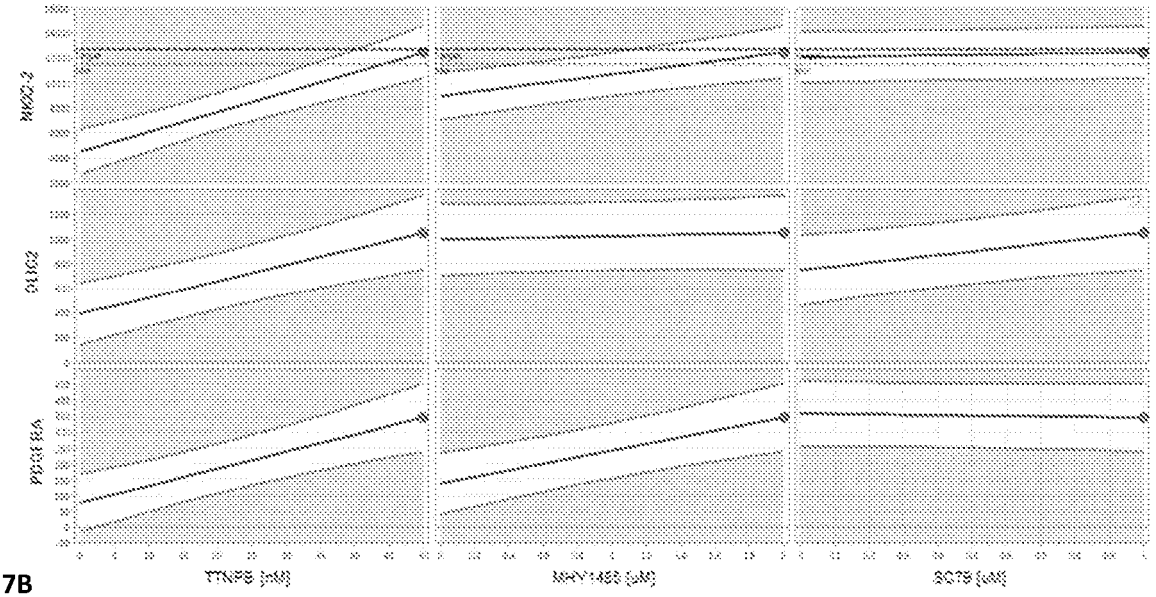

MKN-1
SNU5
PDGFRA
TTNPB [nM]
MHY1485 [uM]
SC79 [uM]

