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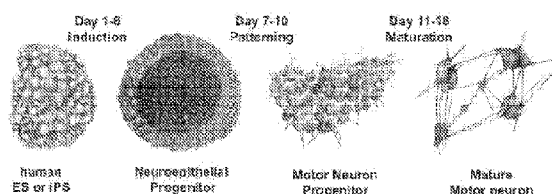
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FIG. 1 A



(57) Abstract: Described herein are suspension culture-based, directed differentiation protocols, methods, and systems useful in producing a highly pure MN population within 3 weeks. Also disclosed are compositions produced by the disclosed methods, protocols, processes, and systems, and compositions useful in their implementation. The resulting MN populations express surface markers that may be consistent with a motor neuron phenotype. In many embodiments, the phenotype may be specific for motor neurons from the thoracic and lumbar intersection of a mammal. Also disclosed is an effective model system for the investigation of RNA transcript localization in MN soma and neurites in an unbiased high-throughput manner.



## **A FAST AND SCALABLE MAMMALIAN MOTOR NEURON DIFFERENTIATION SYSTEM FOR MODELING NEUROPATHY**

### **CROSS-REFERENCE TO RELATED APPLICATIONS**

**[0001]** This application claims benefit of priority pursuant to 35 U.S.C. § 119(e) of U.S. provisional patent application No. Appln. No.: 62/992,694 entitled “A FAST AND SCALABLE MAMMALIAN MOTOR NEURON DIFFERENTIATION SYSTEM FOR MODELING NEUROPATHY,” filed on March 20, 2020, which is hereby incorporated by reference in its entirety.

### **FIELD**

**[0002]** The disclosed processes, methods, and systems are directed to production and use of stem cells, especially neural stem cells, in the study and treatment of various diseases.

### **BACKGROUND**

**[0003]** Human motor neuron diseases encompass a wide range of progressive disorders including amyotrophic lateral sclerosis (ALS) and diabetic motor neuropathies that lead to severe disabilities and/or death. Motor neurons (MNs) are unique in that their cell bodies are located within the motor cortex, brainstem and spinal cord, and extend long axonal projections to innervate peripheral tissues. In addition, RNA transcripts are transported to these axons where they are translated to provide highly regulated localization of specific functional proteins. Recently, several studies using animal models have suggested that the mislocalization of transcripts is a critical contributor to MN degeneration in several diseases, including Fragile X Syndrome, ALS and Myotonic Dystrophy (Fallini et al, Brain Res, 2012; Wang ET et al., J Neuroscience, 2016). A recent study using human iPSC generated from ALS patients or by mutation of the FUS gene also showed mislocalization of the Fos-B mRNA in ALS samples when compared to controls (Akiyama et al., EBiomedicine, 2019).

**[0004]** Transcript mislocalization could be a common mechanism for the loss of MN function in other disease settings, including ischemic diabetic neuropathy. However, a critical barrier to translating motor neuron studies in animal models to the human context is the lack of a rapid, scalable and functional *in vitro* human motor neuron model.

**[0005]** Human pluripotent stem cells (hPSCs) provide unique opportunities to investigate previously inaccessible aspects of motor neuron disease and can provide functional cell types for disease modeling and cell therapy approaches. Human pluripotent stem cells can be sub-classified into human embryonic stem cells (ESCs; that may be derived from the

inner cell mass of the blastocyst) and induced pluripotent stem cells (iPSCs; that may be generated by introducing dominant reprogramming factors into somatic cells that reverse their differentiated phenotype into a pluripotent state). While iPSCs and ESCs are comparable, iPSCs have the advantage of being patient-specific.

[0006] hPSCs are characterized by two key features: (1) rapid and indefinite cell division, thus having the potential to produce large numbers of cells, and (2) their ability to give rise to many cell types found in the human body when appropriate differentiation signals are provided. Indeed, there has been substantial progress in directed differentiation of hPSCs into diverse functional cell types using step-wise differentiation approaches that employ small molecules, growth factors, and extracellular signals to recapitulate key aspects of organogenesis *in vivo*.

[0007] Motor neurons (MNs) have been generated *in vitro* from hPSCs. However, current protocols are inadequate in that they require long maturation times and/or generate heterogeneous cell populations that contain non-MN cell types. The presence of these non-MN cell types complicate both experimental set-up and data interpretation. In addition, most differentiation protocols are conducted in conventional attachment cultures that are not amendable to the upscaling necessary for large experiments, unbiased genetic screens, and cell replacement therapies.

## SUMMARY

[0008] Disclosed herein are methods of producing mature motor neuron cells comprising aggregating a population of stem cells to form stem cell clusters; culturing the cell clusters in a progenitor media for a differentiation duration to differentiate the stem cell clusters into neuroepithelial progenitor cell clusters, culturing the neuroepithelial progenitor cells in an induction media that activates the Sonic Hedgehog and Retinoic Acid pathways for an induction duration to induce the neuroepithelial progenitor cells to be motor neuron progenitor cells, culturing the motor neuron progenitor cells in culture plates coated with an extracellular matrix-like substance in a maturation media for a maturation duration to mature the progenitor motor neuron cells into mature motor neuron cells; and thereby producing a mature motor neuron cell. In some embodiments, the stem cells may be selected from embryonic stem cells or induced pluripotent stem cells, in some embodiments the cells may be human cells, and the progenitor media may comprise one or more of Compound C (AMP-kinase inhibitor, BMP type I receptor inhibitor), SB431542 (activin receptor-like kinase receptor inhibitors), CHIR99021 (GSK-3 inhibitor) and Rock Inhibitor (RI), and the progenitor duration may be from 4 to 8 days. In some embodiments, the induction media may comprise one or more of Compound C, SB431542, SAG (Smoothed agonist, Hedgehog activation),

TTNPB (Retinoic Acid agonist), and Rock Inhibitor (RI), and the induction duration may be between 2 and 6 days. In some embodiments, the maturation media may comprise one or more of glucose, NEAA (non-essential amino acids), RI, SAG, TTNPB, BDNF (brain derived neurotrophic factor), and GDNF (glial derived neurotrophic factor), and the maturation duration may be between 2 and 40 days, in some embodiments about 6 days. In many embodiments, the methods may include a step of culturing the mature motor neuron cells in culture plates coated with an extracellular matrix-like substance in a maintenance media for a maintenance duration, wherein the maturation media may comprise one or more of a  $\gamma$ -Secretase Inhibitor, BDNF, and GDNF, and the maintenance period may be between 0 and 40 days. In many embodiments, the individual neuroepithelial progenitor cells may be frozen after the induction step and before the maturation step, and/or culturing the motor neuron progenitor cells may include plating, non-frozen individual neuroepithelial progenitor cells at about 20,000 cells to about 120,000 cells per  $\text{cm}^2$ , in some embodiments cells may be plated on an extracellular matrix or similar structure, in some embodiments the extracellular matrix or similar structure may be MATRIGEL, and frozen individual neuroepithelial progenitor cells may be plated at about 50,000 cells to about 200,000 cells per  $\text{cm}^2$ , in some embodiments cells may be plated on an extracellular matrix or similar structure, in some embodiments the extracellular matrix or similar structure may be MATRIGEL. In many embodiments, the methods may include dissociating the neuroepithelial progenitor cell clusters into individual neuroepithelial progenitor cells, before culturing the neuroepithelial progenitor cells. In many embodiments, the mature motor neuron cells may express the HOXC8 gene at least about 2 to 10-fold higher than any of HOX A1, B3, C4, C5, C6, B8 and D8, and/or the mature motor neurons may be phenotypically identified as lateral motor column motor neurons typically found in the thoracic/lumbar region of the spinal cord.

**[0009]** Also disclosed are methods of producing a population of motor neuron progenitor cells, comprising, aggregating a population of stem cells to form cell clusters, culturing the cell clusters in a progenitor media for a differentiation duration to differentiate the cells into neuroepithelial progenitor cell clusters, dissociating the neuroepithelial progenitor cell clusters into individual neuroepithelial progenitor cells, culturing the individual neuroepithelial progenitor cells in an induction media that activates the Sonic Hedgehog and Retinoic Acid pathways for an induction duration to induce the neuroepithelial progenitor cells to be motor neuron progenitor cells, collecting and freezing the motor neuron progenitor cells; and thereby, producing a mature motor neuron cell, wherein the stem cells may be mammalian stem cells, such as human cells. In some embodiments, wherein the cells may be human cells, the human may be a patient suffering from a disease or disorder related to one or more of motor neuron dysfunction selected from one or more of amyotrophic lateral sclerosis

(ALS), viral poliomyelitis, Guillain-Barre syndrome, Fragile X syndrome and diabetic motor neuropathies.

**[0010]** Also disclosed are cell compositions comprising a population of induced pluripotent stem cell-derived cells, wherein at least 70% to 99% of the cells express ISL1, OLIG2, and MNX; and the population of cells express greater than about 2-fold or more HOXC8 transcript than HOX A1, B3, C4, C5, C6, B8 and D8. In many embodiments, the disclosed cell composition may display global gene expression profiling resembling functionally mature MNs at the molecular level, for example when compared with published datasets from in vitro-derived MNs (Ziller et al., 2018) (Figure 2A) as evidenced by Spearman rho correlation coefficients values greater than 0.75. In many embodiments the presently disclosed motor neurons at d20 maybe defined as mature based on specific gene expression levels for one or more of ISL1, OLIG2, MNX, HOXC8, HOX A1, B3, C4, C5, C6, B8 and/or D8, or on global gene expression profiling similar to published datasets and having a rho correlation coefficient of greater than 0.75. In some embodiments the induced pluripotent stem cells may be derived from a subject having or at risk of developing a disease or disorder related to motor neuron dysfunction selected from one of more of amyotrophic lateral sclerosis (ALS), viral poliomyelitis, Guillain-Barre syndrome, Fragile X syndrome and diabetic motor neuropathies.

**[0011]** Also disclosed are methods for analyzing effects on motor neurons, comprising growing a first population of cells on a first substrate in a first medium, growing a second population of cells on a second substrate in a second medium, altering a growth condition for the first population of cells, wherein the first and second population of cells may be derived from stem cells, and wherein at least 70% of the cells in the population express ISL1, OLIG2, and MNX, and the population of cells express 10-fold more HOXC8 transcript than HOX A1, B3, C4, C5, C6, B8 and D8, allowing an amount of time to elapse, collecting a first cell material from a first surface of the first and second substrates, collecting a second cell material from a second surface of the first and second substrates, wherein the first cell material may be substantially cell soma, and the second cell material may be substantially cell neurite, analyzing the first cell material and the second cell material to determine an amount or concentration for at least one nucleic acid, peptide, compound, or biomarker; and comparing the amounts of the at least one nucleic acid, peptide, compound, molecule, or biomarker in the cell materials. In some embodiments, the cells of the first or second population may be derived from, human stem cells, pluripotent stem cells, embryonic stem cells, and progenitor cells, and/or may be matured in to MNs in less than about 21 days, and/or derived from a subject having or at risk of developing a disease or disorder related to motor neuron dysfunction selected from one of more of amyotrophic lateral sclerosis (ALS),

viral poliomyelitis, Guillain-Barre syndrome, Fragile X syndrome and diabetic motor neuropathies.

### BRIEF DESCRIPTION OF THE DRAWINGS

**[0012]** FIG. 1 shows suspension culture based direct differentiation approach of pluripotent stem cells into human motor neurons. Panel (A) Schematic outlining the direct differentiation approach. Panel (B) Representative images taken at the indicated magnification at key stages of the differentiation protocol. Panels (C and D) Immuno-fluorescence analysis for neuronal markers of d20 MNs. Scale bar= 20um. Panel (E) Quantification of HB9 and ISL1 positive cells over DAPI at day 20.

**[0013]** FIG. 2 shows direct differentiation results in functional, spatially defined human motor neurons. Panel (A) Global gene expression analysis of differentiated MNs in comparison to previously published datasets of hESC derived motor neurons (Ziller *et al.*, 2018). Gene expression values from each sample were correlated. Values represent Spearman rho correlation coefficients. Panel (B) Quantitative PCR analysis of pluripotency markers NANOG and OCT4 normalized to TTNPB at subsequent stages of differentiation. n=3-4, data is presented as Standard Error of the Mean (SEM). Panel (C) Quantitative PCR for reveals expression of mature MN markers OLIG2 and MNX1 at d14 which is maintained at d23, at which point ISL1 expression also becomes highly enriched. n=3-4, data is presented as SEM. Panels (D-D'') Representative images of calcium imaging before, during and after depolarization. Panel (E) Whole-cell patch clamp recordings of cultured MNs. An example of evoked action potentials with a 50pA step. Four sweeps overlaid. Y-axis=mV (millivolts), X-axis=msec. Panels (E' and E'') Additional example of evoked single or multiple action potentials from MNs. Action potentials were evoked by 25-50pA steps. Y-axis=mV (millivolts), X- axis=msec. Panel (F) Quantitative PCR analysis for HOX gene expression of mature MNs reveals a strong enrichment of HOXC8. Schematic showing the developmental distribution of HOX genes in humans. n=4-8, data is presented as SEM.

