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Original Article

Human induced pluripotent stem cells generated neural cells behaving like brain and spinal cord cells: An insight into the involvement of retinoic acid and sonic hedgehog proteins

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ABSTRACT

Objectives: The previous studies generated neural progenitor cells (NPCs) from human induced pluripotent stem cells (hiPSCs) using different protocols. However, the nature of the temporal or regional specificity of NPCs derived using these protocols is not well defined. Therefore, this study aimed to generate age- and region-specific NPCs from hiPSCs, which mimic in vivo fetal brain (FNPC-B) or spinal cord (FNPC-SC) tissues, in the absence or presence of retinoic acid (RA) and sonic hedgehog (SHH).

Materials and Methods: Ventral, caudal and posterior neural cells were generated from hiPSCs with morphogens (RA and SHH), or dorsal, rostral, and anterior neural cells by the withdrawal of these morphogens from the NPC-media. NPCs generated from hiPSCs were compared to FNPC-B or FNPC-SC using immunocytochemical staining assays and global microarray for the evaluations of general and region-specific neural cells markers of neural induction, differentiation, and maturation. Microarray profiling results were analyzed using quantitative unpaired *t*-test ($P < 0.05$).

Results: Immunocytochemical analyzes showed that generated NPCs expressed general neural cells markers (PAX6 and MUSASHI-2). Furthermore, FNPC-B and anterior NPCs were characterized with marked expression of cortical neural cells marker (SOX1) when compared to FNPC-SC and posterior NPCs. Microarray profiling results showed the up-regulation of brain cells markers (EMX2 and PAX6) in FNPC-B and anterior NPCs. Similarly, spinal cord cells markers (COL5A2, HOXB5, HOXB7, HOXB8, HOXC4, and HOXD4) were up-regulated in FNPC-SC and posterior NPCs.

Conclusion: NPCs that mimic *in vivo* brain and spinal cord cells can be generated from hiPSCs in the absence or presence of RA and SHH.

Keywords: Fetal brain tissues, fetal spinal cord tissues, human induced pluripotent stem cells, neural progenitor cells, molecular analyses

Introduction

Human embryonic stem cells (hESCs) have the capacity to divide and differentiate into all body cells types (pluripotent).[1-5] In theory, these cells hold potential for the possible treatment of all human diseases caused by the loss of or defective body tissues.[1-5] However, the use of hESCs for research raises ethical concerns as the embryos are normally destroyed when employed for research purposes. Such concerns appeared to have been avoided with the generation of human induced pluripotent stem cells (hiPSCs) derived from the reprogramming of adult somatic cells into pluripotent stem cells with the aid of retroviral mediated transfection of transcription factors such as Oct3/4, SOX2, c-Myc, and Klf4.[1-4] hiPSCs, therefore, have great relevance in cell therapy, drug screening, and toxicology experiments. Furthermore, the successful generation of neural progenitor cells (NPCs) from hiPSCs, introduced the possibility of generating neural structures devoid of host – immune rejection issues in cells or tissues replacement therapies.[1-4]

Human nervous system development begins with neural induction converting ectodermal cells into neuroectodermal cells leading to the formation of the neural plate and finally the neural tube.[6,7] The molecular bases of neural induction are not completely understood, however, bone morphogenetic protein (BMP) antagonists (such as chordin, follistatin, and noggin) and fibroblast growth factor (FGF) agonists are

believed to regulate this process.[6-9] Temporal patterning and regionalization first along anterior-posterior and then the dorsal-ventral (D-V) axes occur post-neural induction with exposure to extrinsic cues.[6-12] In the absence of extrinsic factors, the default fate is suggested to be anterior or rostral.[6-12] Dorsal patterning is believed to be regulated by transforming growth factor – β proteins such as BMP – 4 and 7, dorsalin and activin.[6,7] Ventral patterning is believed to be regulated by sonic hedgehog (SHH) assumed to be secreted by the notochord and retinoic acid (RA) believed to be secreted by the surrounding somites.[6,7] Posterior identities are achieved with exposure to caudalizing agents such as Wnts, RA, and FGFs while further ventral fate is achieved with concentration gradient exposures to SHH.[6-13]

