Application Note

Neural Induction Medium Efficiently Generates Multipotent Neural Progenitors from Human iPS Cells

Introduction

The discovery that somatic cells could be converted into induced pluripotent stem (iPS) cells with the expression of four transcription factors (Oct-4, Klf-4, Sox-2, and c-Myc) has created an exciting new area of stem cell biology research[1]. Many human diseases that previously lacked a comprehensive *in vitro* cellular model can now be modeled using patient-derived iPS cells. Neurological diseases, such as Alzheimer's disease, Parkinson's disease, and autism were some of the first diseases modeled using an iPS cell approach. Highly enriched and scalable neural progenitor cells (NPCs) derived from iPS cells are attractive alternatives to tissue-isolated primary cells and immortalized neural stem cell lines for biomedical research. These iPS cell-derived NPCs offer an exciting opportunity to examine neuronal development and the commitment to specific neural cell types without ethical concerns associated with the use of human embryonic stem cells.

Dual SMAD inhibition is a well-established method to derive neural progenitor cells from both human ES and iPS cells[2]. This protocol uses two SMAD inhibitors, Noggin and SB431542, to drive the rapid differentiation of ES/iPS cells into a highly enriched population of NPCs[2]. Noggin acts as a BMP inhibitor and SB431542 inhibits the Lefty/Activin/TGFβ pathways by blocking the phosphorylation of ALK4, ALK5, and ALK7 receptors. In an effort to make a more defined and optimized neuronal differentiation protocol, Li and colleagues modified the original protocol to establish a completely small molecule-based differentiation method, which relies on three small molecules to inhibit GSK-3β

(CHIR99021), TGFβ (SB431542), and Notch (compound E) signaling pathways, along with human LIF[3]. This new small molecule-based neural differentiation protocol increased neural differentiation kinetics and allowed the derivation of truly multipotent neural stem cells that respond to regional patterning cues specifying forebrain, midbrain, and hindbrain neural and glial subtypes.
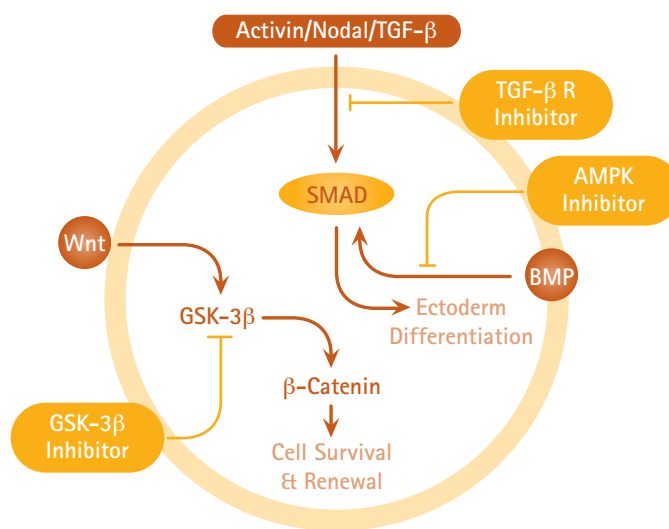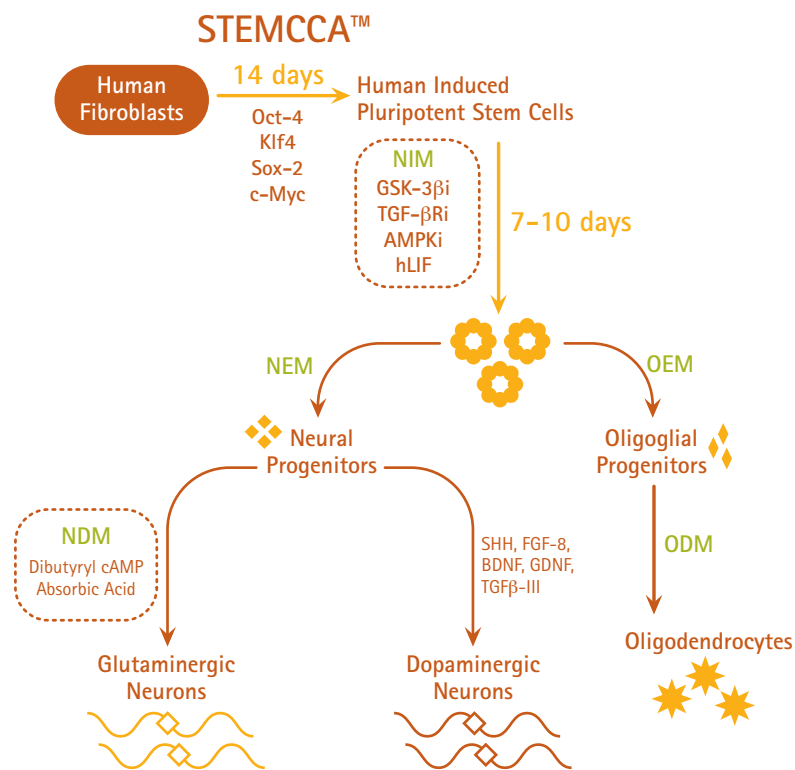