**[0014]** FIG. 3. shows global analysis of motor neuron soma and neurite enriched RNAs. **Panel (A)** Fractionation schematic. Neurons are plated on porous membranes that allow neurite growth through the membrane but restrict soma to the top of the membrane. Cells are then mechanically fractionated, and RNA and protein from each fraction is isolated and analyzed. Panel (B) Dot blot of soma and neurite protein fractions from three independent fractionation experiments. Beta-actin is present in both fractions while histone H3 is restricted to the soma fraction. Panel (C) Hierarchical clustering of gene expression values from motor neuron RNA fractionations from ES derived and Panel (D) control iPS and T1D iPS. Values represent Spearman rho correlation coefficients. Panel (E) Comparison of gene

expression values in soma and neurite samples for ES, iPS control Panel (E') and T1D iPS cells Panel (E''). Genes significantly enriched ( $p < 0.01$ ,  $\log_2$  fold change  $> 1.5$ ) in either fraction are represented in purple. Three known neurite-enriched genes (NGRN, ACTB, and RANBP1) are highlighted and are neurite-enriched in this data. Panel (F) LR values for genes from two gene ontology categories: electron transport chain and structural constituent of ribosome in all three cell types Panels (F, F', F''). These ontologies categories have been previously observed to contain many neurite-enriched genes. Panel (G) Comparison of LR values from motor neuron data with previously published LR values from E15.5 mouse cortical neurons. Only one-to-one human-to-mouse orthologs are considered.

**[0015]** FIG. 4 shows hypoxia induces significant changes in gene expression and RNA localization in motor neurons. Panel (A) Principal component analysis (PCA) of gene expression values from soma and neurite samples of normoxia and hypoxia-treated ES, Panel (A') iPS control and Panel (A'') iPS T1D motor neurons. Panel (B) LR values from normoxia and hypoxia-treated motor neurons. Genes with significant ( $p < 0.05$ ) changes in LR between conditions are colored in purple. Panels (C-E) Nuclear-encoded genes related to mitochondrial function, including those involved in the electron transport chain and the mitochondrial translation machinery are more localized to neurites upon hypoxia treatment, while those involved in cytosolic translation show no change in localization. In ES derived MNs, mitochondrially-encoded genes are significantly less localized to neurites but show no significant changes in control iPS Panel (D) and T1D iPS Panel (E). P-values are Wilcoxon rank sum values. Panel (F) Representative images and Panel (G) quantification of mitochondrial stain in normoxic and hypoxic conditions show a significant reduction of mitochondria stain intensity in the neurites cultured under hypoxic conditions.

**[0016]** FIG. 5 shows generation of patient specific induced pluripotent stem cells. Panel (A) Schematic outlining the reprogramming approach and micrograph of emerging clonal, patient specific iPSC colony. Panel (B) Immunofluorescence analysis of pluripotency markers NANOG, OCT4 and SOX2 in established iPSC and control hESC cells. Panel (C) Flow based quantitative analysis for the pluripotency marker TRA1-60 and the endodermal marker gene SOX17 in iPSC and control hESC. Panel (D) qPCR analysis for pluripotency markers NANOG, OCT4 and SOX2 of iPSC and control hESC. Panel (E) Established iPSCs exhibit a normal karyotype as assayed by G-Band karyotyping.

**[0017]** FIG. 6 shows suspension culture based direct differentiation approach of in-house reprogrammed, patient specific iPSCs into human motor neurons. Panel (A) Representative images taken at the indicated magnification at key stages of the differentiation protocol. Panel (B) Quantitative PCR analysis of pluripotency markers NANOG and OCT4 normalized to TTNPB at subsequent stages of differentiation.  $n=2$ , data is presented as SEM. Panel (C)

Quantitative PCR reveals expression of MN progenitor marker OLIG2 peaks at d14 and becomes significantly reduced by d23. Panel (C') Mature MN markers ISL1 and MNX1 expression is detectable at d14 and is maintained at d23, at which point CHAT expression also becomes highly enriched. n=2, data is presented as SEM. Panel (D) Immuno-fluorescence analysis for neuronal markers of d20 MNs.

**[0018]** FIG. 7 shows quality control of soma / neurite fractionation. mRNAs that encode ribosomal proteins and components of the electron transport chain are enriched in neurites. Panel (A) mRNAs that encode ribosomal proteins and electron transport chain components Panel (B) are strongly enriched in neurite samples. Significantly enriched ( $p < 0.01$ , log2 fold change  $> 1.5$ ) genes are in purple. Both of these categories have been repeatedly seen as neurite-enriched in previous neuron fractionation studies, demonstrating the validity of these fractionations and samples.

**[0019]** FIG. 8 shows changes in gene expression in response to hypoxia. Cellular responses to hypoxia at the level of RNA metabolism. Panel (A) Gene ontology analysis of genes that were differentially expressed between normoxia and hypoxia conditions. Terms related to the response to hypoxia and glycolysis were the two most highly enriched categories. Panel (B) Hierarchical clustering of LR values from normoxia and hypoxia-treated ES derived and Panel (C) iPS control and T1D motor neurons. Panel (D) LR values from normoxia and hypoxia-treated iPS control and Panel (E) iPS T1D motor neurons. Genes with significant ( $p < 0.05$ ) changes in LR between conditions are colored in purple. Panel (F) Differentially expressed genes between normoxia and hypoxia samples. Significantly differentially genes ( $p < 0.01$ , log2 fold change  $> 1.5$ ) are in purple. Panel (G) Differentially expressed genes that contain the GO term "response to hypoxia". Panel (H) Differentially expressed genes that contain the GO term "glycolytic process". Panels (I, J) Gene expression differences between normoxia and hypoxia conditions in neurite Panel (I) and soma Panel (J) samples. In neurite samples, genes involved in the electron transport chain and mitochondrial translation are more expressed in hypoxia conditions relative to normoxia. However, in soma samples, there is no difference in expression. Genes involved in cytosolic translation are upregulated in response to hypoxia in both neurite and soma compartments. Mitochondrially-encoded genes are downregulated in both compartments, but the level of downregulation is greater in neurite than in soma.

**[0020]** FIG. 9 is a schematic diagram of one embodiment of the presently disclosed compositions, methods, processes, and systems.

## DETAILED DESCRIPTION

[0021] Described herein is a suspension culture-based, directed differentiation protocol that produces a highly pure MN population within about 3 weeks or less, for example 23 days or less. The resulting MN population expresses surface markers consistent with the phenotype of motor neurons, and specifically for motor neurons from the thoracic and lumbar intersection. Also disclosed is an effective model system for the investigation of RNA transcript localization in MN soma and neurites in an unbiased high-throughput manner.

[0022] Human motor neuron diseases encompass a spectrum of disorders including amyotrophic lateral sclerosis, viral poliomyelitis, Guillain-Barre syndrome, Fragile X, and diabetic motor neuropathies. A critical barrier to dissecting the mechanisms of these diseases affecting motor neurons is the lack of a rapid, scalable, and functional human motor neuron models *in vitro*.

[0023] The described compositions, methods, protocols, and systems provide for scalable suspension-based generation of large numbers of human pluripotent stem cell (hPSC)-derived neuronal progenitors. The disclosed progenitors may be differentiated into functional human motor neurons (MNs), and the entire process requiring less than about 3 weeks. Using this human motor neuron model, Applicants show that experiments disclosed herein show that mRNA mis-localization plays a role in disease development and progression to the human context. Specifically, a membrane-based culture system was created that allows efficient fractionation of MN cell soma and neurites in normal versus hypoxic growth conditions that mimic hypoxia seen in diabetic neuropathies caused by microvascular damage in the periphery. In response to hypoxia, it was discovered that MNs upregulated mitochondrial transcripts, predominantly in neurites. In contrast, the amount of mitochondrial proteins decreased, suggesting that hypoxia may disrupt translation of mitochondrial mRNA into mitochondrial proteins, potentially leading to neurite damage and development of neuropathies.

[0024] Human motor neuron diseases encompass a wide range of disorders including ALS, viral poliomyelitis, Guillain-Barre syndrome and diabetic motor neuropathies. These progressive diseases are debilitating and often irreversible; thus, increasing understanding of mechanisms of motor neuron disease may be useful in reducing patient mortality and morbidity. Described herein are compositions, methods, processes, and systems to reliably and quickly generate highly pure populations of spatially defined, functional human motor neurons that can be used to assess mechanisms associated with various motor neuron diseases. The disclosed methods, processes, systems, and compositions are useful for translating animal studies of motor neurons into the human context, and investigating disease mechanisms, as well as various therapies, optionally in a patient-specific manner.

[0025] The present study improves upon protocols for production of MNs from hPSCs with surprisingly unexpected results. The presently disclosed methods, processes, and systems were useful in generating surprisingly large numbers of, and purer populations of, MN progenitor cells, which may be frozen for future use. Moreover, the compositions, methods, processes, and systems are able to generate mature motor neurons in just 18 days or in some embodiments less than about 25 days, 24 days, 23 days, 22 days, 21 days, 20 days, 19 days, 18 days, 17 days, 16 days, 15 days, or 14 days, and more than about 13 days, 14 days, 15 days, 16 days, 17 days, 18 days, 19 days, 20 days, 21 days, 22 days, 23 days, or 24 days. This accelerated (compared to prior techniques) differentiation of hPSC-derived motor neurons produced cells with a global RNA expression signature comparable to previously reported MNs, and also expressed MN-characteristic proteins, including HB9 and ISL1. Direct evaluation of the cells' functional properties (e.g. by intracellular calcium imaging and direct patch clamping) verified that these *in vitro* generated MNs were also functional. In addition, the protocol produced a spatially defined subpopulation of MNs expressing high levels of HOXC8 that are usually located in the thoracic/lumbar region of the spinal cord, and specifically innervate muscles of the legs. These thoracic/lumbar MNs provide an ideal model system to study neuropathies of the lower extremities.

**Suspension culture-based generation of highly pure, spatially-defined, functional human motor neurons from pluripotent stem cells**

[0026] To generate motor neurons in a high throughput and rapid fashion from human pluripotent stem cells (hPSC), a scalable three-dimensional suspension culture system was employed. Independent of culture method (feeder-dependent or defined, feeder-free media) or hPSC type (ESC or iPSC), single cell suspensions were aggregated overnight to form individual clusters of cells containing approximately 5,000 cells. In other embodiments, the culture system may be other than a suspension system. In some embodiments, the cells may be cultured as adherent cells, for example starting from a single cell suspension at about 100k-500k cells per cm<sup>2</sup>, for one embodiment about 300,000 cells per cm<sup>2</sup>. In many embodiments, the cells may be pluripotent stem cells and may be cultured in a growth media (for example mTeSR<sup>TM</sup>1 or mTeSR<sup>TM</sup>plus). In many embodiments, the culture media may comprise about 10uM RI. The cells may be cultured, for example in growth media comprising 10µm RI, for from about 18 to 100 hours, for example 24-96 hours. In most embodiments, the cells may be cultured as adherent cells in tissue culture plates. After about 24-96 hours, the cells may be cultured in a progenitor media, for example Progenitor Media, as described below.

[0027] In many embodiments, the clusters may contain between about 300 and 30,000 cells, for example 1,000 to 10,000. In some embodiments the clusters may contain more

than about 300 cells, 400 cells, 500 cells, 600 cells, 700 cells, 800 cells, 900 cells, 1000 cells, 1500 cells, 2000 cells, 2500 cells, 3000 cells, 3500 cells, 4000 cells, 4500 cells, 5000 cells, 10,000 cells, 20,000 cells, or 30,000 cells and less than about 40,000 cells, 30,000 cells, 20,000 cells, 10,000 cells, 5000 cells, 4500 cells, 4000 cells, 3500 cells, 3000 cells, 2500 cells, 2000 cells, 1500 cells, 1000 cells, 900 cells, 800 cells, 700 cells, 600 cells, 500 cells, 400 cells, or 300 cells. In some embodiments, the cells may be grown in an AggreWell system. In many embodiments, cell clusters may be defined as masses of cells that require mechanical or enzymatic disaggregation.

**[0028]** Aggregated or adherent culturing was followed by a stepwise differentiation scheme as described at FIG.1 Panel A. Briefly, during the first 6 days (FIG. 1 Panel B), cultures were differentiated into neuroepithelial (e.g. epithelium that has the capacity to further differentiate into nerve tissue and is marked by expression of neuroepithelia markers known to those of skill in the art, without wishing to be limited, for example the genes PAX6 and SOX1, and cells may also be marked by the loss of expression of pluripotency markers, again without wishing to be limited, e.g. the gene OCT4) progenitors by dual sMAD inhibition. sMAD inhibition may be achieved by culturing in a progenitor media. In many embodiments, the progenitor media is Progenitor Media comprising Knock Out media or regular DMEM (in many embodiments the media may comprise about 25% or more DMEM), 0.1-10x N2-A or N21 or SM1 or B27 Supplement (for example in one embodiment about 1x), about 20nM to 20uM Glutamine, and about 10uM to 1 M ascorbic acid and one or more of Compound C(AMP-kinase inhibitor, BMP type I receptor inhibitor), SB431542 (activin receptor-like kinase receptor inhibitors), CHIR99021 (GSK-3 inhibitor) and Rock Inhibitor (RI). In some embodiments, differentiation into neuroepithelial cells, or differentiation duration, may be more than about 4 days, and less than about 8 days, for example more than 4, 5, 6, or 7 days, and less than about 8, 7, 6, or 5 days. In one embodiment, the cells and/or clusters may be cultured for about 6 days. In most embodiments a one day duration may be about 18 to about 30 hours, a two day duration from about 40 to 56 hours, a three day duration from about 60 to about 84 hours, a four day duration from about 84 to about 108 hours, a five day duration from 108 hours to about 132 hours, a six day duration from about 132 to about 156 hours, a seven day duration from about 156 to about 180 hours, and an eight day duration from about 180 to about 204 hours.

