A unifying neural differentiation platform to direct lineage-specific neurons and glial cells from human pluripotent stem cells

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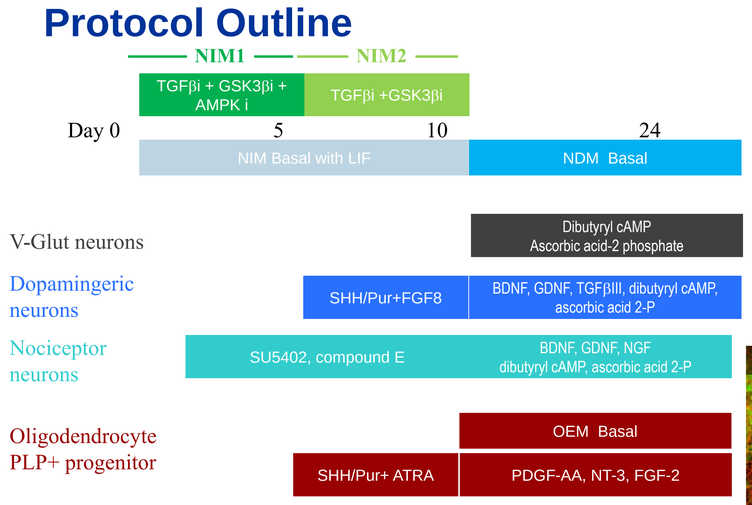
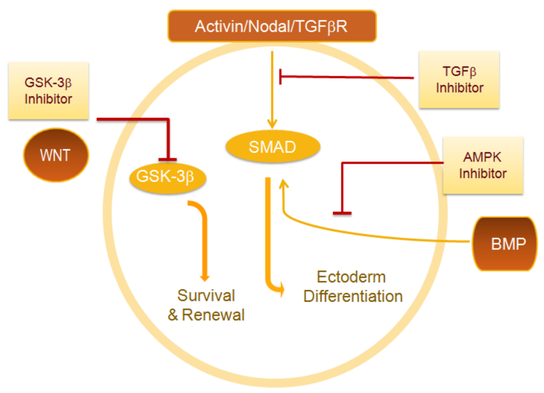
Abstract

Previously, we reported a robust neural differentiation protocol that can efficiently generate greater than 90% neurons from starting pluripotent cultures. We now extend this neural differentiation platform to direct differentiation to lineage-specific neural cells including mid-brain dopaminergic neurons, glutaminergic neurons, nociceptor neurons and oligodendrocytes. Starting with PAX6 and N-Cadherin positive neural rosettes obtained from the 10-day differentiation protocol, the effects of extrinsic signal modulating factors and small molecules that are known to play a role in patterning neural development were examined. Purmorphamine, an activator of the sonic hedgehog signaling pathway and FGF8, yielded approximately 70% TH positive dopaminergic neurons. The PKA pathway activator, dibutyryl cyclin AMP, directed neural cells to greater than 80% V-GLUT2 positive glutaminergic neurons while the introduction of Notch and FGF receptor inhibitors facilitated significant nociceptor neuronal differentiation. Additionally, our results suggest that oligodendrocyte progenitor cells could be more efficiently obtained from the more primitive PAX6$^+$/ N-CAD$^+$ neural rosette populations than from the later Nestin-positive neural progenitor cell stage.
In summary, we suggest that the PAX6$^+$/ N-CAD$^+$ cell population may be more primed for neural cell patterning and may thus be a useful platform from which to study effects of developmental growth cues and signaling regulators on cell lineage specification.

Protocol Outline



The basic platform of neural induction is a 10-day protocol to derive primitive neural progenitor from pluripotent stem cells independent of starting culture method. Switching pluripotent stem cells to NIM will rapidly initiate differentiation toward to neuroectoderm lineage. Additional small molecules or growth factors were added at the indicated timeline to promote specific lineage of neurons or glials.



Signaling pathways targeted by neural induction medium. Merck 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.