Figure 1.

Signaling pathways targeted by neural induction medium. EMD Millipore's Neural Induction Medium uses a combination of three small molecule inhibitors (GSK3β, TGFβR, and AMPK inhibitors) to induce neural differentiation into multipotent neural progenitors from human iPS cells.

Combining the above-described methodologies, we developed a new neuronal induction medium (NIM) and neuronal differentiation (NDM) medium to efficiently produce neural rosettes, expandable neural progenitors, end-stage neural (glutaminergic and dopaminergic) and glial subtypes (oligodendrocytes) from human ES/iPS cells. These media rely on a combination of commonly used neural supplements along with small molecule inhibitor combinations to achieve highly enriched population of multipotent NPCs. Many reports have demonstrated the propensity of the NPC's differentiation toward a single lineage, either primarily neuronal or glial, but not both. Using our newly developed NIM and NDM media, iPS cells-derived NPC population responded well to appropriate inductive patterning signals and could be directed towards both neural and glial cell lineages. In combination with STEMCCA™ reprogramming technologies, these new media provide an efficient solution for studying neurological diseases using an iPS cell model system (Figure 2).

Figure 2.

Workflow showing all steps in iPS cell generation and subsequent differentiation to neural lineages. In as few as four steps, adult fibroblasts can be converted to neural lineages using media formulations for modulating cell fate. Along with iPS cell-generating reprogramming technologies (STEMCCA™ kits), EMD Millipore now offers media to generate different neural and glial subtypes from iPS cells for "disease-in-a-dish" researchers.

NIM: Human ES/iPS Neural Induction Medium (Cat. No. SCM110)

NEM: ENStem™-A Neural Expansion Medium (Cat. No. SCM004)

NDM: Human ES/iPS Neuronal Differentiation Medium (Cat. No. SCM111)

OEM: Human OPC Expansion Media Kit (Cat. No. SCM107)

ODM: Human OPC Spontaneous Differentiation Media Kit (Cat. No. SCM106)

Materials and Methods
Generation of iPS Cells using STEMCCA™ reprogramming kits

Human iPS cells were established from human fibroblasts (Cat. No. SCC058) using STEMCCA™ Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus kit (Cat. No. SCR545) following the manufacturer's protocol. Human foreskin fibroblasts between P3 to P6 were seeded on 0.1% gelatin-coated 6-well plate at 10^4 cells per well with FibroGRO® LS Complete Medium (Cat. No. SCMF002). Lentivirus between 20 to 50 multiplicity of infection (MOI) was added to the growth medium 24 hours after seeding. Viruses were removed after 24 hours of incubation and the growth medium was replaced every day. After five days, transfected fibroblasts were replated on mouse embryonic fibroblast (MEF) feeder cells, together with Human iPS Cell Boost Supplement II (Cat. No. SCM094), for an additional 11 to 20 days with daily medium replacement. iPS cells were clonally expanded on MEF feeder cells with 20% KOSR-based medium (Life Technologies; 80% DMEM/F12, 20% KOSR, 1% βME, 1% nonessential amino acids) supplemented with 8 ng/mL FGF-2 (Cat. No. GF003).