Region-specific neural cells markers such as anterior transcription factor SOX1, OTX2, Forse 1, EMX2 and telencephalic transcription factor FOXG1 for forebrain neural cells,[12,14] and hindbrain or spinal cord transcription factors such as COL5A1, COL3A1, HOXA9, HOXB4, THY1, and EN1 have been established.[12,14] NPCs were previously generated from hESCs using similar protocols from embryoid bodies (EBs),[10] co-culturing of hESCs with stromal cell-derived inducing activity (SDIA) on bone-marrow derived stromal cells; co-culturing of hESCs with PA6 cells, low density culturing of hESCs in a serum-devoid medium but with exposure to insulin,[8] and culturing of hESCs in a serum-devoid medium.[9,12] These established protocols, however, present different challenges requiring new modifications.

Co-culturing with SDIA may present other non-human stromal factors making the understanding of the mechanisms involved unclear. Subsequent cell transplantation may also be hindered by the need to remove the stromal cells due to their tumorigenic properties.[10] Similarly, co-culturing of hESCs with PA6 was mainly established with the expression of Nestin by generated NPCs. Nestin is, however, ubiquitously expressed by non-neural cells and cannot be used for the sole characterization of neural stem cells.[8] In addition, cortical or anterior neurospheres were generated in a highly efficient serum-free pluripotent stem cell neural induction medium that can induce human pluripotent stem cells into primitive NPCs in 7 days[2] or in a supplemented knockout serum replacement medium with 10% CO_2, which doubled the expression of general neural markers (NESTIN and PAX6) as well as brain cells marker (FOXG1) genes compared with those cultured with 5% CO_2.[3] However, these studies did not generate caudal or posterior neurospheres. Serotonin neurons of the hindbrain were generated from human pluripotent stem cells through manipulation of the Wnt pathway,[4] however, no cortical neurons were generated in the study. Different attempts have been made to overcome some of these challenges, yet, contributions toward the production of suitable protocols for age- and region-specific comparable generation of NPCs from hiPSCs to fetal brain-derived NPCs (FNPC-B) or fetal spinal cord-derived NPCs (FNPC-SC) are still required.

iPSCs were observed to have epigenetic memory, with the generated cells having more capacity to differentiate into the cells of origin than other cells types.[15] Similarly some differences in gene expression profiling, microRNA profiling and DNA demethylation have been observed between ESCs and iPSCs in the undifferentiated state.[15] However, continued passaging consistently drives iPSCs closer to ESCs, whether this is due to further reprogramming is not yet well defined.[15] Hence, there is a possibility that improvements to reprogramming technology will reach a point at which no molecular differences will be observed between iPSCs and ESCs. Furthermore, transcriptional differences observed between iPSCs and ESCs in the undifferentiated state were largely dissipated in the differentiated state as the progeny of these pluripotent cells were essentially identical, except for just a handful of genes.[15] Transcription factor-based reprogramming is, therefore, nearly sufficient for complete reprogramming and it is possible that culture conditions or pharmacological manipulation of the epigenome may generate IPSCs that are indistinguishable from ESCs.[15]

Hence, more work is required to produce the most suitable protocols for the generation of age- and region-specific neural cell types from hiPSCs, and to identify specific candidate genes involved in the development of human nervous system using hiPSCs. In addition, RA is an established morphogen for fate caudalization of NPCs. Similarly, SHH is an established morphogen for fate ventralization of NPCs. It is, therefore, relevant to establish the generation of anterior or posterior NPCs from hiPSCs in the presence or absence of RA and SHH. Therefore, this study aimed to: Generate age- and region-specific neural cell types from hiPSCs in the presence or absence of RA and SHH, understand and classify the cell types present in NPCs generated from hiPSCs, and determine if the neural cells generated from hiPSCs are comparable to FNPC-B and FNPC-SC.