Table 1. Antibodies and Concentrations Used In This Study

Polyclonal Marker	Cat. No.	Target	Dilution
SOX2	AB5603A4	Neural progenitor	1:200
PAX6	AB2237	Neural progenitor	1:400
TUJ1	AB15708	Neuron	1:200
SOX10	AB5727	Oligoglial progenitor	1:200
TH	AB152	Dopaminergic neuron	1:200
Substance P	AB926	Sensory neuron	1:200
RET	07-1237	Nociceptor neuron	1:200

Monoclonal Marker	Cat. No.	Target	Dilution
N-Cadherin	05-915	Neural progenitor	1:100
Nestin	MAB5326C3	Neural progenitor	1:400
GFAP	MAB3402	Astrocyte	1:200
MAP2	MAB3418	Neuron	1:200
V-GLUT2	MAB5504	Glutaminergic neuron	1:200
GAD65	MAB351R	GABAergic neuron	1:200
O4	MAB345	Oligoglial progenitor	1:25
A2B5	MAB312RX	Glial progenitor	1:100
OCT4	MAB4401C3	Pluripotent cell	1:100

Rapid and Robust Neural Induction

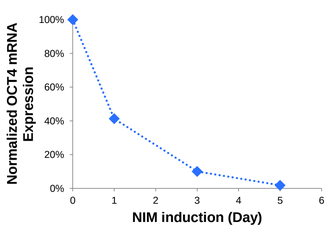


Fig 1. Rapid down regulation of pluripotent maker , OCT4, in NIM protocol. The level of OCT4 mRNA in H9 cells cultured in serum-free medium was analyzed by qRT-PCR, n=3, and normalized with GAPDH expression. OCT4 expression was drastically down regulated as early as 24 hour post neural induction.

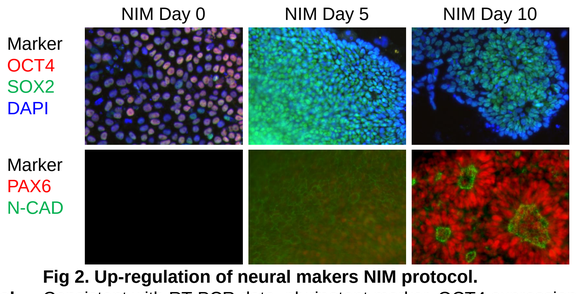


Fig 2. Up-regulation of neural makers NIM protocol. Consistent with RT-PCR data, pluripotent marker, OCT4 expression diminished after 5 days of NIM **(A, B, C)** while SOX2 expression showed moderate increase during neural induction time course. Neural marker, PAX6 and N-cadherin **(D, E, F)** are up-regulated. After 10 days, cells form polarized rosettes structure **(C, F)** suggested that these cells are primitive neural stem cells .

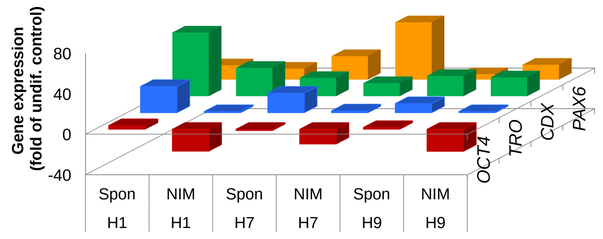


Fig 3. NIM overcome inner cell propensity for differentiation. Human ESCs H1, H7, H9 lines cultured on MEF were subjected to either spontaneous differentiation (**Spon)** by 14 days in EB or NIM induction (**NIM)** by10 days in NIMs. Gene expression of pluripotent (OCT4), neural (PAX6), mesoderm (Troponin-1, TRO), and endoderm (CDX) markers were analyzed by qRT-PCR. N=2, Cq normalized with GAPDH and standardize with undifferentiated sample. All three lines showed increased neural lineage compare to endo/mesoderm lineage when differentiated spontaneously.

iPS to Expandable NPCs

Schematic workflow to generate expandable NPC

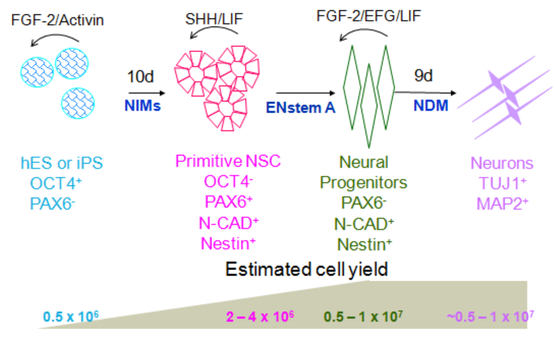


Fig 4. Direct pluripotent stem cells to neural lineage by NIM method. Pluripotent stem cells in monolayer can be direct to neural lineage with NIMs . The induction can apply to the traditional KOSR or serum-free culture with no additional adaptation steps.
10 days after induction, primitive neural stem cells in polarized rosettes can be either maintained in primitive stage with the addition of sonic hedgehog (SHH) in NIM basal with limited expansion or move to neural progenitor cells with the addition FGF-2 for 7 to 10 passages. At least a 20-fold expansion ratio was expected.