Neural induction of iPS cell using NIM1 and NIM2 media

iPS cell-derived neural progenitor and differentiated neuronal cells were generated using the Human ES/iPS Cell Neurogenesis kit (Cat. No. SCR603). Undifferentiated iPS cell colonies were seeded on to MEFs for 48 hours with 20% KOSR medium supplemented with FGF-2 (8 ng/mL). After 48 hours, growth medium was replaced by neural induction medium 1 (NIM1) for five days with medium changed every other day. On day six, cells were dissociated with non-enzymatic buffer (Cat. No. S-004-C) and plated on 0.5 mg/mL Matrigel® (BD Biosciences) plates with neural induction medium 2 (NIM2) for an additional five days with medium changed every other day. After 10 days, neural progenitor cells were harvested with Accutase® reagent (Cat. No. SCR005) and expanded with ENStem™-A medium (Cat. No. SCM004) supplemented with 20 ng/mL FGF-2 and 2 mM glutamine on the flask or plates coated with 0.1 to 0.2 mg/mL of Matrigel® at a density of 0.5 to 1 x 10^5 cells/cm^2.

Differentiation of neural progenitor cells into neurons

Neural progenitors between P3 to P8 were harvested with Accutase® reagent and NPCs were plated on poly-L-ornithine/laminin (10 µg/mL each; Poly-L-ornithine Sigma P4957; Laminin, EMD Millipore Cat. No. CC095) at 10 to 20 thousand cells/cm^2 with neuronal differentiation medium (Cat. No. SCM111) that was supplemented with 0.5 mM dibutytryl cAMP and

0.2 mM ascorbic acid phosphate. About 80% of the medium was refreshed every two to three days for a total of 9-14 days. Cells were fixed with 2% paraformaldehyde before immunofluorescent staining. Percentage of differentiated cells was determined by visual estimation.

Differentiation of neural progenitor cells into dopaminergic neurons

To derive dopaminergic neurons, the Dopaminergic Differentiation Growth Factor Sampler kit (Cat. No. SCR128) was incorporated into NIM-based neurogenesis protocol. 200 ng/mL of sonic hedgehog and 100 ng/mL FGF-8 were included in the NIM2 medium and cells were cultured for five days. Cells were then harvested with Accutase® reagent into single cells, and 3 x 10^4 NPCs (from either iPS or ES cells) were plated on poly-L-ornithine- and laminin-coated plates at 10 µg/mL each with neuronal differentiation medium containing 20 ng/mL BDNF and GDNF and 1 ng/mL of TGFβIII for 14 days. Tyrosine hydroxylase-positive cells representing dopaminergic neurons were characterized by immunofluorescent staining.

Differentiation of neural progenitor cells into oligodendrocytes

Oligoglial differentiation from iPS cells was performed by culturing neuronal progenitors at P1 in suspension culture with oligodendrocyte expansion medium (Cat. No. SCM107, basal medium with PDGF-AA, NT3, FGF2, T3) and retinoic acid for 14 days. The glial fate-primed neurospheres were then plated on poly-L-ornithine- and laminin-coated plated at 10 µg/mL each to further mature with Human OPC Spontaneous Differentiation Media (Cat. No. SCM106) for 14 days, with medium changes every two to three days.

Region-specific patterning of neuronal progenitor cells

iPS cells were induced to the neuronal lineages as described in the NIM user guide. On day five, cells were cultured for 10 more days in NIM2 (for spontaneous differentiation), NIM2 containing 200 ng/mL sonic hedgehog and 100 ng/mL FGF8 (SHH/FGF8; to prime cells for midbrain fate), or 200 ng/mL sonic hedgehog and 1 µM retinoic acid (SHH/RA; to prime cells for hindbrain fate). After 10 days, cells were harvested and the gene expression pattern was analyzed by qRT-PCR. GAPDH was used to normalize gene expression between samples. Fold changes in gene expression were calculated based on ΔCt values (normalized to GAPDH) and normalized to the spontaneous sample (spontaneous, n = 2).