FIG. 7B

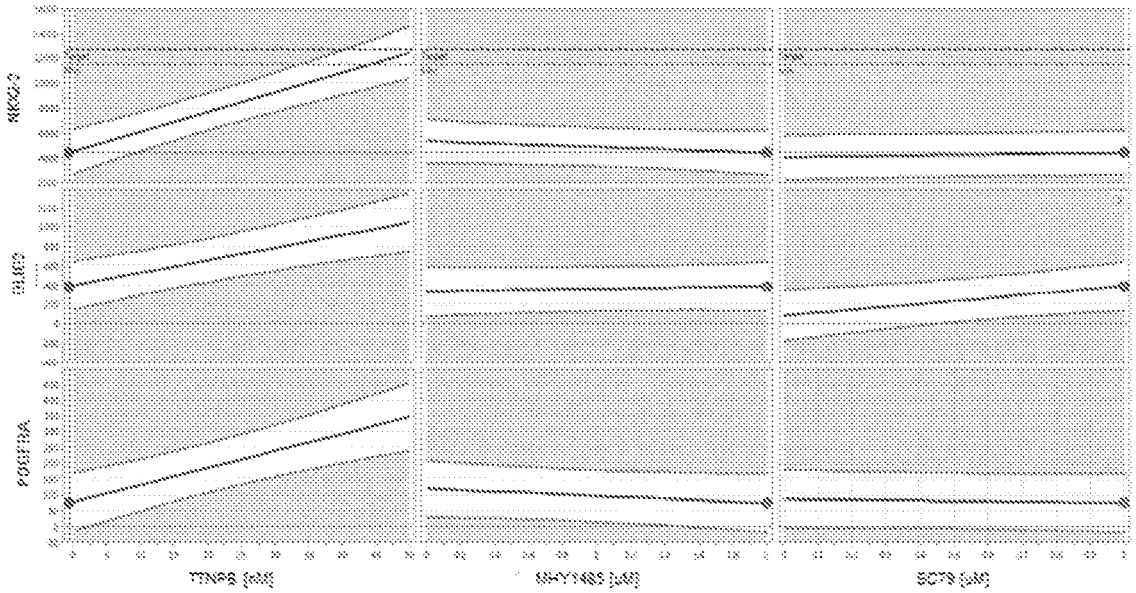

MKN-1
SNU5
PDGFRA
TTNPB [nM]
MHY1485 [uM]
SC79 [uM]

FIG. 7C

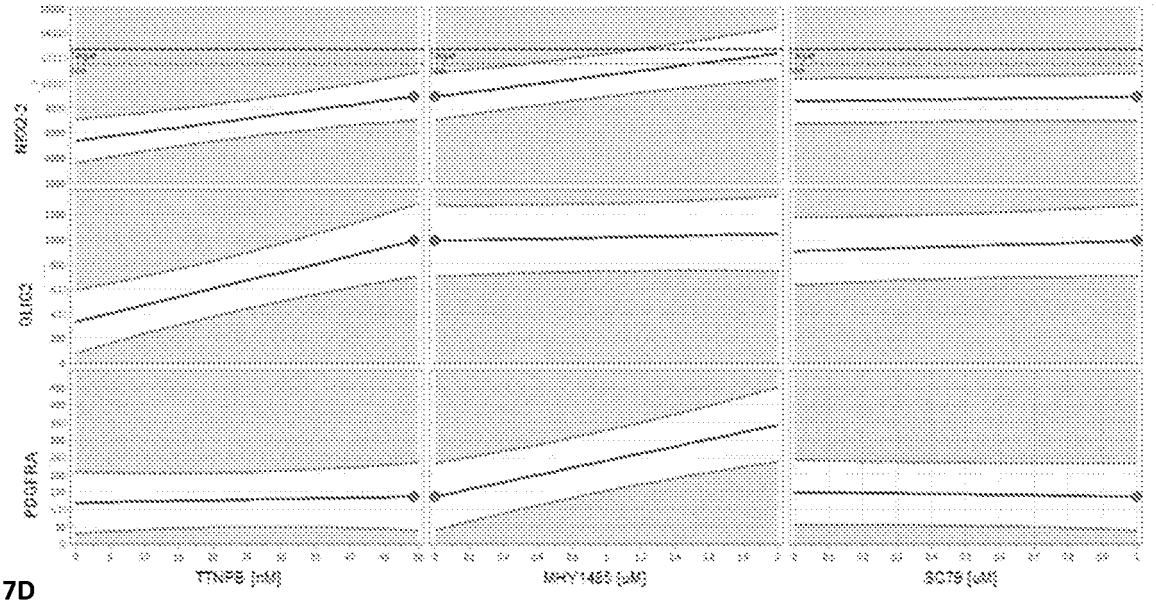

KDR
GUCY
PDGFRA
TTNPB [nM]
MHY1485 [uM]
SC79 [uM]