Materials and Methods

This research study was conducted in accordance with the ethical guidelines of the Helsinki Declaration of 1975, as revised in 2000 with respect to the use of human cells for research purposes. NPCs were generated from hiPSCs, fetal brain and spinal cord tissues, compared and classified based on modifications of the following protocols as previously described:[16] Generation of EBs from hiPSCs, floating of EBs in neurobasal (N2/B27) base media with or without RA, differentiation in rosette media (with or without RA and SHH), maintenance of NPCs in basic FGF (bFGF) and epithelia growth factor (EGF), splitting of NPCs, fixing of NPCs, freezing of NPCs, immunofluorescence staining assay, and RNA extraction for microarray analyses.

Generation of EBs

hESCs colonies cultured with mouse embryonic fibroblasts (MEFs) feeder layer were received from the Stem Cell Core of

the University of Los Angeles (UCLA) and expanded in hESCs media for seven (7) days. EBs were subsequently generated from hESCs as earlier described.[16] Collagenase solution was prepared by 1:10 dilution of one vial of 1 ml collagenase (stored in −80°C) with 9 ml of Dulbecco's modified eagle medium (DMEM)/F12 (Invitrogen 11330-32, USA). The prepared collagenase solution was further filtered through a 2.0 µm membrane to obtain a sterile solution. hiPSCs media was aspirated from Mouse Embryo Fibroblasts (MEFs) feeder-plated hiPSCs. 1 ml of collagenase solution was added per well of hiPSCs. The plates containing colonies of hiPSCs were incubated at 37°C until colonies were lifted completely off from the feeder layer (about 8-10 min). Equal ml volume of EBs media (hiPSCs media without bFGF) was added per well to neutralize the effects of the added 1 ml collagenase solution.

The mixed collagenase solution, EBs media, and hiPSCs were transferred to a 50 ml tube. 3 ml of EB media was added per well to rinse any remaining colonies from the wells. This rinse was equally transferred to the 50 ml tube. The resulting mixed solution was gently mixed in the 50 ml tube avoiding the break-up of colonies. Colonies were allowed to settle on their own for about 10 min, during which 1 ml of EB media was added to new low-attached plates. The supernatant was removed using a pipette while the cell pellets were re-suspended in 1 ml per well of EB media depending on the number of plates of EBs to be prepared. Re-suspended cells were gently mixed between wells without breaking-up colonies, and 1 ml of EB medial plus cells (EBs) was added to each well.

Floating of EBs in neurobasal (N2/B27) base media with or without RA

Spent EB media was carefully removed using 5 ml pipette leaving EBs on low-attached plates intact. EBs were floated in new (minus RA or plus RA) N2 base media for 7 days (days 0-7) while feeding with new media was carried out every 3 days at 2 ml per well. The N2 base media (minus RA) contained 500 ml DMEM: F12 (Invitrogen 11330-32, USA), 5 ml N2 supplement (×100), liquid (Invitrogen 17502-048, USA), 10 ml B27 Minus vitamin A (×50), liquid (Invitrogen 12587-010, USA), 5 ml ×100 Pen-Strep, and 500 µl ×1000 Primocin (optional). The N2 base media (plus RA) contained 500 ml DMEM: F12 (Invitrogen 11330-32, USA), 5 ml N2 supplement (×100), liquid (Invitrogen 17502-048, USA), 10 ml B27 (containing vitamin A) (×50), liquid (Invitrogen 17504-044, USA), 5 ml ×100 Pen-Strep, and 500 µl ×1000 Primocin (optional).