Antibody	Cat. No./Host	Target	Dilution
Sox-2	AB5603/ Rabbit	Neural progenitor	1:200
Pax6	AB2237/ Rabbit	Neural progenitor	1:400
Tuj1	AB15708/Rabbit	Neuron	1:200
Sox10	AB5727/Rabbit	Oligodendrocyte progenitor (OPC)	1:200
Olig-2	AB9610/Rabbit	OPC	1:200
NG2	AB5320/Rabbit	OPC	1:200
N-Cad	05-915/ Mouse	Neural progenitor	1:100
Nestin	MAB5326/Mouse	Neural progenitor	1:400
GFAP	MAB3402/Mouse	Astrocyte	1:200
MAP2	MAB3418/Mouse	Neuron	1:200
VGLUT2	MAB5504/Mouse	Glutaminergic neuron	1:200
GAD65	MAB351R/Mouse	GABAergic neuron	1:200
O4	MAB345/Mouse	OPC	1:25
CNPase	MAB326/Mouse	OPC/Oligodendrocyte (OL)	1:200
GalC	MAB342/Mouse	OPC/OL	1:200
MBP	05-675/Mouse	OL	1:20
A2B5	MAB312/ Mouse	Glial progenitor	1:100
Tyrosine Hydroxylase	AB152/Rabbit	Dopaminergic neuron	1:400

Table 1. Antibodies used in the study.

Results

Over 80% Pax6-positive, polarized neural rosettes were generated from Oct-4-positive human iPS cells using NIM1 and NIM2 media after 10 days of differentiation. Pax6 expression increased in as little as 5 days, while mature neural rosettes structures, expressing both Pax6 and N-cadherin were observed after 10 days of culture (Figure 3).

NIM1 5d
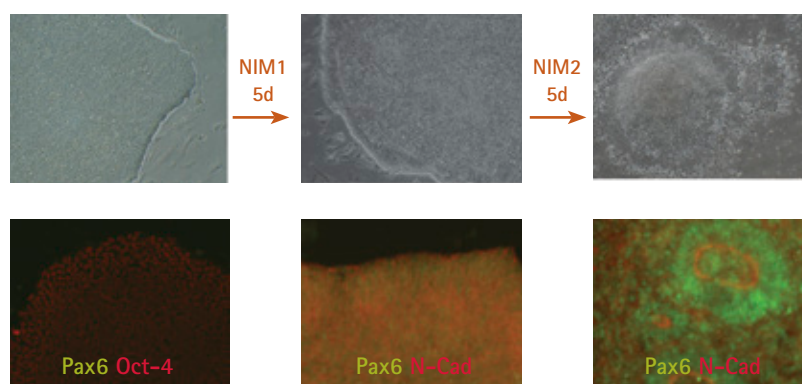
NIM2 5d

Pax6 Oct-4   Pax6 N-Cad   Pax6 N-Cad

Figure 3.

Time course of neural induction from iPS cells. Brightfield (top) and fluorescent micrographs show STEMCCA™-derived iPS cells as well as five-day-old and ten-day-old neural rosettes. iPS cells were positive for Oct-4 (red). Five-day-old rosettes were Pax6-positive (green), and ten-day-old rosettes were both Pax6- and N-cadherin-positive (green and red)

Neural rosettes could be dissociated and expanded up to 10 passages on Matrigel®-coated cultureware in ENStem™-A expansion medium, while maintaining a high level of Sox-2, Nestin, and N-cadherin expression (Figure 4).
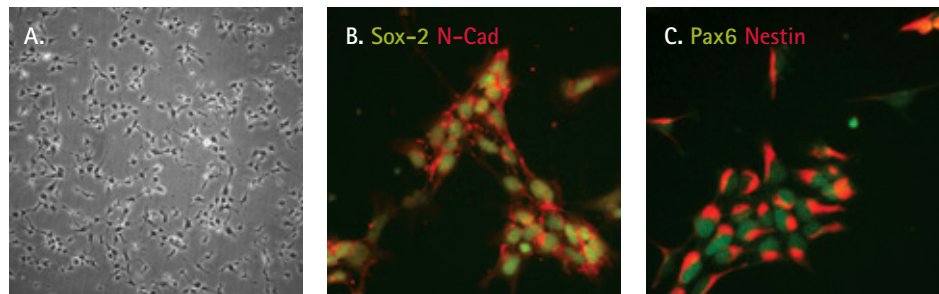

Figure 4.