FIG. 7D

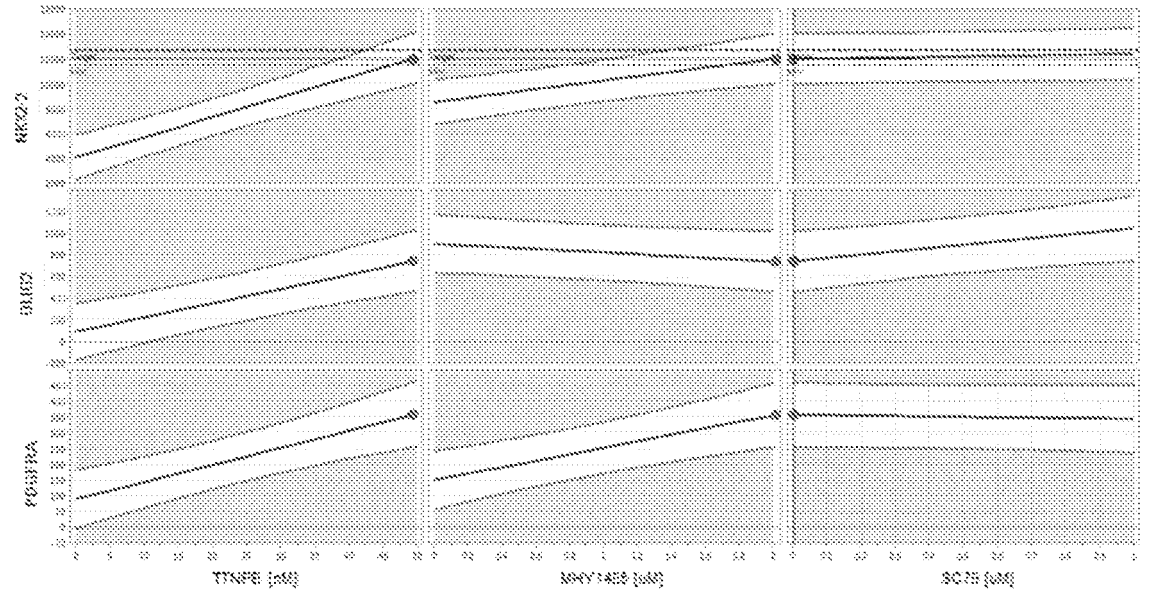

KDR
GUCY
PDGFRA
TTNPB [nM]
MHY1485 [uM]
SC79 [uM]