**[0029]** In one embodiment, at about day 7 (d7), clusters were observed to exhibit obvious morphological changes that included visible cell layers. In other embodiments, these changes may be observed at day 4 to day 10. The organization of cell layers indicates distinct cell organization and potential polarization of the developing epithelium (FIG. 1 Panel B'). Thereafter, efficient patterning into motor neuron progenitor cells (MNPs) was achieved

by activating the Sonic Hedgehog (SHH) and Retinoic Acid (RA) pathways and inhibiting the ROCK pathway by incubation in an induction media. In some embodiments, this may be referred to as induction of differentiation, for example induction of differentiation into MN progenitor cells. In many embodiments, the induction media is Induction Media comprising Knock Out media or DMEM, about 0.1-10x N2-A, N21, SM1, or B27 Supplement (in some embodiments the supplement is 1x in the Induction Media), about 20nM to 20uM Glutamine, and about 10uM to 1 M ascorbic acid, and may comprise one or more of Compound C(AMP-kinase inhibitor, BMP type I receptor inhibitor), SB431542 (activin receptor-like kinase receptor inhibitors), CHIR99021 (GSK-3 inhibitor), Rock Inhibitor (RI), SAG, TTNBP, AMPK, and TGFb. In many embodiments, the duration for culturing in an induction media, the induction duration, may be from about 2 days to about 6 days, in some embodiments about 3 days.

**[0030]** Induction of differentiation into MN progenitor cells may be complete, in one embodiment, at about d10 (day 10). At this point, the cell clusters were dissociated into single cells, yielding ~10e6 MNPs per seeded 1e6 hPSCs (i.e. ~ten-fold cell expansion). In some embodiments, the cells may be expanded more than about 5 fold, 6 fold, 7 fold, 8 fold, 9 fold, 10 fold, 15 fold or 20 fold and less than about 25 fold, 20 fold, 15 fold, 10 fold, 9 fold, 8 fold, 7 fold or 6 fold. In many embodiments, dissociation of the progenitor cells may not be performed and the clusters remain substantially intact (i.e. they may lose less than about 20% of their cells, volume, or mass). At this stage, MNPs (single cells or clusters) may be frozen down or processed for further maturation for example into motor neurons. Cells or clusters processed for further maturation into motor neurons (MN) may be plated onto a culture receptacle where the receptacle may be coated with a matrix- or extracellular matrix-like substance in a maturation media. In some embodiments the maturation media may be Maturation Media comprising Knock Out media or DMEM, about 0.1-10x N2-A, N21, SM1, or B27 Supplement (in many embodiments the supplement may be 1x), about 20nM to 20uM Glutamine, and about 10uM to 1 M ascorbic acid, and may include one or more of glucose (about 10nM to 1m), non-essential amino acids, RI, SAG, TTNBP, BDNF, and GDNF. In some embodiments, after about 2-4 days in Maturation Media, the cells may be cultured in a maturation media that may exclude one or more of glucose, non-essential amino acids, RI, SAG, or TTNBP. In many embodiments, the matrix like substance may be MATRIGEL™. In many embodiments, a maturation media may comprise one or more of gamma secretase inhibitors, brain derived neurotrophic factor (BDNF) and glia cell derived neurotrophic factor (GDNF). The duration the cells or clusters are in maturation media, the maturation duration, may be from about 1 day to about 40 days. In many embodiments, the maturation duration may be 1-5 days, for example 3 days.

**[0031]** Plated MNPs gave rise to neurite outgrowths after about 3 days culture in differentiation media (FIG. 1 Panel B"). Neurite outgrowth indicates differentiation of progenitors into mature neurons with native-like motor neuron function (see below). These mature motor neurons further self-organize into discrete structures of dense MN cell body clusters that are highly interconnected through extended neurites (FIG. 1 Panel B").

**[0032]** Growth of fresh MNPs and frozen/thawed MNPs was compared. These tests showed that fresh MNPs can be plated at a density of about 20,000 cells to about 120,000 cells, in one example about 53,000 cells, per cm<sup>2</sup> for MN differentiation, whereas d10 MNPs that were frozen may be thawed and plated at a higher density of about 50,000 cells to about 200,000 cells, in one embodiment about 106,000 cells, per cm<sup>2</sup> (i.e. 2x, for example from greater than about 1.5x, 2.0x, 2.5x, 3.0x, 3.5x, or 4.0x and less than about 4.5x, 4.0x, 3.5x, 3.0x, 2.5x, or 2.0x). In some embodiments, the fresh MNPs may be plated at a density of greater than about  $2.0 \times 10^4$ ,  $2.5 \times 10^4$ ,  $3 \times 10^4$ ,  $3.5 \times 10^4$ ,  $4 \times 10^4$ ,  $4.5 \times 10^4$ ,  $5 \times 10^4$ ,  $5.5 \times 10^4$ ,  $6 \times 10^4$ ,  $6.5 \times 10^4$ ,  $7 \times 10^4$ ,  $7.5 \times 10^4$ ,  $8 \times 10^4$ ,  $8.5 \times 10^4$ ,  $9 \times 10^4$ ,  $9.5 \times 10^4$ ,  $10 \times 10^4$ ,  $10.5 \times 10^4$ ,  $11 \times 10^4$ , or  $11.5 \times 10^4$  and less than about  $12 \times 10^4$ ,  $11 \times 10^4$ ,  $10 \times 10^4$ ,  $9.5 \times 10^4$ ,  $9 \times 10^4$ ,  $8.5 \times 10^4$ ,  $8.0 \times 10^4$ ,  $7.5 \times 10^4$ ,  $7.0 \times 10^4$ ,  $6.5 \times 10^4$ ,  $6.0 \times 10^4$ ,  $5.5 \times 10^4$ ,  $5.0 \times 10^4$ ,  $4.5 \times 10^4$ ,  $4 \times 10^4$ ,  $3.5 \times 10^4$ ,  $3 \times 10^4$ , or  $2.5 \times 10^4$  cells per cm<sup>2</sup> and frozen/thawed MNPs may be plated at a density of greater than about  $5 \times 10^4$ ,  $6 \times 10^4$ ,  $7 \times 10^4$ ,  $8 \times 10^4$ ,  $8.5 \times 10^4$ ,  $9 \times 10^4$ ,  $9.5 \times 10^4$ ,  $10 \times 10^4$ ,  $11 \times 10^4$ ,  $12 \times 10^4$ ,  $13 \times 10^4$ ,  $14 \times 10^4$ ,  $15 \times 10^4$ ,  $16 \times 10^4$ ,  $17 \times 10^4$ ,  $18 \times 10^4$ ,  $19 \times 10^4$ , or  $20 \times 10^4$  and less than about  $20 \times 10^4$ ,  $19 \times 10^4$ ,  $18 \times 10^4$ ,  $17 \times 10^4$ ,  $16 \times 10^4$ ,  $15 \times 10^4$ ,  $14 \times 10^4$ ,  $13 \times 10^4$ ,  $12 \times 10^4$ ,  $11 \times 10^4$ ,  $10 \times 10^4$ ,  $9 \times 10^4$ ,  $8 \times 10^4$ ,  $7 \times 10^4$  or  $6 \times 10^4$  cells per cm<sup>2</sup>. Plating previously frozen clusters or cells at higher density may help counter cell loss, for example from freezing and thawing, however, freezing and/or high density plating does not substantially impair subsequent differentiation into MNs.

#### **hPSC culture**

**[0033]** Human embryonic stem cells were grown as previously described (Russ *et al.*, 2015) or in Nutristem (Corning) or mTeSR™1 or mTeSR™plus (Stemcell Technology Inc.) media on culture plates. In some embodiments, the culture plates may be coated with one or more substances. In some embodiments, the substance is an extracellular matrix-like composition, for example a MATRIGEL™ such as hES-qualified MATRIGEL cell culture plates (Corning). Induced pluripotent stem cells were grown in mTeSR™1 or mTeSR™plus (Stem Cell Technology) media on hES-qualified Matrigel (Corning) coated cell culture plates. Clusters were formed using AggreWells800. To do so, 70-100% confluent pluripotent stem cell cultures were washed once with PBS (GIBCO) followed by incubation for 6 min at 37°C in TrypLE (GIBCO) to generate a single-cell suspension. Digested cultures were quenched with a growth media (mTeSR™1 or mTeSR™plus available from StemCell Technologies Inc.), and live cells counted using a MOXI Z cell counter (Orflow Technologies). The disclosed cells may be added at various concentrations, for example in some embodiments from about

300 to 30,000 cells per microwell may be added to wells of a 24-well AggreWell400 or 800 plate (Stem Cell Technology), for example about 3000 cells. In some embodiments greater than about 300 cells, 500 cells, 1000 cells, 1500 cells, 2000 cells, 2500 cells, 3000 cells, 3500, 4000 cells, 5000 cells, 6000 cells, 7000 cells, 8000 cells, 9000 cells, 10,000 cells, 15,000 cells, 20,000 cells, or 25,000 cells, and less than about 30,000 cells, 25,000 cells, 20,000 cells, 15,000 cells, 10,000 cells, 9,000 cells, 8,000 cells, 7,000 cells, 6,000 cells, 5,000 cells, 4,000 cells, 3500 cells, 3000 cells, 2500 cells, 2000 cells, 1500, 1000 cells, 900 cells, 800 cells, 700 cells, 600 cells, or 500 cells may be added to the wells of a 24-well AggreWell400 or 800 plate (Stem Cell Technology). The cells in the AggreWell400 or 800 plate may be spun down for about 3 min at about 1,200 rpm and incubated overnight in the presence of about 10uM Rock Inhibitor Y- 27632 (RI, Rock Inhibitor, or Rock Y-27632; Tocris Bioscience or Selleck Chemicals) to allow cluster formation before proceeding with direct differentiation. hPSC cultures were regularly tested for mycoplasma contamination and found negative. In some embodiments, the concentration of Rock Inhibitor may be about 0.1  $\mu$ M to 50  $\mu$ M, and in one embodiment the concentration is about 10  $\mu$ M.

### **Motor neuron differentiation**

#### **Base Media**

[0034] KO DMEM (Gibco), 1x N2-A Supplement (Stem Cell Technologies), 1x SM1 (Stem Cell Technologies), 1x Glutamax (Gibco) and 50mM Ascorbic acid (Sigma).

#### **Differentiation protocol**

[0035] Clusters were washed once with base media and subsequently placed in a Progenitor Media, for example Progenitor Media may comprise Base Media Compound C, SB431542, CHIR99021 and RI ("Rock Inhibitor" or "Rock Y-27632"). Clusters were then placed on a shaker for about 6 days. Afterwards, clusters were placed in Induction Media, which may for example comprise Base Media comprising Compound C, SB431542, RI (Rock Inhibitor or Rock Y-27632), SAG and TTNPB for 3 days. At about day 10, the clusters were dissociated using Accumax for 12min at 37C. Live cells were counted, and about 53,000 or 106,000 cells per  $\text{cm}^2$  of fresh or frozen MNPs, respectively, were plated on Matrigel (Corning) coated dishes in Maturation Media, which may comprise, for example, Base Media comprising glucose, non-essential amino acids (NEAA: 10.0 mM glycine, 10.0 mM L-alanine, 10.0 mM L-asparagine, 10.0 mM L-aspartic acid, 10.0 mM L-glutamic acid, 10.0 mM L-proline, 10.0 mM L-serine), RI, SAG, TTNPB, BDNF and GDNF for about 4 days. At about day 14, cells were switched into base media containing gamma-secretase inhibitor, BDNF/GDNF and RI for about 3 days after which, cells were cultured in Base Media with BDNF/GDNF alone until about d18-21 when they were analyzed. See Table 1, below, for

company and concentration details for reagents for use with one embodiment of the disclosed methods and compositions.

Table 1

| Reagent                          | Company               | Cat#     | Final Concentration |
|----------------------------------|-----------------------|----------|---------------------|
| Knock Out DMEM F12               | Gibco                 | 12660012 |                     |
| Glutamax                         | Gibco                 | 35050061 | 1X                  |
| 2-Phospho-L-Ascorbic Acid        | SIGMA-ALDRICH         | 49752    | 50mM                |
| N2 Supplement A                  | Stemcell technologies | 7152     | 1X                  |
| SM1 supplement                   | Stemcell technologies | 5710     | 1X                  |
| SAG                              | Stemcell technologies | 73412    | 200nM               |
| Dorsomorphin (Compound C)        | Stemcell technologies | 72102    | 1uM                 |
| TTNPB                            | Stemcell technologies | 72892    | 1.5uM               |
| BDNF                             | Stemcell technologies | 78005    | 0.02ug/mL           |
| GDNF                             | Stemcell technologies | 78058    | 0.02ug/mL           |
| CHIR99021                        | Stemcell technologies | 72052    | 4uM                 |
| SB431542                         | Stemcell technologies | 72232    | 10uM                |
| $\gamma$ -Secretase Inhibitor XX | Calbiochem            | 565789   | 2uM                 |
| Rock Y-27632                     | Selleck Chemical LLC  | S1049    | 5uM                 |

#### Quantitative PCR

[0036] RNA was isolated using the Qiagen RNA MINI extraction kit. cDNA was synthesized using the iScript (Biorad) kit. 4ng of cDNA was used per reaction and reactions were performed in duplicate. Data was normalized to control housekeeping genes ACTIN or GAPDH using the delta Ct method. Primer and probe information can be found in Table 2, below.