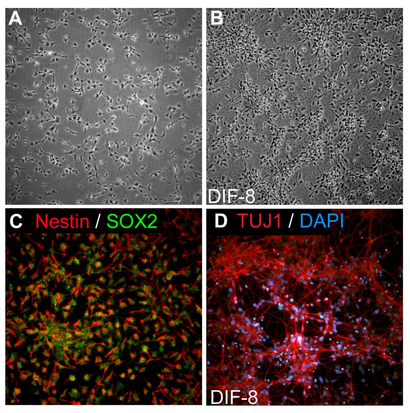


Fig 5. STEMCCA™ derived iPS cells to expandable, highly enriched neural progenitor cells
Xenogene-free, human STEMCCA™ derived iPS lines were cultured with serum-free PluriSTEM™ medium (Cat. No. SCM130), before switching to NIMs for 10 days . After 10 day induction, NPCs were harvested and expanded in ENStem-A™ medium (Cat. No. SCM004) supplemented with FGF-2. Over 90% of progenitors express Nestin and SOX2 **(C)** while over 90% of progenitors differentiated into TUJ1 **(D)** positive neuronal cells upon switching to NDM without dibutyryl cAMP for 8 days.

Lineage-specific Neurons

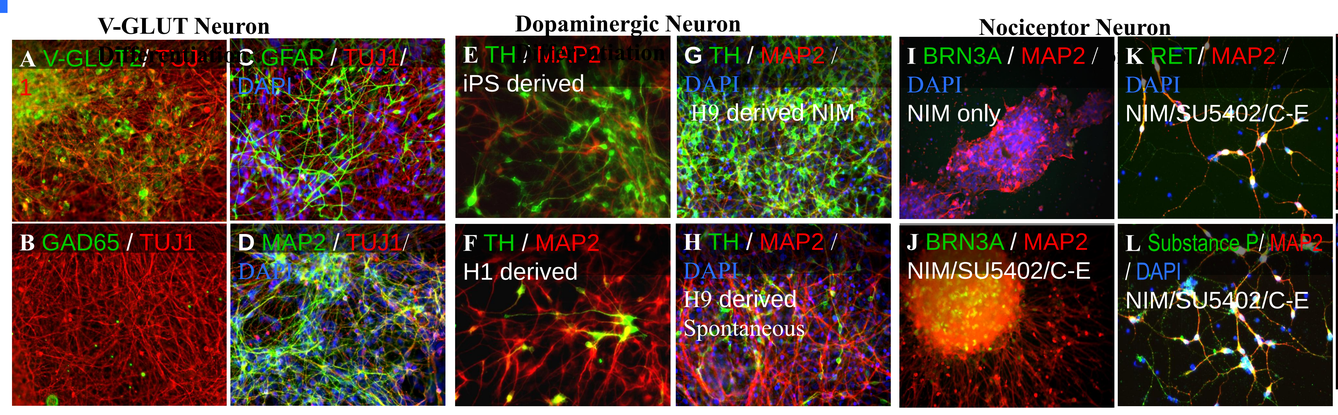


Fig 6. Combine NIM induction with known factors to generate lineage-specific neurons.
NIM-based neural induction was used as a platform to derive lineage-specific neurons as described in Protocol Outline and Materials and Methods. In the presence of dibutyryl cAMP, differentiated neurons are labeled with V-GLUT2 markers but not GAD65 **(A, B)** suggesting the enrichment of excitation neurons. In the same condition, 5 -10% of cells express GFAP markers while over 80% of cells are TUJ1-positive **(C, D).** Combining SHH and FGF-8 in NIM2 stage derived cells to mid-brain lineage and further maturation in BDNF, GDNF, TGFβIII, dibutyryl cAMP, and ascorbic acid generated 10 to 70% tyrosine hydroxylase (TH) -positive neurons **(E, F, G).** The percentage is dependent on the parental line. Without the cocktail to promote dopaminergic neurons, spontaneous differentiation only generated less than 5% of TH-positive neurons **(H)**. Incorporating FGF-2 inhibitor, SU5402, and notch inhibitor, Compound E (C-E), 2 day after NIM induction promoted cells to develop the sensory neuron marker, BRN3A, while NIM only treatment exhibited minimal staining for BRN3A **(I, J)**. These cells, after 14 days maturation, expressed both RET and substance P, suggesting that they had been directed toward nociceptor neuron cell fate.