Characterization of iPS cell–derived neural progenitor cells. Passage 3 NPCs derived from iPS cells using NIM display typical neural progenitor morphologies (A) and were stained for NPC markers, Sox-2, N-cadherin (B), Pax6 and nestin (C). These cells could be expanded for 10 passages while maintaining nestin and Sox-2 expression.

iPS cell-derived neural progenitors were further differentiated into terminally differentiated neurons using Neuronal Differentiation Medium (NDM). Over 70% of differentiated cells were positive for TUJ1 and MAP2, markers of mature neurons, while less than 20% of the differentiated cells were positive for GFAP, a marker of astrocytes. Terminally differentiated neuronal cells were preferentially glutaminergic (VGlut+) rather than GABAergic neurons (GAD65 -) using NDM (Figure 5).
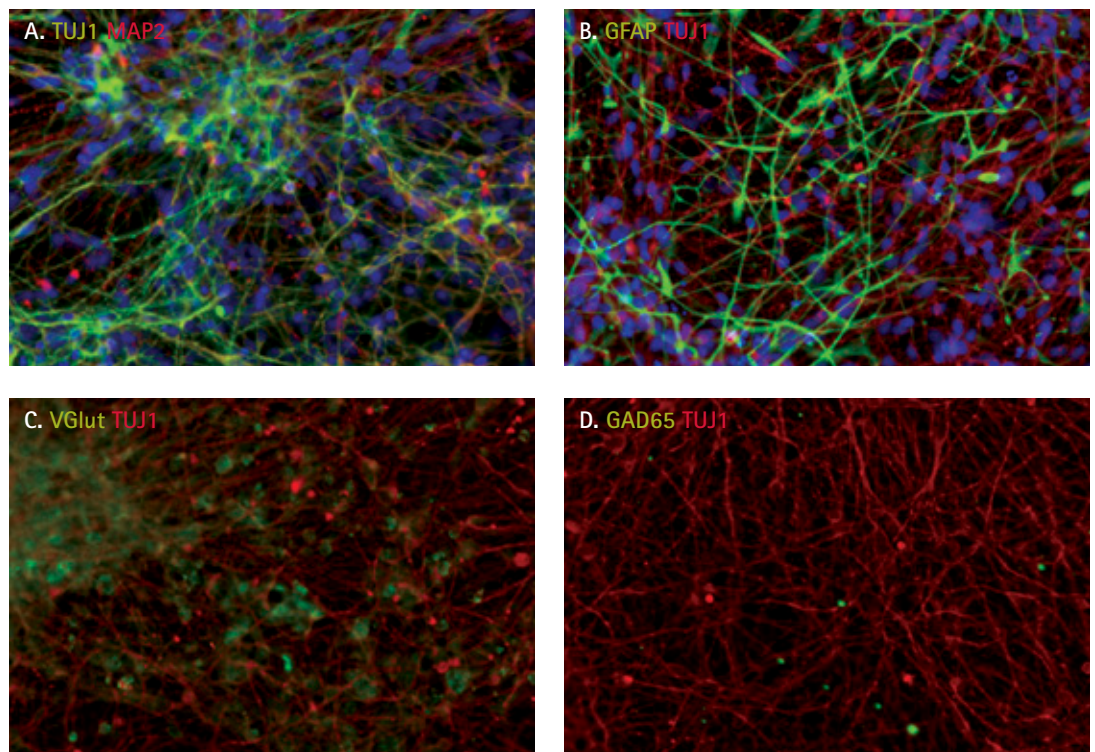

Figure 5.