Differentiation of EBs in rosette derivation media (*in situ*)

EBs were manually removed using 200 µl pipette and plated onto polyornithine-laminin coated plates for 7 days in rosette derivation media (minus RA and SHH or plus RA and SHH). The rosette derivation media (minus RA and SHH) contained N2 base media (minus RA) and 20 ng/mL bFGF. The rosette derivation media (plus RA and SHH) contained N2 base media, 1 µMo SAG (smoothened agonist = SHH, calbiochem), 1 µMo RA, and 20 ng/mL bFGF. 1% fetal bovine serum (FBS) was added per well on the 1st day of attachment of EBs (day 8) to aid the attachment of EBs to polyornithine-laminin coated plates. EBs were taken out of 1% FBS after 24 h. Feeding with new media was carried out every 2 days at 2 ml per well. Quantification of how many EBs was attached or not was carried out on day 9. At the end of 7 days of the differentiation of EBs in rosette derivation media (day 14), neural tube-like rosettes were derived from attached EBs (Passage 0). Quantification of how many attached EBs were able to generate rosettes (neurospheres with ribbon or rounded edges) was carried out on day 14.

Maintenance of NPCs in bFGF and EGF

Rosettes were dissected out on day 14, manually picked and plated onto polyornithine-laminin coated plates as Passage 1 (P1) NPCs and maintained in NPCs media (minus RA or plus RA). The NPC maintenance media contained 50 ng/mL EGF and 20 ng/mL bFGF. Feeding with new media was carried out every 2 days at 2 ml per well. After 3-7 days (days 17-21), P1 NPCs were split and divided into three equal parts: For RNA extraction, for immunocytochemical staining and for further passages as P2-P4 NPCs. For global microarray profiling, P1 NPCs were lysed with 400 µl of Lysis Buffer (Agilent Technologies – 400711-13, USA) per well, collected in 1 ml micro-centrifuge tubes and stored in −80°C. In all cases, once growth factors have been added, prepared media was used within 4-5 days, except for N2 base media containing B27 (minus RA or plus RA) which were used within 7-10 days at 4°C.

Splitting of NPCs

Trypsin solution was prepared by 1:10 dilution of 1 ml of 0.25% trypsin ethylenediaminetetraacetic acid (EDTA) ×1 (Invitrogen/GIBCO – 25200, USA) with 9 ml of phosphate buffer saline (PBS) solution. Polyornithine-laminin coated plates were prepared by adding 1 ml NPC media per well to provide more passages of NPCs. Spent NPC media (minus RA or plus RA) was removed from P1 NPCs and 2 ml of trypsin solution added per well and incubated at 37°C strictly for 5 min. Cells were removed by pipetting up and down using a 5 ml pipette. The resulting mixed trypsin plus cells were transferred to a 15 ml conical tube and spun down at 300 G (RCF) for 6 min. The supernatant was aspirated and the tube tapped to disrupt pellets. NPC media (minus RA or plus RA) was added to re-suspend cells. Cells were divided into three equal parts: For RNA extraction, for immunocytochemical staining, and for further passages as P2-P4 NPCs. NPCs prepared for further passages as P2-P4 NPCs were gently mixed in between and distributed at 1 ml per well on polyornithine-laminin coated plates.

Fixing of NPCs

Passages 2-4 NPCs maintained in NPC media (minus RA or plus RA) were plated on polyornithine-laminin coated glass

cover-slips. 4% paraformaldehyde (PFA) solution was prepared (10 ml of 16% PFA, Ample, India) and added to 30 ml PBS solution. Spent NPC media was sucked up using 1000 µl pipette and replaced with 300-500 µl of 4% PFA solution and incubated at room temperature for 20 min. PFA was sucked up, and cells washed thrice at 5 min interval each with PBS solution with the last wash carried out using 1 ml of PBS. All procedures were carried out under the chemical hood for safety purposes. Cover-slips fixed in 4% PFA (to immobilize antigens) were stored in 4°C.