FIG. 8A

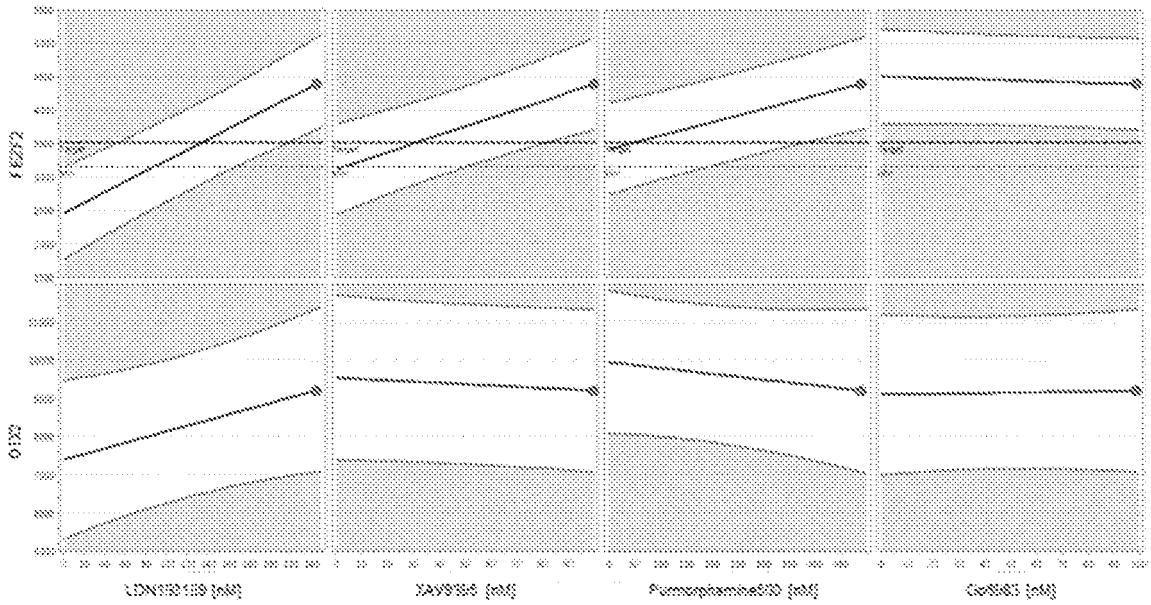


FIG. 8B

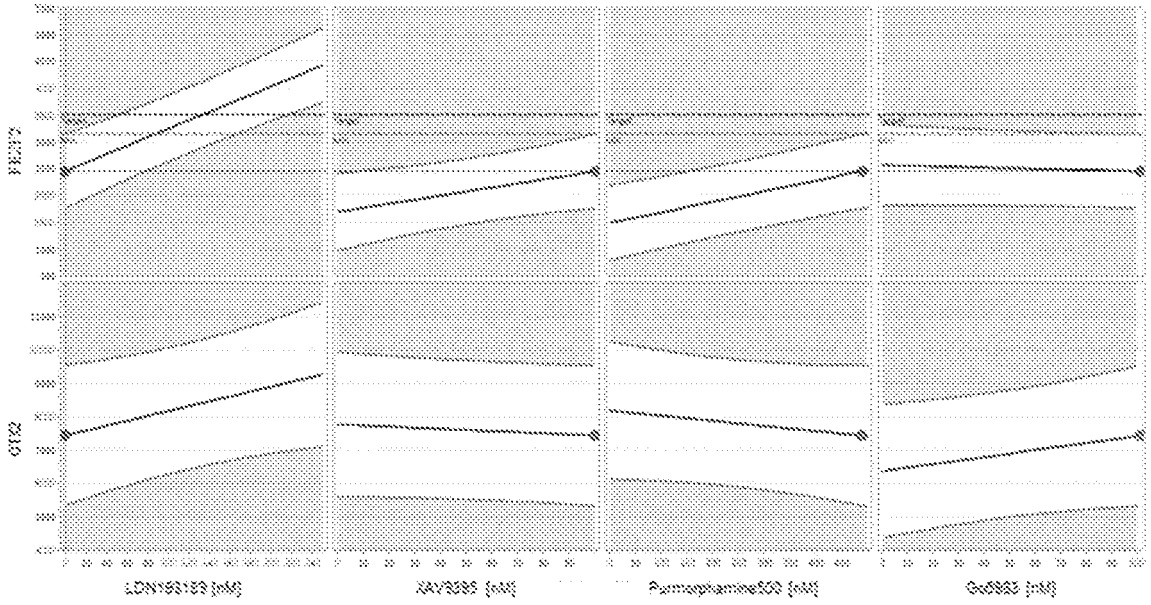