Table 2

| SYBR PRIMERS        |                                   |                                 |
|---------------------|-----------------------------------|---------------------------------|
| Gene                | Forward                           | Reverse                         |
| hTBP                | CCTGCCGATAACTATCTCTGGC            | GTTCCACGGATGCTTTCTCG            |
| hHOXA1              | AAATCAGGACCACACAGAC               | GTAGCCCTCTCCAACCTTC             |
| hHOXB3              | GGCAAACGTCCAAGCTGAA               | CTCCAGCTCCACCAGCTGCG            |
| hHOXC4              | GAGAGAGGTAGTGAGCGTCC              | CTGGCCCAGCTTTTCACAAT            |
| hHOXC5              | ACAGATTACCCGTGGATGAC              | AGTGAGGTAGCGTTAAAGTG            |
| hHOXC6              | GAATGAGGGAAGACGAGAAAGAG           | CATAGGCGGTGGAATTGAGG            |
| hHOXC8              | TTTATGGGGCTCAGCAAGAGG             | TCCACTTCCTTCGGTTCTG             |
| hHOXC9              | AGCACAAAGAGGAGAAGGC               | CGTCTGGTACTTGGTGTAGG            |
| hHOXB8              | TAA GCG GCG AAT CGA GGT AT        | TGT TTC TCC AGC TCC TCC TG      |
| hHOXD8              | CCT GAC TGT AAA TCG TCC AGT GGT A | AGT TTG GAA GCG ACT GTA GGT TTG |
|                     |                                   |                                 |
| <b>Taqman probe</b> | <b>Biorad FAM</b>                 |                                 |
| NANOG               | CEP0050656                        |                                 |
| OCT4                | CEP0041056                        |                                 |
| ISL1                | CEP0049263                        |                                 |
| OLIG2               | CEP0025687                        |                                 |
| MNX1                | CEP0057720                        |                                 |

|      |            |  |
|------|------------|--|
| CHAT | CEP0049559 |  |
| TBP  | CIP0036255 |  |

## RNA Sequencing

### Cell Fractionation

[0037] To fractionate neuronal cells, media was removed and both sides of the membranes were rinsed with PBS. 1 mL PBS was placed on top of the membrane. Cell bodies were scraped in the PBS from the top of the membrane using a cell scraper. The membrane, still containing neurites, was then cut out of its plastic housing and incubated with RNA Lysis buffer (Zymo Research) at room temperature for 15 min. Between 6 and 12 membranes were combined for a single preparation, depending on the cell density on the membranes. RNA was purified from both the soma and neurite fractions using a Quick RNA Microprep kit (Zymo Research). Typically, between 200 and 500 ng total RNA was collected from neurite fractions in a single preparation.

### RNAseq library construction

[0038] Stranded, polyA-selected RNAseq libraries were prepared from 100 ng total RNA using the Kapa mRNA Hyperprep kit (Roche). The final PCR amplification of the libraries consisted of 15 cycles. The libraries were sequenced to a depth of 20-35 million 150bp paired end read pairs per sample on a NovaSeq sequencer (Illumina).

### Analysis of high-throughput sequencing data

[0039] Adapter sequences (AGATCGGAAGAGCACACGTCTGAACTCCAGTCA and AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT) were removed using cutadapt (Martin M, EMBnet.journal, 2011). Reads were then processed to remove the final 75 nt of each read, essentially converting the sequencing data from 2x150 to 2x75. Transcript expression was quantified using Salmon v0.8 (Patro R, *et al*, Nature Methods, 2017) against the human transcriptome (hg38, Gencode 28). Gene-level expression data was calculated from the transcript expression data using txlImport (Soneson C *et al*, F1000 Research, 2015). The extent of neurite-localization of individual genes was calculated by taking the ratio of expression (TPM) of the gene in neurites to its expression in soma. Significantly localized genes were identified using DESeq2 (Love M, *et al*, Genome Biology, 2014). When comparing soma and neurite samples, significantly localized genes had adjusted p-values of less than 0.05 and log2 fold changes of at least 1.5.

[0040] To identify genes whose localization changed across conditions, the R package Xtail was used (Xiao Z *et al*, Nature Communications 2016). This software package is designed for the analysis of ribosome profiling across conditions. Ribosome profiling experiments monitor the ratio of expression data in ribosome footprint and RNAseq datasets (otherwise known as translational efficiency or ribosome occupancy). Xtail asks if this ratio changes for a gene across conditions. This package was modified to ask if localization ratios

(neurite / soma) change across conditions. Gene ontology enrichments were calculated using GOrilla (Eden E, *et al*, BMC Bioinformatics, 2009). The sequencing data has been deposited at GEO, accession number GSE134737.

### **MN characterization**

**[0041]** Immunohistochemical analysis indicated that the hPSC-derived MNs expressed several mature MN markers, including Insulin gene enhancer protein 1 (ISL1), Motor neuron and pancreas homeobox 1 (MNX1/HB9), the neurofilament marker SMI-32, and  $\beta$ III-TUBULIN (FIG. 1 Panels C-D"). Quantification of either ISL1 or HB9 positive nuclei over total nuclei (DAPI positive) revealed that about 80% of the disclosed MN cells expressed one or more of ISL1, OLIG2, and MNX and 63% expressed HB9 (FIG. 1 Panel E). In some embodiments, greater than about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% and less than about 100%, 99%, 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 85%, 80%, or 75%, of the MN cells express ISL1, and greater than about 55%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 75%, 80%, 85%, 90%, or 95%, and less than about 100%, 95%, 90%, 85%, 80%, 75%, 70%, 69%, 68%, 67%, 66%, 65%, 64%, 63%, or 62%, expressed HB9. Global gene expression profiling of d20 MN cells indicated that their final differentiation stage resembled functionally mature MNs at the molecular level when compared to recently published datasets from in vitro-derived MNs (Ziller *et al.*, 2018) (FIG. 2 Panel A).

**[0042]** Quantitative PCR (qPCR) analysis of cells, performed at intermediate stages of the differentiation, showed the rapid loss of pluripotent stem cell markers NOGGIN and POU class 5 homeobox 1 (POU5F1, also known as OCT4) (FIG. 2 Panel B) and confirmed upregulation of the neuronal progenitor marker Oligodendrocyte transcription factor 2 (OLIG2) at days 8-14 (Imayoshi & Kageyama, 2014). Expression of mature neuronal markers ISL1 and HB9 was confirmed at day 20 MNs (FIG. 2 Panel C).

**[0043]** Functional analysis also confirmed the MN identity. Live  $\text{Ca}^{2+}$  imaging using Fluo-4 imaging dye (Grienberger & Konnerth, 2012) demonstrated that day 20 MNs exhibited robust baseline activities, with many active connections between unstimulated cells at basal conditions (FIG. 2 Panel D). Upon depolarization by 60mM KCL, MNs displayed a strong and immediate  $\text{Ca}^{2+}$  influx (FIG. 2 Panel D'), demonstrating the ability of the disclosed cells to mount action potentials. Consistent with the functional MN phenotype, whole-cell patch clamping analysis of iPSC-derived MNs at day 20 revealed single and multiple action potentials in response to 25-50mV (FIG. 2 Panels E-E").

**[0044]** To determine if day 20 MNs represent a distinct regional subpopulation, mRNA expression levels of segmentation marker genes was analyzed. Specifically, expression of HOX A1, B3, C4, C5, C6, C8, B8 and D8 was assayed by qPCR. HOXC8 expression was

significantly higher (for example about greater than about 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, or 9-fold, and less than about 10 fold, 9-fold, 8-fold, 7-fold, 6-fold, 5-fold, 4-fold, 3-fold, or 2-fold) than all the other HOX genes analyzed (FIG. 2 Panel F), suggesting that a subset of lateral motor column MNs, located in the thoracic/lumbar region of the spinal cord, are generated from the disclosed methods, processes, and systems. Paralogues HOXB8 and HOXD8 were expressed at lower levels in the disclosed MNs, which supports previous results showing that different cells within a specific tissue express typically one, but not all, region specific Hox paralogous (Fabre *et al.*, BMC Bio, 2018; Mucenski *et al.*, Sci. Rep, 2019). Taken together, these results evidence that the disclosed, scalable differentiation approach generates relatively pure populations of functional MNs within about 3 weeks.

### **Immunohistochemistry**

**[0045]** Cells were cultured on cover slips starting day 10 and harvested at various time points. For immunohistochemical analysis cells were fixed in 4%PFA for 30 minutes at RT, washed 3 times for 5 min in PBS and then blocked in CAS block (Invitrogen) for one hour at room temp.

**[0046]** The following antibodies were used at the indicated ratios: SMI-32 mouse 1:500 (Calbiochem cat no. NE1023), HB9 mouse 1:50 (DSHB 81.5C10-c), ISL-1 polyclonal 1:100 (Milipore Cat AB4326), B3- Tubulin Rabbit 1:100 (Cell Signaling #5568). Cells were exposed to antibodies and incubated in CAS-block (Invitrogen) overnight at 4 °C. Cells were then washed 3 times for 5 min with PBS and incubated with Alexa secondary antibodies (1:500, Invitrogen) for 1 hour at room temp. Thereafter, the cells were washed 3 times with PBS and incubated with DAPI for 5 min at RT. The cover slips were mounted on slides using Vectashield Mounting media (Vectorlabs Cat # H-1000) for analysis.

**[0047]** To visualize mitochondria, cells were placed on Mattek dishes and incubated in BMHH buffer plus Mitotracker Green FM (Thermofisher M7514) for 30 min. MNs were imaged using a LSM800 Zeiss confocal microscope with a 40x or 63x objective.

**[0048]** Quantification was performed using FIJI software by analyzing 3-5 images from 2 independent experiments. Soma and neurite intensity/area was calculated followed by neurite/soma ratios, which were then graphed. P-value were calculated using standard student t- test.

### **Ca<sup>2+</sup> imaging**

**[0049]** Cells were incubated in BMHH buffer plus Fluo-4 dye (Thermofisher Cat # F14201) for 30-45min at 37C and imaged on a Zeiss LSM 800 confocal microscope. Cells were imaged for 1 min at baseline after which they were subjected to depolarization with 30mM KCl. Calcium dynamics were analyzed using Fiji(Image J).

**Patch clamping**

[0050] MNPs were plated on glass coverslips that were coated with 2% Matrigel and matured for 10 days as described above. Glass coverslips were then placed in a recording chamber and perfused with recording solution which contained (in mM) 120 NaCl, 2.5 KCl, 26 NaHCO<sub>3</sub>, 1 NaH<sub>2</sub>PO<sub>4</sub>, 10 glucose, 1 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, adjusted to 285–290 mOsmol/kg H<sub>2</sub>O. All solutions were bubbled in 95% O<sub>2</sub>, 5% CO<sub>2</sub>. Recording pipettes ranged from 4 to 7 MΩ. Internal solutions contained (in mM) 123 K-gluconate, 2 KCl, 2 EGTA, 2 Na-ATP, 0.5 Na-GTP. Recordings were acquired using Axograph X and a MultiClamp 700B amplifier (Molecular Devices). Data were low-pass filtered at 2 KHz and acquired at 10 KHz.

**mRNA Localization in Motor Neurons****MN-based screen for compounds and/or conditions that affect MNs**

[0051] Disclosed herein are compositions, methods, protocols, and systems for the screening of compounds and conditions that affect motor neuron. In some embodiments, the disclosed screening may comprise one or more neurons, for example a population of progenitor or mature motor neurons. In many embodiments, the disclosed screening may include growth of neuron cells on a substrate (for example, a porous substrate) that may allow for neurite transit but not soma transit through the substrate, wherein the soma are located on a first surface of the substrate (for example a top surface), while the neurites are substantially located on the opposite side of the substrate (for example a bottom surface). The screening may also include a step of separating neurites from soma, for example by scraping of one or more of the two substrate surfaces. In some embodiments, the screening may involve testing for localization and sublocalization of various compounds, for example mRNA.