Freezing of NPCs

Trypsin solution was prepared by 1:10 dilution of 1 ml of 0.25% trypsin EDTA ×1 (Invitrogen/GIBCO – 25200, USA) with 9 ml of PBS solution. Dimethyl sulfoxide (DMSO) plus NPC media solution was prepared by adding 10% DMSO to equivalent NPC media (minus RA or plus RA). For example, 400 µl DMSO was added to 3.6 ml of NPC media. Media was never added to DMSO. Spent media (minus RA or plus RA) was removed from P1 NPCs, and 2 ml of trypsin solution added per well strictly for 5 min. Cells were removed by pipetting up and down using a 5 ml pipette. The resulting mixed trypsin plus cells were transferred to a 15 ml conical tube and spun down at 300 G (RCF) for 6 min. The supernatant was aspirated, and the tube tapped to disrupt pellets. DMSO plus NPC media solution (minus RA or plus RA) was added to re-suspend cells. Cells were distributed at 1 ml per cryovials and stored in −80°C for further use.

Immunocytochemistry/immunofluorescence staining assay

The blocking solution containing 10% goat serum (Invitrogen, USA) (no PBS addition since dilution is not required) or donkey serum (Jackson ImmunoResearch, USA) diluted with PBS (depending on the unrelated species in which the primary antibody was raised) and 0.1% Triton X-100 (detergent) was prepared. The serum blocked any antigenic site not specifically binded by the primary antibody while Triton permeabilized the cells permitting intracellular access of the antibody. The general neural cells markers tested for and analyzed were PAX6 and MUSASHI-2. The region-specific neural cells marker tested for and analyzed was SOX 1 (brain neural cells marker).

RNA isolation from NPCs

RNA isolation from NPCs was according to the protocol described in the strategene absolutely RNA Microprep Kit (Strategene, Denmark).

Microarray analyses

RNA (isolated as described above) in the Elution Buffer was submitted for microarray analyses. Microarray profiling was evaluated using Affymetrix Human HG-U133 2.0 Plus arrays, and the data were normalized with robust multichip algorithm in Genespring. For further analyses, only probe sets expressed at a raw value of >50 in at least 10% of samples were included.

Statistical analyses

Microarray profiling results were analyzed using quantitative unpaired *t*-test ($P < 0.05$). Complete linkage was employed in hierarchical Euclidian clustering while gene expression differences were only classified as significant when the *P* value of the fold change was at least 2.00-fold different between analyzed samples and <0.01.

Results

Generation of neural tube-like rosettes and NPCs from hiPSCs

EBs generated from hiPSCs differentiated into rosettes and NPCs mimicking *in vivo* neural tube formation during the human nervous system development (Figure 1a). Rosettes derived from EBs were composed of radially organized columnar cells (rosettes) with defined lumen and neural processes extending outward (Figure 1b and c). These columnar cells were loosely attached to the petri dish and were morphologically distinguished from their outlining flat peripheral cells which attached strongly to the petri dish. NPCs generated from hiPSCs with or without RA and SHH were non-morphologically distinct from one another, and from FNPC-B and FNPC-SC (Figure 2a-d).

Expression of general and region-specific neural cells markers by NPCs derived from hiPSCs (with or without RA and SHH)

NPCs generated from hiPSCs, FNPC-B and FNPC-SC expressed general neural cells markers (PAX6 and MUSASHI-2) (Figure 3a and b). Further characterizations of anterior or posterior NPCs derived from hiPSCs with or without RA

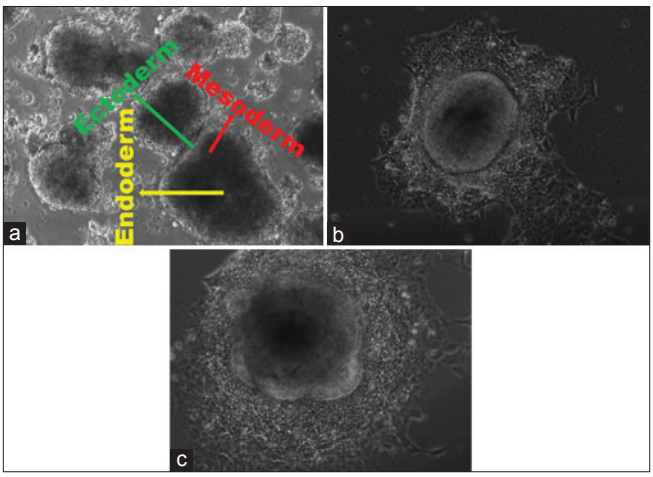

Figure 1: (a) Generated embryoid bodies and (b and c) non-morphologically distinct rosettes generated from human induced pluripotent stem cells with or without retinoic acid and sonic hedgehog