FIG. 8C

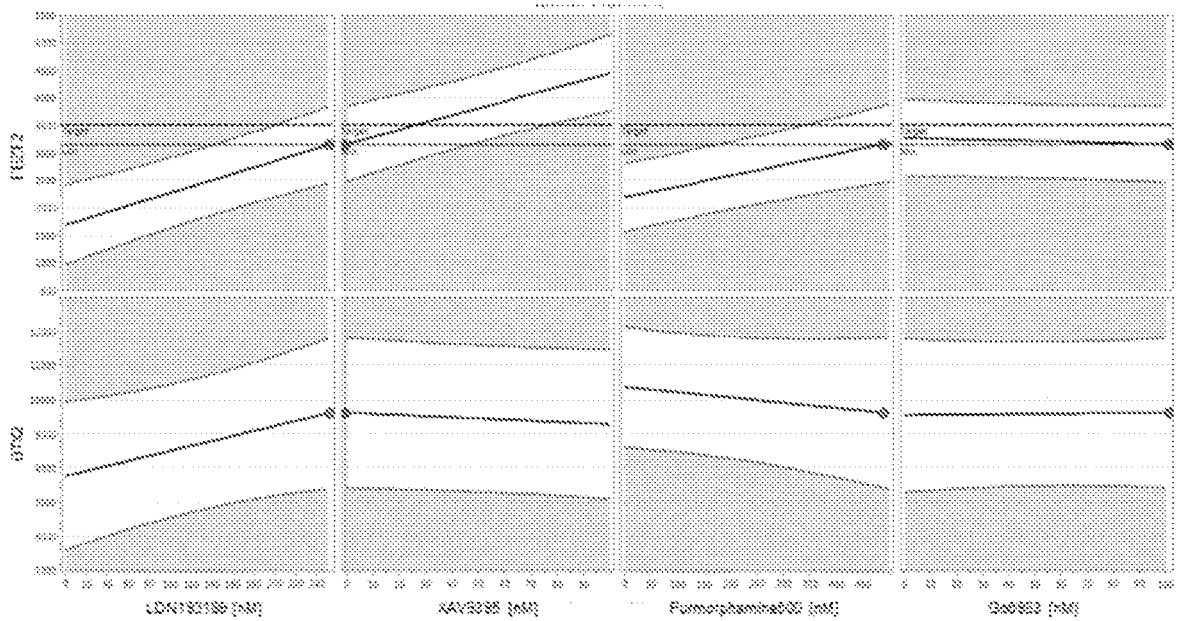
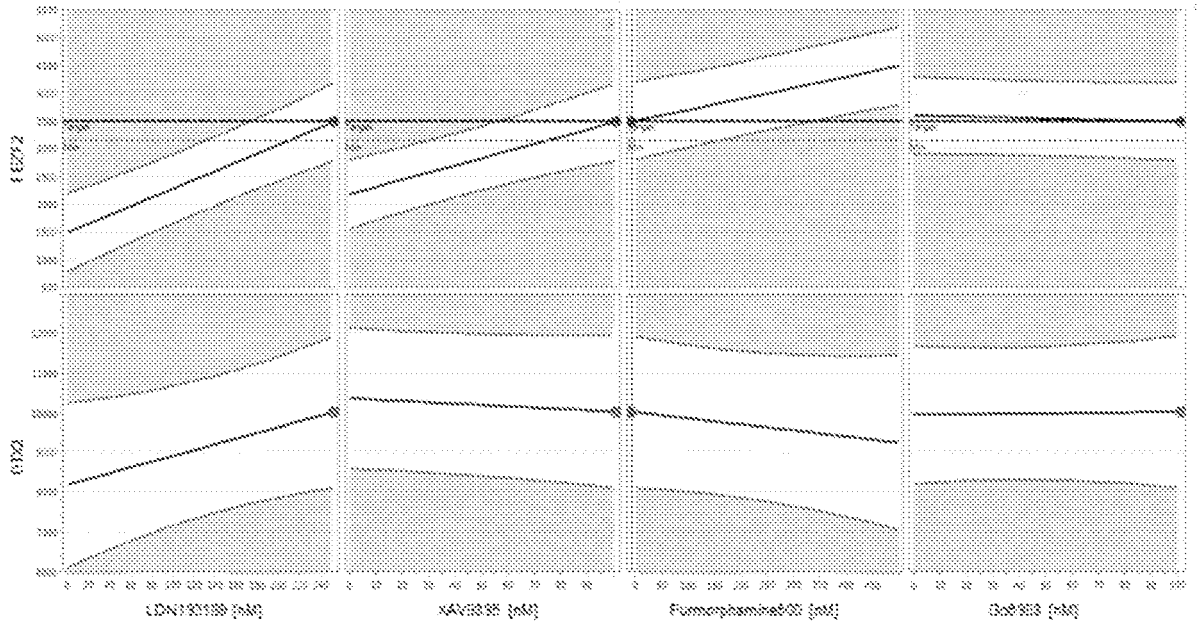