[0052] The subcellular localization of RNA transcripts within neurons allows MNs to spatially regulate gene expression (Sahoo, Smith, Perrone-Bizzozero, & Twiss, 2018), and many studies have identified specific transcripts that are trafficked to neurites (Zivraj *et al.*, 2010; Cajigas *et al.*, 2012; Taliaferro *et al.*, 2016; Zappulo *et al.*, 2017). Recently, mis-regulation of this process has been linked to several neurological diseases including Fragile X mental retardation, Spinal Muscular Atrophy, Myotonic Dystrophy (Fallini *et al.*, 2012; Wang *et al.*, 2016) and ALS (Akiyama *et al.*, EBiomedicine, 2019). To investigate whether the presently disclosed *in vitro*-derived MNs could be employed for the study of RNA localization in the human context, mechanical fractionation method that has been previously used for mouse neuronal cells to separate cell neurites from soma was used (FIG. 3 Panel A, Taliaferro *et al.* 2016). Briefly, in this technique, cells are plated on porous membranes that allow growth of neurites through the membrane, but restrict soma to the top of the membrane. This allows for mechanical separation of cell neurites from soma by simply

scraping the top of the membrane for sample collection. Protein and RNA samples from both the soma and neurite fractions were collected and analyzed. Efficiency of the fractionation approach was validated by assaying for beta-actin, which is present in both soma and neurite fractions, and for histone H3 protein, which is restricted to the nucleus (FIG. 3 Panel B).

**[0053]** High-throughput RNA sequencing was performed on the fractionated samples. For this analysis, samples were collected from three independent ES cell experiments, two independent control iPS, and four independent T1D iPS. Data from these samples was analyzed by hierarchical clustering.

**[0054]** This analysis showed that the soma and neurite samples exhibited distinct transcriptomes, as evidenced by clustering of samples based on cell fraction (FIG. 3 Panels C, D). Gene expression was then compared using the ratio of a specific gene's expression in the neurite versus its expression in the soma, this metric is referred to as the Localization Ratio (LR) (Taliaferro *et al.*, 2016). Neurite-enriched genes have positive log (LR) values, while soma enriched genes have negative log(LR) values. Comparison of soma and neurite expression data using a standard statistical framework (Love, Huber, & Anders, 2014) identified 1380 genes significantly enriched in neurites and 1020 genes significantly enriched in soma ( $p < 0.01$ ,  $\log_2FC > 1.5$ ) in the ES cells, 914 neurite enriched, 823 soma enriched in iPS control and 1335 neurite enriched, 1059 soma enriched in T1D iPS derived MNs.

**[0055]** These data sets included three genes known to be enriched in neurites: ACTB, NGRN, and RANBP1. Of these genes, NGRN and RANBP1 were significantly neurite-enriched and ACTB transcripts were more abundant in neurites than soma (FIG. 3 Panels E-E"). There was also strong enrichment of transcripts encoding ribosomal protein genes and nuclear-encoded members of the electron transport chain, which have been shown to be hallmarks of neurite-associated RNAs (Taliaferro *et al.*, 2016; Gumy *et al.*, 2011) (FIG. 3 Panels F-F", Supp. FIG. S Panels 3A, B). As a further validation, the observed LR values for all genes in ES derived MNs was compared to those previously reported from a fractionation of mouse cortical neurons (Taliaferro *et al.*, 2016) to demonstrate a highly significant correlation of LR values for genes between the two groups (FIG. 3 Panel G).

**[0056]** Effective mechanical separation of cell fractions was verified by specific subcellular protein expression, followed by the generation of global RNA transcript data sets from soma and cell neurites by deep sequencing technology (the data are accessible at GEO accession number GSE134737). Dependent on hPSC cell type derived MNs, between 1380 to 1226 genes were significantly enriched in neurites while 1020 to 582 genes were enriched in soma. A significant amount of genes normally enriched in neurites were enriched in the neurite fraction, including ribosomal proteins and nuclear encoded members of the electron transport chain, additionally verifying the validity of the approach. Thus, the data provided

here represents a novel and comprehensive data set of transcripts of MN soma and neurites in a human setting.

### **Effect of Hypoxia on MN mRNA localization and translation in MN**

**[0057]** Hypoxia is believed to play important roles in the development of neuropathies (Gupta & Agarwal, 2006; Mayer *et al.*, 1999.), particularly those related to diabetes, due to vascularization defects during disease onset and progression (Malik *et al.*, 1990; Hendriksen, Oey, Wieneke, van Huffelen, & Gispen, 1992).

**[0058]** The disclosed rapid MN differentiation protocols, methods, processes, systems, and resulting cells were used to study the effects of hypoxia on MNs. For these studies, d20 MN were cultured under normoxic (atmospheric oxygen concentration) and hypoxic (5% O<sub>2</sub>) conditions for 24 hours on a porous substrate such as filters, as described above. Cells were then fractionated into soma and neurite samples. After verifying efficient fractionation, as described above (see FIG. 3 Panel B-D), global RNA gene expression was analyzed by high-throughput sequencing, again as described above.

**[0059]** Results showed a high degree of reproducibility across treatments and fractionations. Samples were clustered according to hypoxia treatment and subcellular compartment regardless of their ES or iPS origin (FIG. 4 Panels A-A"). Because soma samples contain approximately 99% of the total cellular RNA, soma samples at normoxia versus hypoxia conditions were compared, as a proxy for general gene expression changes in response to hypoxia. Significant ( $p < 0.01$ ,  $\log_2FC > 1.5$ ) expression changes were identified in 2737 genes of the ES MNs. Many of these genes are known to be involved in the hypoxic response (FIG. 8 Panels A-C). Additionally, many genes involved in glycolysis were highly upregulated (FIG. 8 Panels A, D), presumably in response to the increased need for glycolytic energy production due to the decreased efficiency of oxidative phosphorylation.

**[0060]** To determine how RNA localization to neurites was regulated in response to hypoxic conditions, LR values observed in normoxia and hypoxia in ES, iPS control and T1D iPS (FIG. 8 Panels B,C) were compared. Using a software package designed to identify changes in the ratios of gene expression values across conditions (Xiao, Zou, Liu, & Yang, 2016), 1511 genes were identified that became significantly ( $p < 0.05$ ) more neurite-localized upon hypoxia treatment and 718 genes that became less neurite-localized in ES MNs (FIG. 4 Panel B), in T1D cells, 1226 genes were more neurite-enriched in hypoxia and 582 genes were less neurite-enriched (FIG. 8 Panel H). In control iPS cell, 1 gene which was significantly more neurite-enriched in hypoxia and 2 that were less neurite enriched (FIG. 8 Panel G) were detected. This is likely due to the smaller sample size. Surprisingly, nuclear-encoded genes were observed to be involved in mitochondrial function, including those involved in mitochondrial translation and the electron transport chain were strongly more

neurite-localized upon hypoxia treatment in all cell types (FIG. 4 Panels C-E, FIG. 8 Panels I, J). Genes involved in cytoplasmic translation were unchanged in their localization following hypoxia, suggesting that this effect is specific to mitochondria-related genes and not a general stress response.

**[0061]** To assess whether the observed transcript enrichment translates into an increase of functional mitochondria within neurites, MitoTracker was used to directly visualize mitochondria. The number of mitochondria decreased significantly in neurites of hypoxic MNs when compared to controls (FIG. 4 Panels F, G). Consistently, neurite-localization of genes encoded by the mitochondrial genome was also significantly reduced upon hypoxia treatment (FIG. 4 Panel C). Taken together these data suggest, without wishing to be confined by theory, that mitochondria loss in MN neurites occurs in response to hypoxia causing the cells to upregulate nuclear-encoded mitochondrial genes and their transport to neurites in an attempt to compensate for this loss. These data provide novel insight into critical neuronal responses to hypoxic disease condition and provide a framework for future investigations into the development and progression of neuropathies.

### **Neuropathy in Type I Diabetes**

**[0062]** A widely accepted cause for peripheral neuropathies is the development of a hypoxic environment that damages MNs due to the loss of surrounding blood vessels. Indeed, while still poorly understood, this mechanism is believed to be a key feature of many neuropathies, including ones caused by long-term diabetes. Approximately 50% of longstanding T1D patients develop neuropathies of the extremities, independent of glucose control. This suggests a potential genetic component and urged performing fractionation experiments with T1D iPSCs.

**[0063]** To investigate potential skewing of transcript localization in human MNs under diabetic neuropathic conditions, MNs derived from ES, control and T1D patients iPSC were exposed to hypoxic conditions and cell fractionation performed, along with global RNA analysis. RNA samples from hypoxic cell soma were significantly enriched for genes involved in the hypoxic response. Of note, cell soma contain approximately 99% of all cellular RNAs, thus can serve as a proxy for total RNA changes. In addition, an upregulation of genes in the glycolytic pathway was also identified. Thus upregulation is likely a compensatory mechanism to counteract the decrease in energy production through oxidative phosphorylation. Approximately 1500 and 700 genes became significantly up- or down-regulated in neurites, respectively, upon hypoxia. Interestingly, neurite enrichment of mitochondrial translation- and electron transport-associated genes was observed, while cytoplasmic translational genes were not enriched. This indicated an active stress response of the genes localized to neurites, the cell compartment that may be most affected by

hypoxia. Quantitative analysis showed that the compensatory stress response observed at the transcriptomic level did not result in an increase, but rather a decrease of neurite localized mitochondria.

**[0064]** Results from the disclosed screen compositions, methods, protocols, and systems provide first insights into the underlying molecular mechanisms of MN function. In the screens described above, identified molecular mechanisms affected by the hypoxic conditions result in the loss of functional MNs, hence the development of neuropathies.

**[0065]** While no obvious developmental phenotypes or different hypoxia responses in T1D derived MNs was observed, the present experiments provide an important proof of principle of the disclosed compositions, methods, processes, and systems for using patient specific cells.

**[0066]** Mitochondrial dysfunction has been proposed to be a central mediator in the development and progression of diabetes and diabetic neuropathies (Fernyhough, 2015; Fernyhough, Huang, & Verkhatsky, 2003)(Pinti *et al.*, 2019), and provided here is strong evidence that mitochondrial dysfunction occurs specifically in MN neurites. Also disclosed, are compositions, methods, processes, and systems for creating and using patient-specific iPSC from patients suffering from various neuropathies, including but not limited to Type I diabetes. These cells may be compared to healthy patients and/or patients with the same disease (e.g. T1D), but without complications – this may help to illuminate unknown (genetic) disease contributors. In addition, the disclosed compositions, methods, processes, and systems can be effectively employed to test the effects of various existing and novel drugs for treating various neuropathies.

**[0067]** Disclosed herein are rapid and efficient approaches to generate human MNs from pluripotent stem cells and to provide reliable assay systems for investigating various abnormalities in these cells, for example RNA mis-localization. In some cases, skewed subcellular localization of RNAs may prove to be a contributor to various MN diseases. Indeed, Applicant has identified, using patient-specific MNs from Fragile X patients, and healthy controls, differential enrichment of RNAs containing a G-quadruplex sequence (Goering *et al.*, BioRxiv, 2019). These results corroborate the presented theory RNA localization plays a role in MN biology and disease development.

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**[00101]** While multiple embodiments are disclosed, still other embodiments of the present invention will become apparent to those skilled in the art from the following detailed description. As will be apparent, the invention is capable of modifications in various obvious aspects, all without departing from the spirit and scope of the present invention. Accordingly, the detailed description is to be regarded as illustrative in nature and not restrictive.

**[00102]** All references disclosed herein, whether patent or non-patent, are hereby incorporated by reference as if each was included at its citation, in its entirety. In case of conflict between reference and specification, the present specification, including definitions, will control.

**[00103]** Although the present disclosure has been described with a certain degree of particularity, it is understood the disclosure has been made by way of example, and changes in detail or structure may be made without departing from the spirit of the disclosure as defined in the appended claims.

## CLAIMS

We claim:

1. A method of producing mature motor neuron cells, comprising:  
aggregating a population of stem cells to form stem cell clusters in suspension culture or adherent culture;  
culturing the cell clusters in a progenitor media for a differentiation duration to differentiate the stem cell clusters into neuroepithelial progenitor cell clusters;  
culturing the neuroepithelial progenitor cells in an induction media that activates the Sonic Hedgehog and Retinoic Acid pathways for an induction duration to induce the neuroepithelial progenitor cells to be motor neuron progenitor cells;  
culturing the motor neuron progenitor cells in culture plates coated with an extracellular matrix-like substance in a maturation media for a maturation duration to mature the progenitor motor neuron cells into mature motor neuron cells; and thereby  
producing a mature motor neuron cell, wherein the stem cells are selected from embryonic stem cells or induced pluripotent stem cells.
2. The method of any of claim 1, wherein the progenitor media comprises one or more of Compound C(AMP-kinase inhibitor), SB431542 (activin receptor-like kinase receptor inhibitors), CHIR99021 (GSK-3 inhibitor) and Rock Inhibitor (RI), and the progenitor duration is from 4 to 8 days.
3. The method of any of claims 1 to 2, wherein the induction media comprises one or more of Compound C, SB431542, SAG (Smoothed agonist, Hedgehog activation), TTNPB (Retinoic Acid agonist), and Rock Inhibitor (RI), and the induction duration is between 2 and 6 days.
4. The method of any of claims 1 to 3, wherein the maturation media comprises one or more of NEAA(non-essential amino acids), RI, SAG, TTNPB, BDNF(brain derived neurotrophic factor), and GDNF (glial derived neurotrophic factor), and the maturation duration is between 2 and 6 days.
5. The method of claim 4, including a step of culturing the mature motor neuron cells in culture plates coated with an extracellular matrix-like substance in a maintenance media for a maintenance duration, wherein the maintenance media comprises one or more of a  $\gamma$ -Secretase Inhibitor, BDNF, and GDNF, and the maintenance period is between 0 and 40 days.