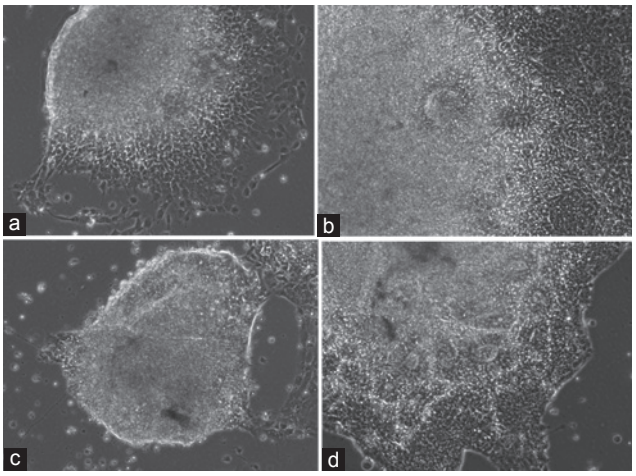

Figure 2: Generated non-morphologically distinct neural progenitor cell (P1) from human induced pluripotent stem cells: (a and b) With retinoic acid (RA) and sonic hedgehog (SHH); (c and d) without RA and SHH

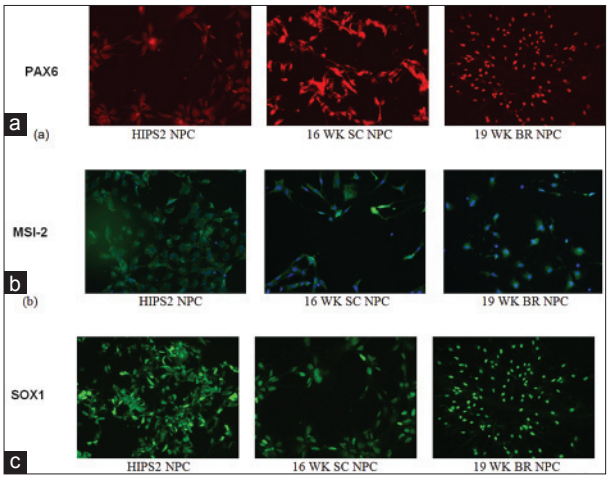

Figure 3: Results of immunofluorescence assay showing the expression of general neural cells markers: (a) PAX6, (b) MUSASHI-2 and differential expressions of brain neural cells marker: (c) SOX1 by neural progenitor cells (NPCs) derived from human induced pluripotent stem cells without retinoic acid and sonic hedgehog (HIPS2), 16 week fetal spinal cord NPC (16 WK SC) and 19 week fetal brain NPC (19 WK BR NPC)

and SHH were evaluated based on the expression of SOX1. Anterior NPCs were characterized with marked expression of SOX1 in NPCs derived from hiPSCs and FNPC-B (Figure 3c) in comparison to decreased expression of SOX1 in NPCs derived from FNPC-SC (Figure 3c).

Global gene analyses of genes up-regulated in NPCs derived from hiPSCs (with or without RA and SHH) in comparison to NPCs derived from 19-week-old fetal brain or 16-week-old Spinal Cord Tissues

Global gene analyses showed the up-regulation of anterior or brain cells markers (EMX2 and PAX6) in 19-week-old brain NPCs and NPCs derived from hiPSCs in the absence of RA and SHH (Table 1). Similarly, posterior or spinal cord cells markers (COL5A2, HOXB5, HOXB7, HOXB8, HOXC4, and HOXD4) were up-regulated in 16-week-old spinal cord NPCs and NPCs derived from hiPSCs in the presence of RA and SHH (Table 1).