FIG. 8D



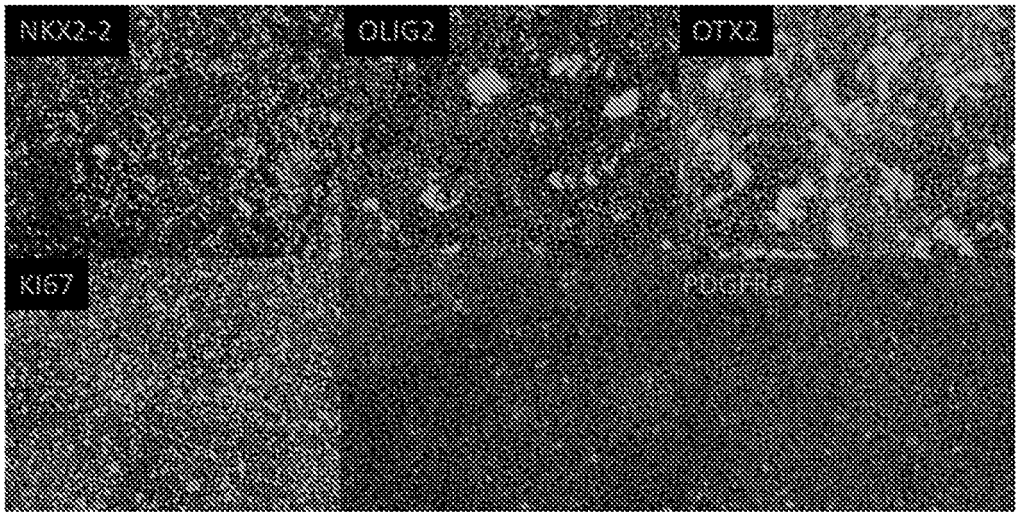


FIG. 9

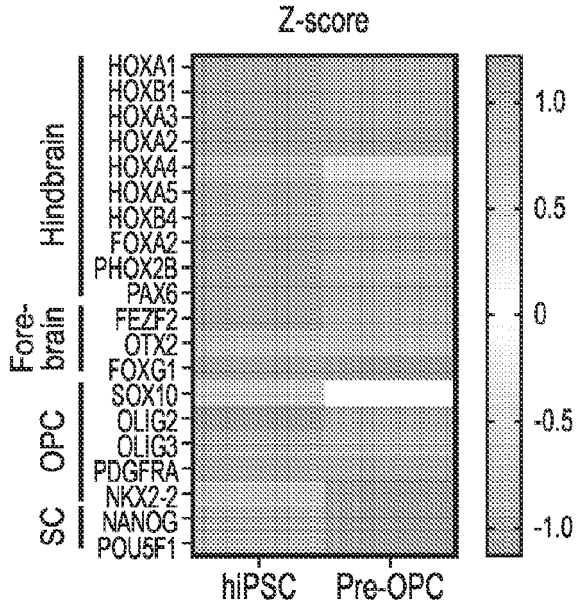


FIG. 10A

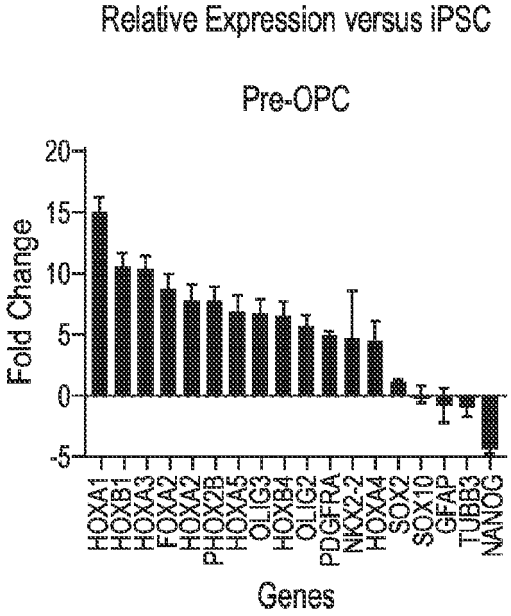


FIG. 10B