Neuronal differentiation of iPSC–derived NPCs. After 9 days of differentiation, cells showed extended, elaborate neurite networks. The majority of the differentiated cells expressed neuronal markers, TUJ1 and MAP2 (A) with some cells differentiating to GFAP+ astrocytes. (B). Terminally differentiated cells were preferentially glutaminergic (C) rather than GABAergic neurons (D).

Both ES and iPS cell-derived neural progenitors can be directed to differentiate into tyrosine hydroxylase-positive dopaminergic neurons using NDM and a combination of growth factors (Cat. No. SCR128; SHH, FGF8, BDNF, GDNF, and TGFβIII). 10-30% of the differentiated culture population was tyrosine hydroxylase-positive after 14 days of differentiation (Figure 6).
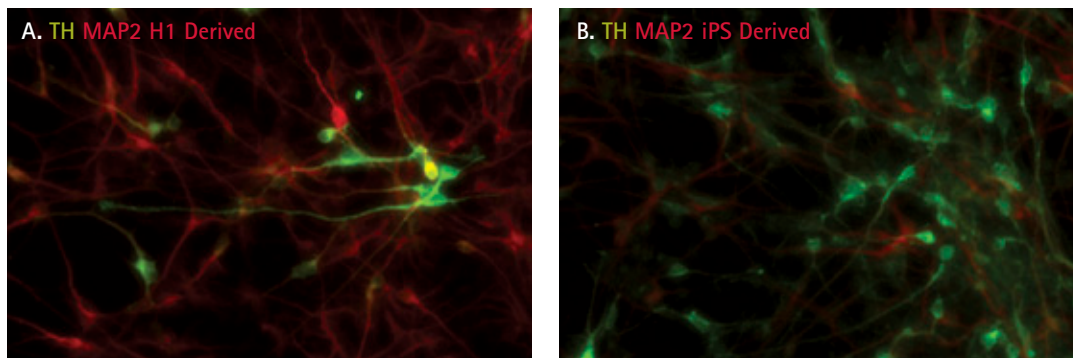

Figure 6.

Dopaminergic differentiation of iPSC–derived NPC. After 14 days of differentiation, dopaminergic neurons were characterized by following the expression of tyrosine hydroxylase (TH). 10-30% cells were positive for tyrosine hydroxylase.

iPS cell-derived NPCs could efficiently differentiate into glial lineage using an proprietary oligodendrocyte differentiation protocol. The differentiated population was >80% positive for oligodendrocyte markers A2B5, O4, and Sox10, 10% GFAP positive, and 30% positive for GalC (Figure 7).
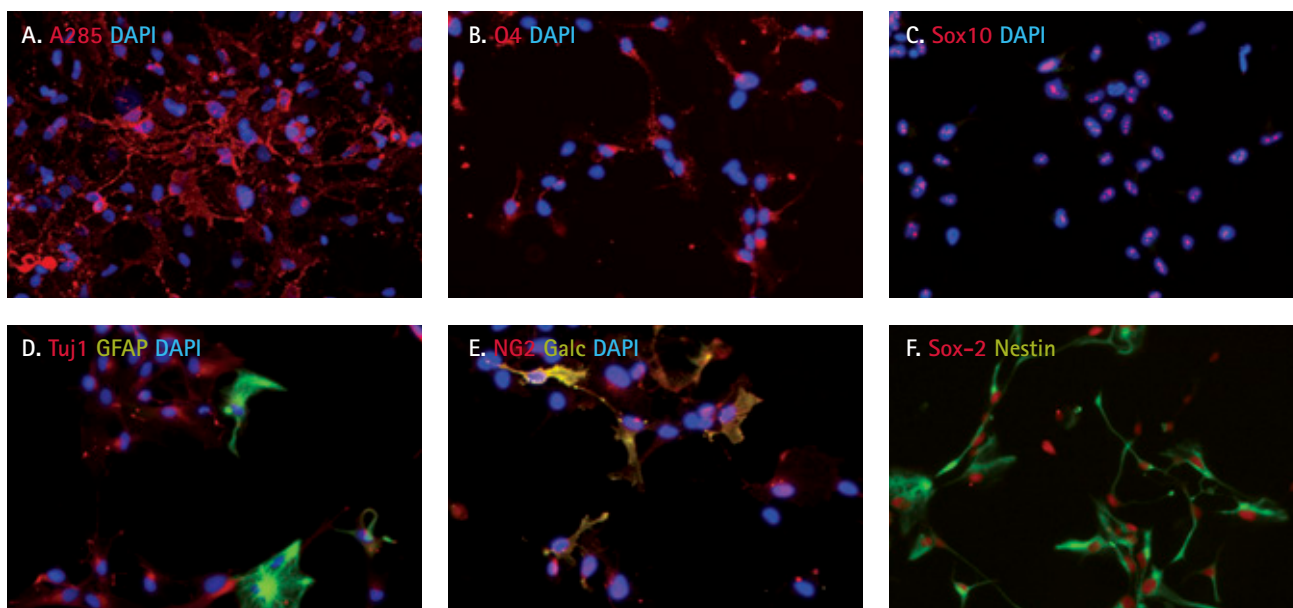