Discussions

This study demonstrated that hiPSCs have the capacity to differentiate into age- and region-specific anterior or posterior NPCs using chemically-defined serum-free medium (N2/B27) with or without RA and SHH. EBs generated from hiPSCs were composed of the three germ layers (ectoderm, mesoderm, and endoderm), akin to *in vivo* gastrulation undergone by the gastrula in human embryogenesis. Subsequently, the EBs differentiated into neural tube-like rosettes (based on morphological identification) mimicking *in vivo* nervous system development. These rosettes were composed of radially organized columnar cells with defined lumen and neural processes extending outward (Figures 1 and 2) as previously established.[14,16] These columnar cells were loosely attached to the petri dish and were morphologically distinguished from their outlining flat peripheral cells which attached strongly to the petri dish as previously described.[8-10,14,16-18]

Neural tube-like rosettes generated from human pluripotent stem cells have been previously established to have defined apico-basal polarity based on the expression of neural cells markers with accompanied functional properties.[17] NPCs derived in this study from hiPSCs, FNPC-B and FNPC-SC expressed established general neural cells markers such as PAX6 and MUSASHI-2 (Figure 3a and b) as confirmed with immunofluorescence staining assays, and in agreement with the previous studies.[8-10,14,16-18] In addition, generated NPCs differentiated into anterior, dorsal or cortical neural cells in the absence of caudalizing (RA) or ventralizing (SHH) agents. NPCs, however, differentiated into posterior, ventral or caudal neural cells in the presence of RA and SHH. Further characterization using immunofluorescence assays observed marked expression of SOX1 by FNPC-B and NPCs differentiated from hiPSCs without RA and SHH, when compared to FNPC-SC (Figure 3c). SOX1, a transcriptional factor expressed generally by precursor neural cells becomes dissipated and limited to later stage rosettes or cortical neural cells with further differentiation.[17] The data from this study further demonstrated that the default fate in the absence of exogenous factors following neural induction is anterior, dorsal or cortical.

The differentiation of NPCs into anterior neural cells had been previously characterized with the significant increased expression of cortical neural cells markers such as LHX2,[12] FOXG1, EMX2, and PAX6 distinguishing anterior from posterior precursor fates.[8-10,17,18] Global gene expression analyses showed that NPCs derived in the absence of RA and

Table 1: Up-regulation of anterior/brain or posterior/spinal cord genes in NPCs generated from hiPSCs with or without RA and SHH: Brain versus spinal cord; and (RA−and SHH−) versus (RA+and SHH+)

Gene ID	19 WK BR P1 NPC	16 WK SC P1 NPC	Fold Change	HIPS2 P1 NPC 4th Expt RA- and SHH -	HIPS2 P1 NPC 4th Expt RA+and SHH+	Fold change	NPC of gene up-regulation
EMX2:(BR CM)	2697.71	12.47	121.10	576.34	270.35	2.13	Anterior NPCs
PAX6:(BR CM)	3510.10	377.01	13.40	467.51	135.45	3.45	Anterior NPCs
COL5A2:(SC CM)	527.80	2387.41	4.52	5980.22	12593.96	2.11	Posterior NPCs
HOXB5:(SC CM)	108.29	708.85	6.54	612.97	2388.21	3.90	Posterior NPCs
HOXB7:(SC CM)	51.32	698.74	13.62	123.69	637.12	5.15	Posterior NPCs
HOXB8:(SC CM)	27.41	648.43	23.66	441.14	3889.18	8.82	Posterior NPCs
HOXC4:(SC CM)	21.87	3194.03	146.05	221.80	2484.53	11.20	Posterior NPCs
HOXD4:(SC CM)	16.75	40.44	2.41	172.99	1595.08	9.22	Posterior NPCs