6. The method of any of claims 1 to 5, wherein the individual neuroepithelial progenitor cells are frozen after the induction step and before the maturation step.
7. The method of any of claims 1 to 6, wherein culturing the motor neuron progenitor cells includes plating, non-frozen individual neuroepithelial progenitor cells at about 20,000 cells to about 120,000 cells per cm<sup>2</sup> in MATRIGEL, and frozen individual neuroepithelial progenitor cells are plated at about 50,000 cells to about 210,000 cells per cm<sup>2</sup> in MATRIGEL.
8. The method of any of claims 1 to 7, including dissociating the neuroepithelial progenitor cell clusters into individual neuroepithelial progenitor cells, or cell clusters of about 2 to 500 cells before culturing the neuroepithelial progenitor cells.
9. The method of any of claims 1 to 8, wherein the mature motor neuron cells express the HOXC8 gene at least about 2 to 10-fold higher than any of HOX A1, B3, C4, C5, C6, B8 and D8.
10. The method of any of claims 1 to 8, wherein the mature motor neurons are phenotypically identified as lateral motor column motor neurons typically found in the thoracic/lumbar region of the spinal cord.
11. A method of producing a population of motor neuron progenitor cells, comprising:
  - aggregating a population of stem cells to form cell clusters;
  - culturing the cell clusters in a progenitor media for a differentiation duration to differentiate the cells into neuroepithelial progenitor cell clusters;
  - dissociating the neuroepithelial progenitor cell clusters into individual neuroepithelial progenitor cells;
  - culturing the individual neuroepithelial progenitor cells in an induction media that activates the Sonic Hedgehog and Retinoic Acid pathways for an induction duration to induce the neuroepithelial progenitor cells to be motor neuron progenitor cells;
  - collecting and freezing the motor neuron progenitor cells; and thereby producing a mature motor neuron cell.
12. The method of any of claims 1-12, wherein the stem cells are mammalian stem cells.
13. The method of any of claims 1-13, wherein the mammal is a human.
14. The method of any of claims 1-14, wherein the human is a patient suffering from a disease or disorder related to motor neuron dysfunction selected from one of more of

amyotrophic lateral sclerosis (ALS), viral poliomyelitis, Guillain-Barre syndrome, Fragile X syndrome and diabetic motor neuropathies.

15. A cell composition comprising:

a population of induced pluripotent stem cell-derived cells, wherein at least 70% to 99% of the cells express ISL1, OLIG2, and MNX; and

the population of cells express greater than about 2-fold or more HOXC8 transcript than HOX A1, B3, C4, C5, C6, B8 and D8.

16. The cell composition of claim 15, wherein the induced pluripotent stem cells are derived from a subject having or at risk of developing a disease or disorder related to motor neuron dysfunction selected from one of more of amyotrophic lateral sclerosis (ALS), viral poliomyelitis, Guillain-Barre syndrome, and diabetic motor neuropathies.

17. A method for analyzing effects on motor neurons, comprising:

growing a first population of cells on a first substrate in a first medium;

growing a second population of cells on a second substrate in a second medium;

altering a growth condition for the first population of cells, wherein the first and second population of cells are derived from stem cells, and wherein at least 70% of the cells in the population express ISL1, OLIG2, and MNX, and the population of cells express between about 1.1-fold and 10-fold more HOXC8 transcript than HOX A1, B3, C4, C5, C6, B8 and D8;

allowing an amount of time to elapse;

collecting a first cell material from a first surface of the first and second substrates;

collecting a second cell material from a second surface of the first and second substrates, wherein the first cell material is substantially cell soma, and the second cell material is substantially cell neurite;

analyzing the first cell material and the second cell material to determine an amount or concentration for at least one nucleic acid, peptide, compound, or biomarker; and

comparing the amounts of the at least one nucleic acid, peptide, compound, molecule, or biomarker in the cell materials.

18. The method of claim 17, wherein the cells of the first or second population are derived from, human stem cells, pluripotent stem cells, embryonic stem cells, and progenitor cells.

19 The method of any of claims 17 or 18, wherein the cells of the first or second population are matured in to MNs in less than about 21 days, and exhibit spontaneous action potentials.

20 The method of any of claims 17 to 18, wherein the cells of the first or second population are derived from a subject having or at risk of developing a disease or disorder related to motor neuron dysfunction selected from one of more of amyotrophic lateral sclerosis (ALS), viral poliomyelitis, Guillain-Barre syndrome, Fragile X syndrome and diabetic motor neuropathies.

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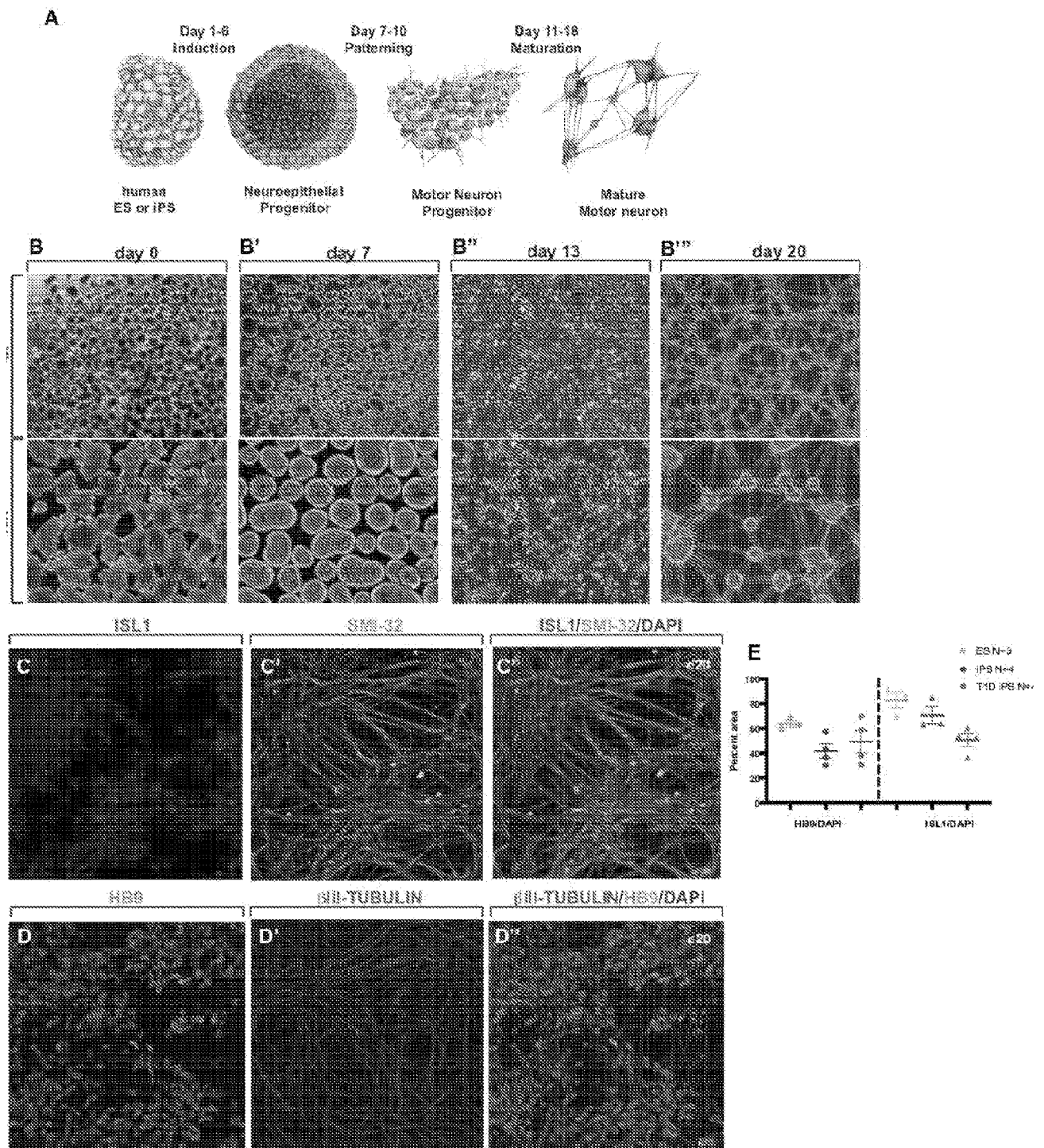