Figure 7.

Oligodendrocyte differentiation of iPS cell–derived NPCs. Cells were cultured as neurospheres in oligodendrocyte expansion medium and small molecules for 14 days before plating on poly-L-ornithine- and laminin-coated plates for immunocytochemical analyses for A2B5 (A), O4 (B), Sox10 (C), TUJ1 and GFAP (D), NG2, GalC (E), Sox-2 and Nestin (F) . Over 80% cells were A2B5-, O4- or Sox10-positive, 10% GFAP-positive, and 30% positive for GalC.

iPS cell-derived NPCs could be patterned into midbrain, forebrain or hindbrain neurons using NDM and specific patterning inducers (SHH/FGF8 or SHH/retinoic acid, Figure 8). Successful patterning was determined by using qRT-PCR to measure the expression of region-specific markers.
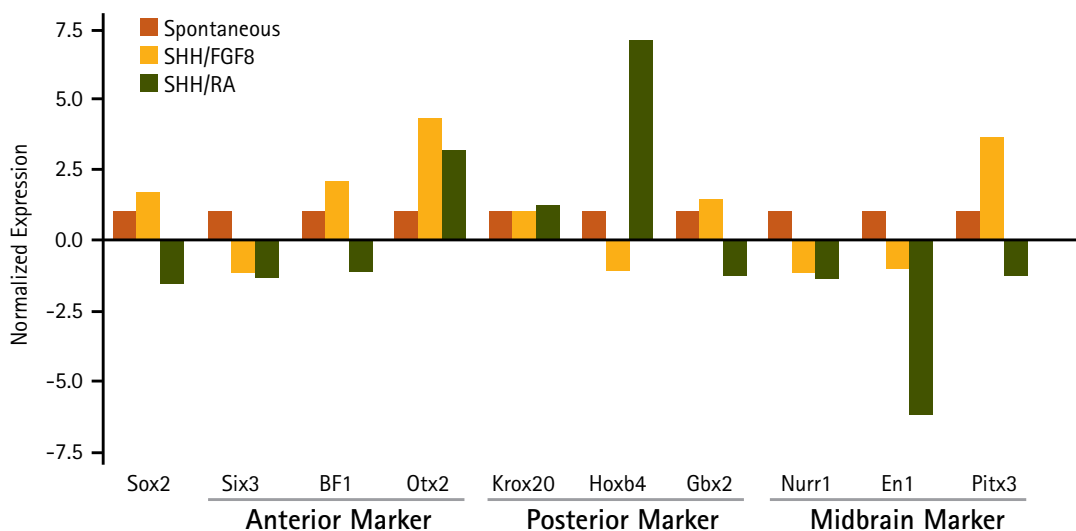

Figure 8.