WK: Week, BR: Brain, SC: Spinal cord, CM: Cells marker, Expt: Experiment, HIPS2 NPC: Neural progenitor Cells of hiPSCs, RA: Retinoic acid, hiPSCs: Human induced pluripotent stem cell

SHH induced the up-regulation of anterior or brain neural cells markers (EMX2 and PAX6) with a significant comparison to 19-week-old FNPC-B (Table 1). In addition, the significant increased expression of HOX genes has been established for the characterization, segmentation and rostro-caudal organizations of hindbrain and spinal cord neurons.[6-10,14,16-18] Data from this study showed that NPCs derived with the addition of RA and SHH in the growth medium induced the up-regulation of established posterior or spinal cord neural cells markers (COL5A2, HOXB5, HOXB7, HOXB8, HOXC4, and HOXD4) with significant comparison to 16 week FNPC-SC, using transcriptional or global gene expression analyses (Table 1).

Furthermore, FGF though previously established as required for induction and maintenance of NPCs in culture[10,12] has also been established to direct fate caudalization of neural cells.[8] Therefore, the previous studies generated and maintained NPCs *in vitro* in the absence of any exogenous factors or morphogens including FGF/bFGF.[12] Following the withdrawal of FGF from the growth medium in this study, NPCs differentiated into anterior neurons as previously noted.[19] However, the data from this study suggested that the maintenance of NPCs *in vitro* in lower concentrations of FGF/bFGF did not direct fate caudalization of neural cells. Hence, N2/B27 with FGF/bFGF can be used as a possible protocol for the generation of anterior or cortical neural cells.

In addition, different attempts have been made to generate hiPSCs without the use of viral vectors due to the possible concerns of genomic integration of viral vectors in clinical applications.[20] These alternative methods include the use of adenovirus vectors to introduce transient gene expression in target cells. Adenoviral vectors are believed to integrate into the genome of target cells at extremely low frequencies, but the reprogramming efficiency is significantly lower than that with a retrovirus.[20] Cre-deletable lentivirus system was also used to generate hiPSCs, however, there is the possibility of gene breaks being introduced near the insertion site.[20] The Sendai virus was similarly used to generate hiPSCs due to the fact the Sendai virus genome is negative-sense single-stranded RNA, and since replication is cytoplasmic, concerns about genomic integration are reduced.[20] Other non-viral vector methods include the use of RNAs or proteins, with this method achieving higher hiPSCs generation efficiency than the original retrovirus system.[20] MicroRNAs have also been used to successfully generate hiPSCs more efficiently than previous retrovirus systems. This method avoids genomic disruption and integration, and is believed to aid clinical application of hiPSCs.[20]

Therefore, the development of suitable protocols for the generation of hiPSCs, neural cells and other cell types from hiPSCs; and their clinical applications are still of strong research interests to the global scientific community. However, the differentiation of hiPSCs into anterior/brain or posterior/spinal cord neural cells in the absence or presence of RA and SHH in this study addresses the concept of developmental neurobiology. Hence, the protocol developed in this study is applicable to hiPSCs derived using viral or non-viral vector methods.

Conclusion

The successful generation of NPCs from hiPSCs introduced the possibility of generating neural structures devoid of host – immune rejection issues in cells or tissues replacement therapies. Human neurodegenerative diseases or injuries affecting the nervous system (brain and spinal cord) are region-specific requiring the *in vitro* generation of age- and region-specific neural cells which mimic *in vivo* neural cells. This study generated a suitable protocol for the production of age- and region-specific neural cell types from hiPSCs. In addition, obtained data demonstrated that the differentiation of hiPSCs into age- and region-specific anterior/brain or posterior/spinal cord neural cells is achievable in the absence or presence of RA and SHH.

Limitations of the Study and Future Perspectives

This study did not compare NPCs generated from hiPSCs produced using viral and non-viral vector methods. Future studies shall attempt to compare NPCs generated from hiPSCs

produced using viral and non-viral vector methods to validate the best protocol for clinical applications.

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