FIG. 1

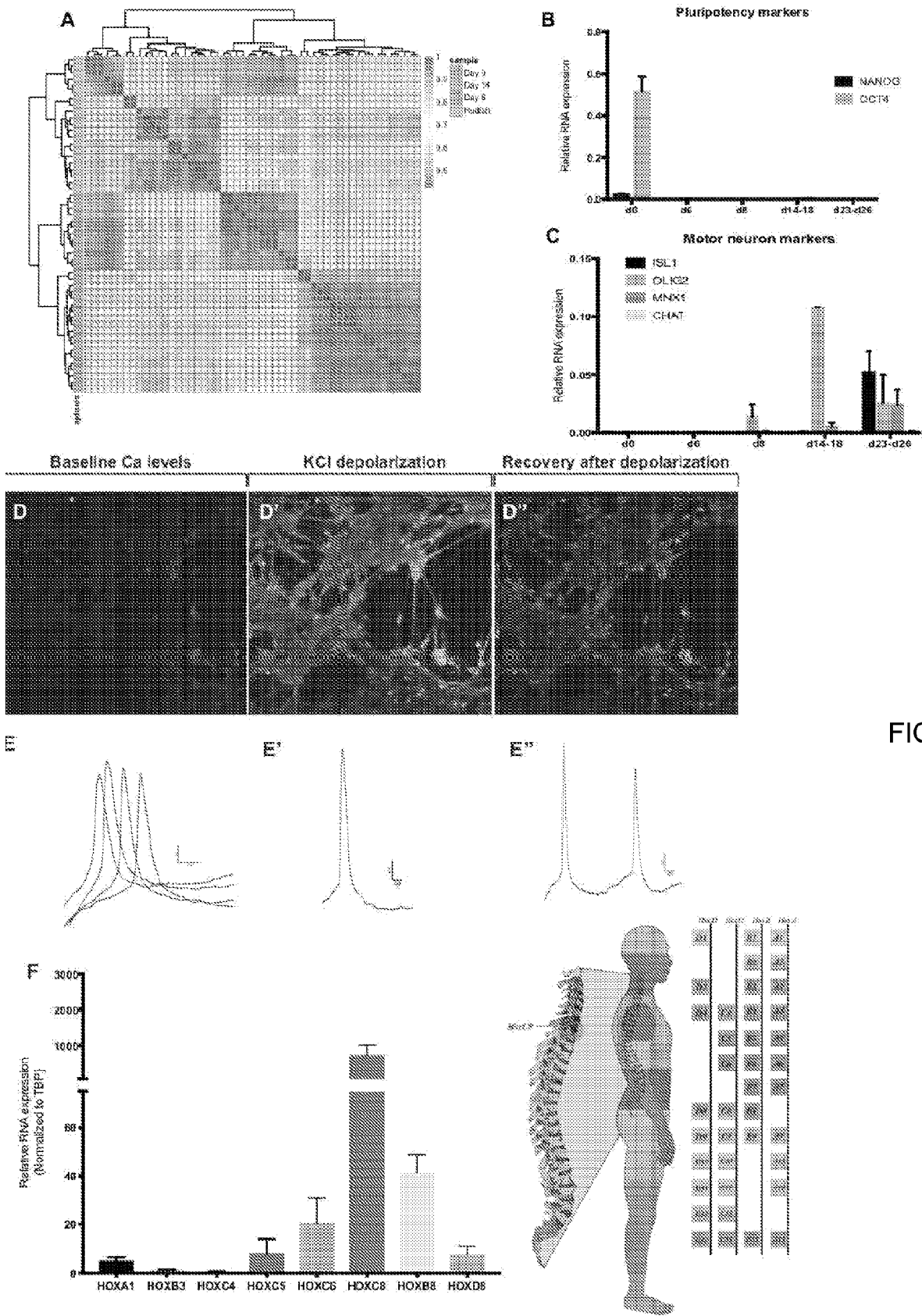


FIG. 2

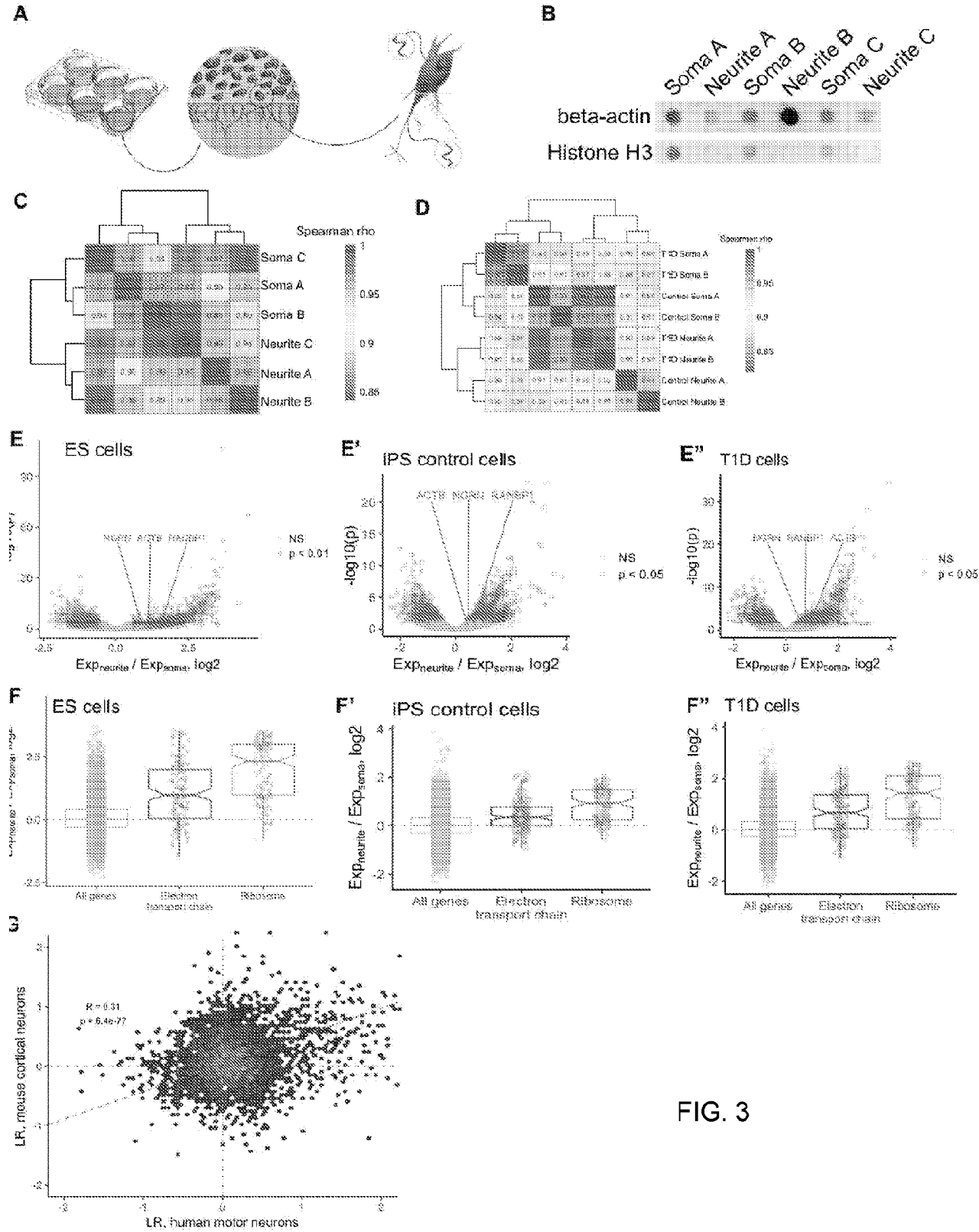


FIG. 3

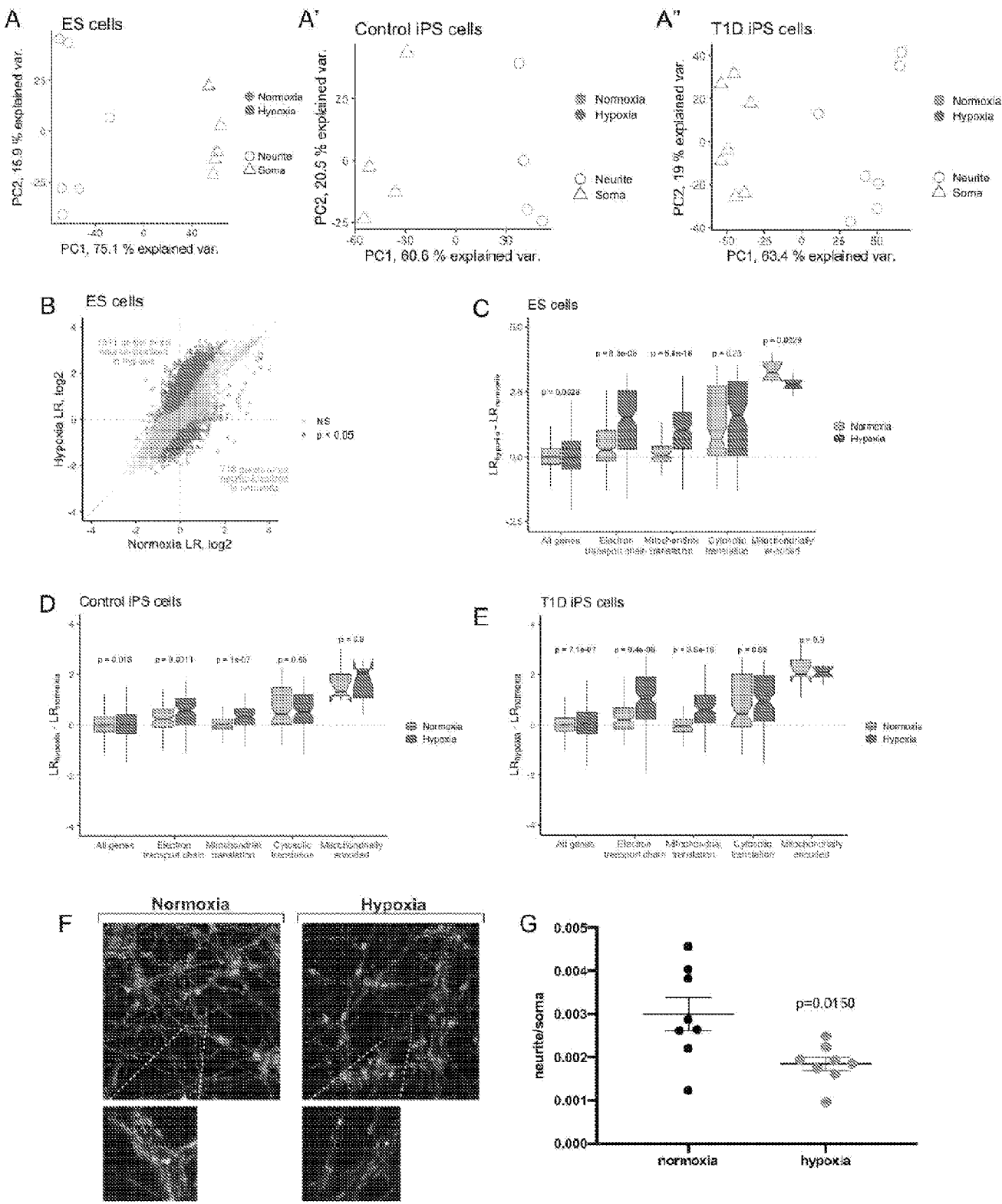


FIG. 4

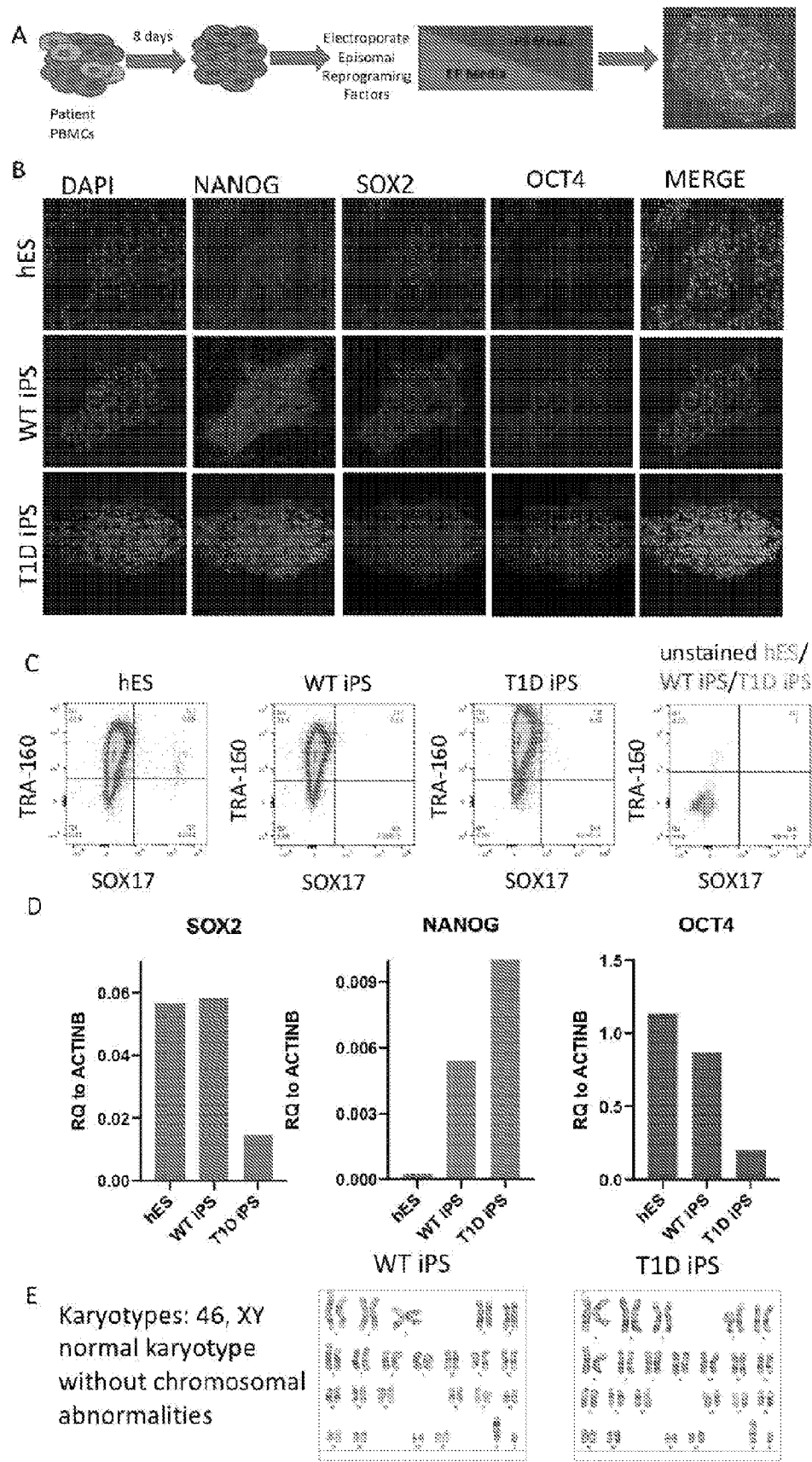


FIG. 5

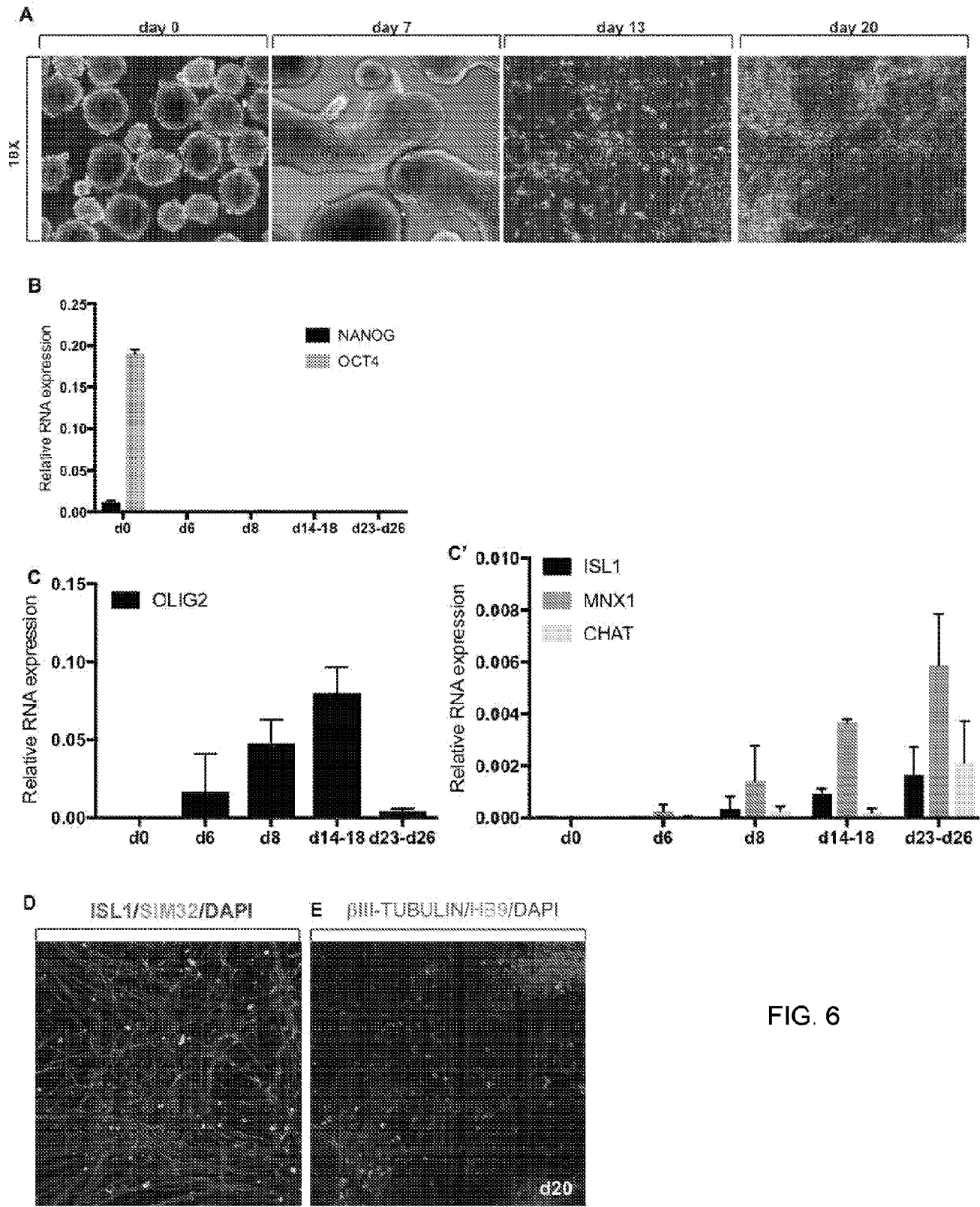


FIG. 6

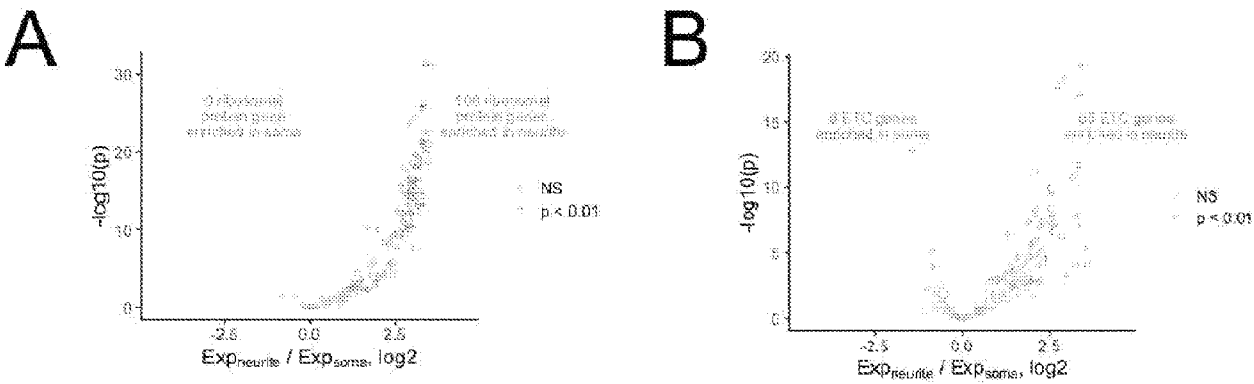


FIG. 7

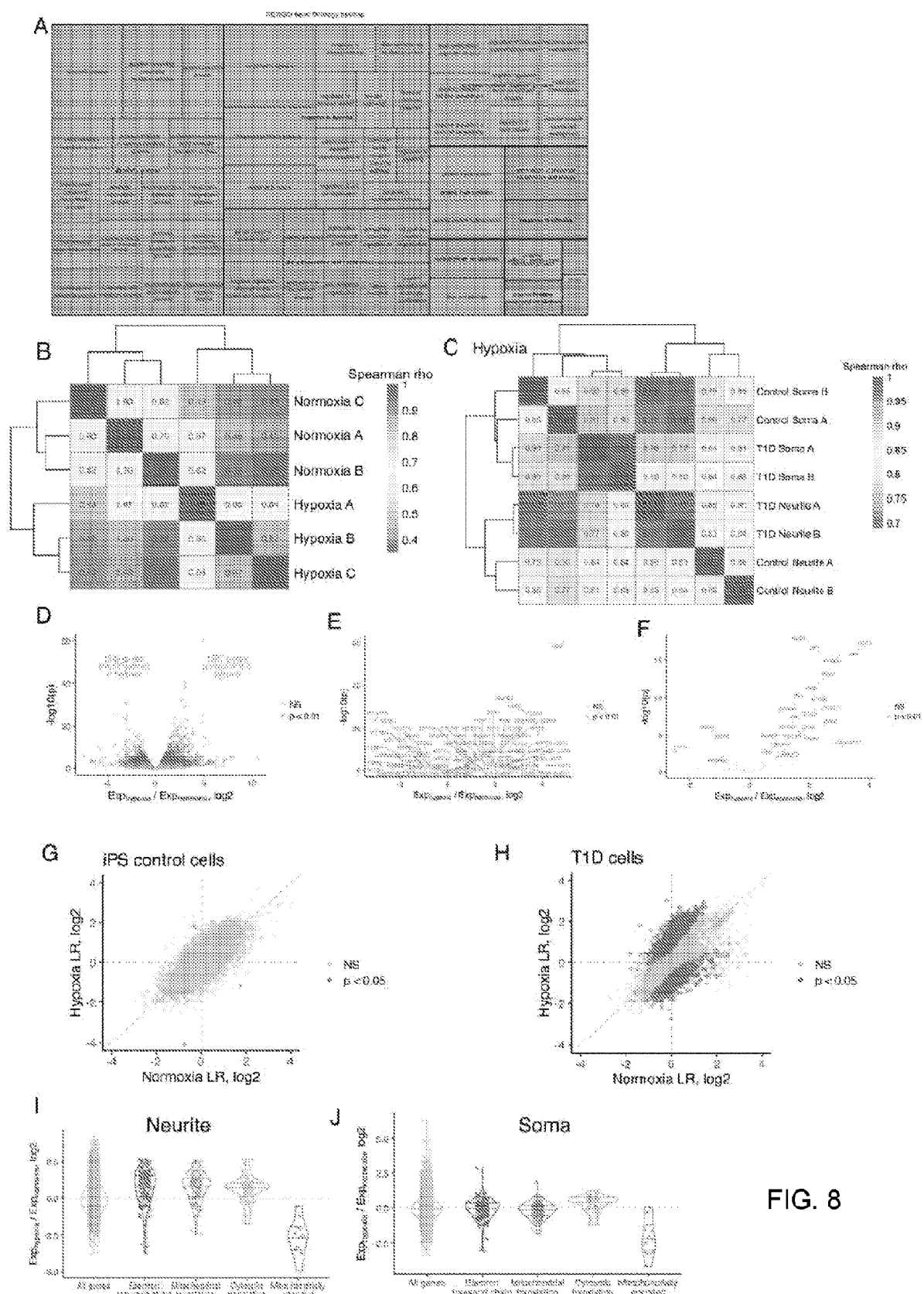


FIG. 8

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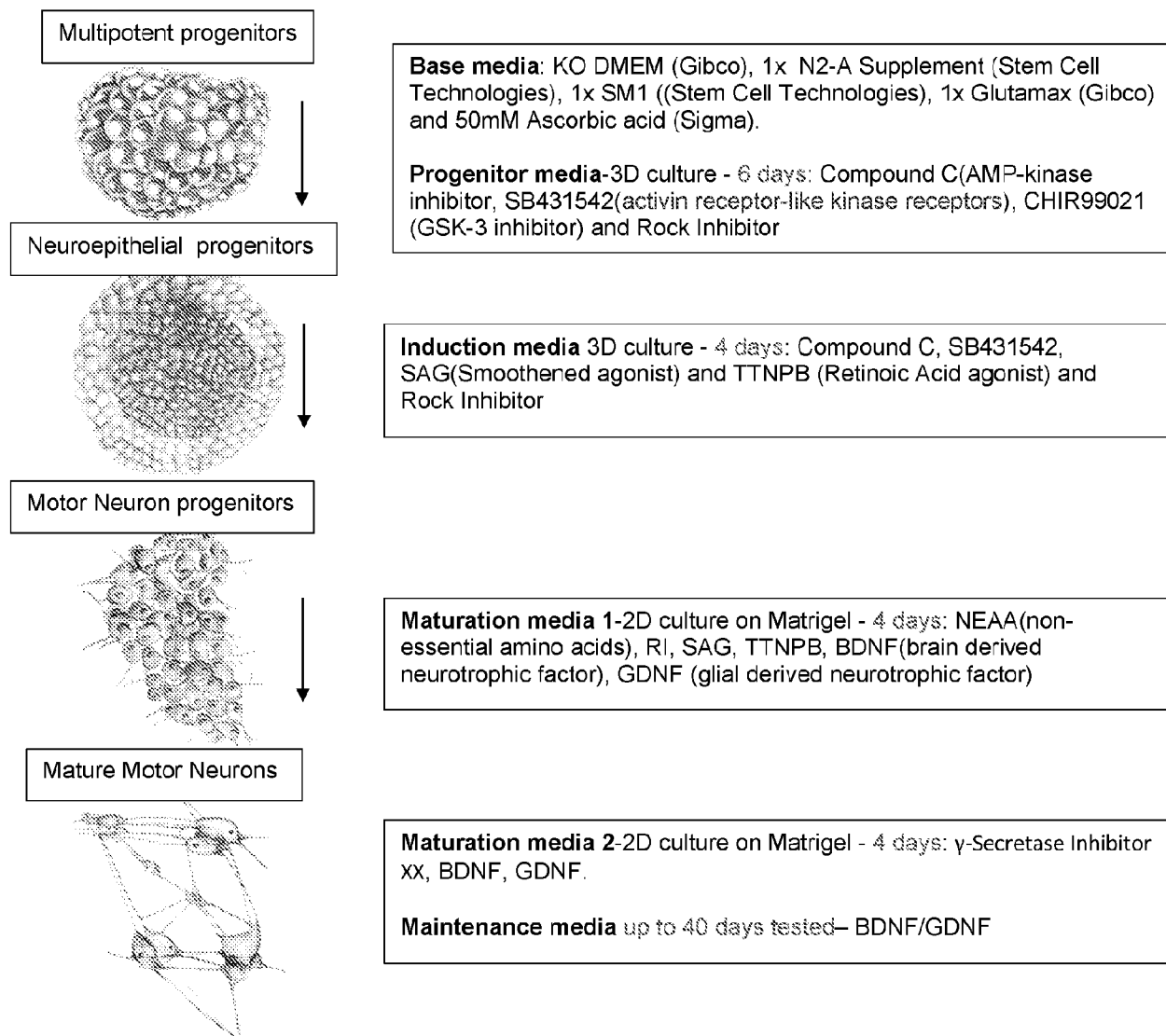


FIG. 9

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/23474

## A. CLASSIFICATION OF SUBJECT MATTER

IPC - C12N 5/0797; C12N 5/0793; C12N 5/0735; C12N 9/12; C07K 14/78 (2021.01)

CPC - C12N 5/0619; C12N 5/0619; C12N 5/0696; C12N 5/0606; C12N 2501/41; C12N 2501/16; C12N 2501/727; C12N 2533/52

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                       | Relevant to claim No. |
|-----------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | US 2014/0248696 A1 (Wisconsin Alumni Research Foundation) 4 September 2014 (04.09.2014). Especially para [0012], [0014], [0030], [0031], [0036], [0041], [0061], [0063], [0066], [0067], sheet 1, fig 1. | 1-3, 11               |
| A         | US 2016/0046905 A1 (Kyoto University) 18 February 2016 (18.02.2016). Especially para [0146], [0299]                                                                                                      | 1-3, 11               |

☐ Further documents are listed in the continuation of Box C.☐ See patent family annex.

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&amp;" document member of the same patent family

Date of the actual completion of the international search

5 August 2021

Date of mailing of the international search report

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/23474

**Box No. I**      **Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
  - a. ☐ forming part of the international application as filed:
    - ☐ in the form of an Annex C/ST.25 text file.
    - ☐ on paper or in the form of an image file.
  - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
  - c. ☒ furnished subsequent to the international filing date for the purposes of international search only:
    - ☒ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
    - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/23474

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

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