Glial Differentiation

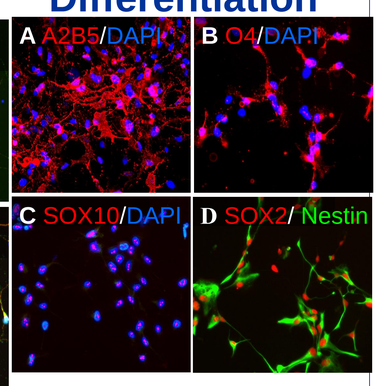


Fig 7. Combine NIM induction to generate oligoglial cells.
Human STEMCCA™ iPS cells were induced to neural progenitor with NIMs with 200 ng/mL Sonic hedgehog and 10 µM ATRA, followed by the oligoglial lineage with oligodendrocyte expansion medium for 14 days before plating on poly-L-ornithine and laminin coated plate for analyzing the expression of **(A)** A2B5 **(B)** O4 **(C)** SOX10 **(D)** Nestin and SOX2. >80% cells were A2B5, O4,or SOX10 positive.

Materials and Methods

Generation of iPS Cells Using STEMCCA™ Vectors
Human induced pluripotent stem (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 and 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 (20-50 MOI) was added to growth medium 24 h after seeding. Virus were removed after 24 h of incubation and the growth medium was replaced every day till day 5. After 5 days, transfected fibroblasts were replated on mouse embryonic fibroblast feeder (MEF) together with Human iPS Cell Boost Supplement II (Cat. No. SCM094) for additional 11 to 20 days with MEF medium. IPS cells were then clonally expanded on MEF with 20% KOSR-based medium (80% DMEM/F12, 20% KOSR, 1% βME, 1% NEAA) supplemented with 8 ng/mL FGF-2 (Cat. No. GF003). Excision of transgenes was achieved by incubating iPS cells with 5 µM of TAT-Cre recombinase (Cat. No. SCR508) following manufacturer's instructions.
Neural induction of iPS Cell Using NIM1 and NIM2
Neural progenitor and neuron cells differentiated from iPS cells were generated by Human ES/iPS Neurogenesis kit (Cat No. SCR603). Undifferentiated iPS colonies were passaged with the culture medium and desired method. After 48 h, growth medium was replaced to neural induction medium I for 5 days with medium changed every other day. On day 6, cells were dissociated with non-enzymatic buffer (Cat No. S-004-C) and plated on 0.5 mg/mL Matrigel® matrix-coated plate with neural induction medium 2 for additional 5 days with medium changed every other day. After 10 days, neural progenitors 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 plate coated with 0.1 to 0.2 mg/mL of Matrigel® matrix at 0.5 to 1x10^5 cells/cm^2.
Quantitive RT-PCR analysis
Human ES lines cultured via the traditional MEF method or serum-free PluriSTEM™ medium were subjected to NIM by switching culture medium. The time course of OCT4 expression was analyzed by TaqMan® probes (Human OCT4: Hs00999634_gH; Huamn GAPDH: Hs02758991_g1). The line propensity was analyzed by Bio-Rad iQ™ SYBR® Green Supermix against indicated genes.
Differentiation of Neural Progenitor Cells into Neurons
Neural progenitors between p3 and p8 were harvested with Accutase™ reagent and plated on poly-L-ornithine/laminin (10 µg/mL each; Poly-L-ornithine, Cat. No. A-004-C; laminin Cat. No. CC095) at 10-50K cells/cm^2 with neuronal differentiation medium (Cat. No. SCM111) supplemented with 0.5 mM dibutytryl cAMP and 0.2 mM ascorbic acid phosphate. Around 80% of medium was refreshed every two to three days for 9-14 days. Cells were fixed with 2% paraformaldehyde before immunofluorescent staining.
Differentiation of Neural Progenitor Cells into Dopaminergic Neurons
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 was included in NIM2 medium and culture cells for 5 days. Cells were then harvest with Accutase™ reagent into single cells and plated on poly-L-ornithine and laminin-coated plate at 10 µg/mL each with neuronal differentiation medium containing 20 ng/mL BDNF and GDNF and 1 ng/mL TGFβIII for 14 days. TH positive cells that represented dopaminergic neurons were characterized by immunofluorescent staining.
Differentiation of Neural Progenitor Cells into Nociceptor Neurons
10 µM of SU5402 (Tocris) and 10µM of Compound E (Cat. No. 565790-1MG) were included in NIM 1 starting at day 2 of induction until day 10. After day 10, cells were harvested with non-enzymatic buffer and plated on poly-L-ornithine/laminin plate for further maturation in NDM with 10 ng/mL of NGF (Cat. No. GF 307), BDNF and GDNF for 14 days prior to fixation.

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