Region-specific patterning of NPC neuronal differentiation. STEMCAA™-derived iPS cells were induced to the neural lineages as described. At day 5, cells were cultured in either NIM2 (spontaneous), or NIM2 with 200 ng/mL sonic hedgehog, 100 ng/mL FGF8 (SHH/FGF8), or 200 ng/mL sonic hedgehog, 1µM retinoic acid (SHH/RA) for 10 days to prime cells for midbrain and hindbrain fate, respectively. After 10 days, cells were harvested and the gene expression pattern was analyzed by qRT-PCR.

Discussion

We have developed an efficient new neural induction and differentiation medium to generate highly expandable and multipotent neural progenitors and end-stage neuronal and glial cells from iPS cells. These media use established small molecule inhibitors and neural media supplements to produce rapid differentiation kinetics and efficiencies. We show that iPS cell-derived NPCs can be expanded for up to 10 passages while maintaining proper growth kinetics and marker expression. These NPCs are multipotent, differentiating into neurons, astrocytes, and oligodendrocytes depending on the environmental cues introduced into the culture conditions. Specific neural subtypes, such as TH+ dopaminergic and VGlut+ glutaminergic neurons could also be generated from the NPCs using commonly used growth factor inducers. When used with STEMCCA™ reprogramming technology and supporting products, these media provide a complete solution for researchers looking to model neurological diseases, such as Alzheimer's disease, Parkinson's disease and autism, using an iPS cell model system.

References:

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

2. Chambers, S.M., Fasano, C.A., Papapetrou, E.P., Tomishima, M., Sadelain, M., and Studer, L. Highly efficient neural conversion of human ES and iPS cells by dual inhibition of SMAD signaling. Nat. Biotechnol. 2009; 27:275–280

3. Li, W., Sun, W. S., Zhang, Y., Wei, W., Ambasuhan, R., Xia, P., Talantova, M., Lin, T., Kim, J., Wang, X., Kim, W. R., Lipton, S. A., Zhang, K., and Ding, S. Rapid induction and long-term self-renewal of primitive neural precursors from human embryonic stem cells by small molecule inhibitors. Proc. Natl. Acad. Sci. USA 2011; 108:8299-8304.

Related Products

For a complete guide to media, cells and kits, please visit us at: **www.millipore.com/stemcells**

Description	Cat. No.
Human ES/iPS Neural Induction Medium	SCM110
Human ES/iPS Neuronal Differentiation Medium	SCM111
Human ES/iPS Neurogenesis Kit	SCR603
Human IPS Derived Neural Progenitors	SCC035
Human IPS Derived Neural Progenitor Kit	SCR131
Human STEMCCA™ Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit	SCR544
Human STEMCCA™ Cre-Excisable Constitutive Polycistronic (OKSM) Lentivirus Reprogramming Kit	SCR545
PluriSTEM™ Human ES/iPS Media	SCM130
ENStem™-A Neural Expansion Medium	SCM004
FibroGRO® Xeno-Free Human Foreskin Fibroblasts	SCC058
FibroGRO®-LS Complete Media Kit for Culture of Human Fibroblasts	SCMF002
Dopaminergic Differentiation Growth Factor Sampler	SCR128
Human OPC Spontaneous Differentiation Media Kit	SCM106
Human OPC Expansion Media Kit	SCM107
Human Oligodendrocyte Differentiation Kit	SCR600

Related Antibodies

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Description	Cat. No.
OCT-4	MAB4401, MAB4419
Sox-2	MAB4423, AB5603
Nestin	MAB353, MAB5326
Pax-6	AB2237
N-Cad	04-116, 05-915
Tuj1	AB15708
GFAP	MAB3402
MAP2	MAB3418
VGLUT2	MAB5504
GAD65	MAB351R
Tyrosine Hydroxylase	AB152
A2B5	MAB312
O4	MAB344
Sox10	AB5727
NG2	AB5320
Galc	AB342

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Description	Cat. No.
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EGF	GF144, 01-107
SHH	GF174
FGF8	GF110
BDNF	GF029, GF301
GDNF	GF030
TGFβIII	GF176, GF317
PDGFaa	GF142
NT3	GF031, GF308

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