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: 4-10, 12-14  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

This International Searching Authority found multiple inventions in this international application, as follows:

-----Go to Extra Sheet for continuation-----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
Claims 1-3, 11

### Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 21/23474

### Continuation of Box III: Observations where Unity of Invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I: Claims 1-3, 11, drawn to a method of producing mature motor neuron cells or motor neuron progenitor cells.

Group II: Claims 15-20, drawn to a composition or method of analyzing a population of cells for specific cell markers.

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

#### Special Technical Features:

Group I has the special technical feature of method steps that include progenitor media, induction media, and a maturation media, not required by Group II.

Group II has the special technical feature of a population of cells, wherein at least 70% of the cells express ISL1, OLIG2, and MNX; and the population of cells express greater than between 1.1-fold and 10-fold more HOXC8 transcript than HOX A1, B3, C4, C5, C6, B8 and D8, not required by Group I.

#### Common Technical Feature:

Groups I and II share the common technical feature of pluripotent stem cell derived cells, motor neurons, and culturing cells in media.

However, said common technical feature does not represent a contribution over the prior art, and is disclosed by US 2016/0046905 A1 to Kyoto University (hereinafter "Kyoto University").

As to the common technical feature, Kyoto University discloses pluripotent stem cell derived cells (para [0002]; "The present invention relates to a method of generating a neuron or a motor neuron from a pluripotent stem cell"), and culturing motor neurons (abstract; para [0066]).

As the common technical feature was known in the art at the time of the invention, this cannot be considered a common special technical feature that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Groups I and II lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature.

Item 4 (cont.): Claims 4-10 and 12-14 are multiple dependent claims and are not drafted according to the second and third sentences of PCT Rule 6.4(a).