Journal of Stem Cell Research & Therapy

Fan et al., J Stem Cell Res Ther 2019, 9:5
DOI: 10.4172/2157-7633.1000451

Research Article Open Access

Regionally-Derived Second-Trimester Primary hfNSCs Have Different Neurogenic Capacity for Neuronal Differentiation

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Received date: May 22, 2019; **Accepted date:** May 31, 2019; **Published date:** June 10, 2019

Abstract

Parkinson disease is a debilitating neurodegenerative disease, which is incurable and treatment has been focused on reducing the symptoms and delaying disease progression. Cellular therapy involving grafting of fetal dopaminergic (DA) neurons or fetal mesencephalic tissues rich in DA neurons has been promising. Human fetal neural Stem Cells (hfNSCs) have the potential to be an ideal cell source and hence, we aimed to investigate DA differentiation capacity of eight regionally-derived hfNSCs while defining the differences between regionally-derived hfNSCs.

The differences in regionally-derived hfNSCs response to brain-derived neurotrophic factor, dopamine, forskolin and retinoic acid (DM2) or interleukin 1β and fetal bovine serum (DM1) indicated different intrinsic neurogenic potentials within the developing fetal brain. DM2 induced more efficient DA differentiation (Tyrosine Hydroxylase (TH)+) than DM1 in the spinal cord (SC), brain stem (BS) and sub-ventricular zone (SVZ) derived hfNSCs, although statistical significance was reached only for SC ($p=0.02$). Similarly, DM2 induced more efficient neuronal differentiation (myelin-associated protein 2a and b (MAP2ab)+) than DM1 in SC, posterior cerebrum, SVZ, thalamus, and BS NSCs, with statistical significance reached only for SC-NSCs ($p=0.03$). Collectively for all eight regional NSCs, TH and MAP2ab positive neuronal differentiation with DM2 was higher than DM1 (10.4 vs 4.6%, $p=0.01$, and 27.6 vs 11.6%, $p=0.01$ respectively). Whole genome expression array showed that BS and SC-NSCs are transcriptionally most similar, while the SVZ and cerebellum-derived NSCs have the largest differences in differentially regulated genes compared to the BS-NSCs. Compared to BS-NSCs, anterior cerebrum and hippocampal NSCs exhibited differences in all three gene ontology (growth factor binding, cytokine binding, and neurogenesis) interrogated, while SC, cerebellum and thalamus only exhibited significant differences in neurogenesis pathway compared to BS-NSCs.

By defining the basic neurogenic differentiation capacity and key molecular differences of regionally-derived hfNSCs, our data may facilitate the choice of regionally-derived hfNSCs for different clinical scenarios such as neurodegenerative or traumatic brain injuries.

Keywords: Dopaminergic differentiation; Human neural stem cells; Parkinson disease

Introduction

Parkinson Disease (PD) is a debilitating neurodegenerative disease that affects more than a million Americans [1]. It is estimated there will be an increase from 4.1 million PD patients in 2005 to 8.7 million by the year 2030 [1], making it particularly important to find a long-term effective therapy for it. It is characterized by a loss of the midbrain dopaminergic (DA) neurons, resulting in a characteristic movement disorder. Studies involving grafting of fetal DA neurons have yielded positive repair in a non-human primate [2,3] and clinical transplantation of fetal ventral mesencephalic tissues rich in DA neuron progenitors in Parkinsonian patients has also shown favorable results up to 18 years post-transplantation, with no further treatment with levodopa [4,5]. Since 2015, TRANSEURO, a trial in Europe have engrafted at least eleven patients with human fetal mesencephalic allografts although the lack of adequate samples have resulted in only 20 out of the intended 90 surgeries actually being realized [6]. In addition, dopaminergic cell therapy is potentially useful as the disease progression occurs over a long time, with grafted neurons unaffected even after a decade [7]. However, dyskinesis have been encountered by some of the graft recipients, highlighting the lack of control of the grafts [8]. In addition, the use of fetal mesencephalic tissue has been encumbered by the need for multiple fetal samples per transplantation [9,10], the limited duration of storage before transplantation [11], and ongoing ethical, safety and quality concerns have curtailed its

implementation as a mainstream treatment for Parkinsons disease (PD) [12]. Nonetheless, PD remains one of the primary targets for cell regenerative therapies as only a limited number of neurons degenerate in a specific brain region, the substantia nigra, with a unique biochemical deficit [12]. There is a need to find a well-defined neural cell population to address the efficacy and safety for clinical purposes such as Neural Stem Cells (NSCs), which will be more controlled and homogenous than fetal neural tissue allografts. Induced pluripotent stem cell (iPSCs) has also been suggested as a viable cell source, with efficient directed differentiation shown, and engraftment into animal models of PD [13,14]. However, the use of pluripotent stem cells has been beset by risks of tumor formation [13] and integration events [15]. While purification of desired cells can be done to reduce tumorigenicity [16,17], this technology is still relatively new and requires further testing. Moreover, the genetic component for PD will remain with the use of autologous iPSCs, although emerging technological tools through CRISPR/cas9 editing may be able to efficiently circumvent that [18].

NSCs have been proposed to be an alternative cell source as they can be generated in large quantities in suspension bioreactors under standardized conditions [19]. Cryopreservation also permits the long-term storage of NSCs with a post-thaw ability of 70-95% and no reduction in neuronal differentiation capacity [20,21]. Finally, long-term expanded NSCs have been shown to be non-tumorigenic after transplantation in the murine striatum. In addition to its potential in neuronal replacement therapy, NSCs have been shown to rescue dysfunctional endogenous neurons through chaperon and trophic effects and may serve as cellular gene delivery vehicles for growth factors such as Glial-Derived Neurotrophic Factor (GDNF), leading to functional improvements [22-24].

While adult NSCs has only been found in the sub-ventricular zone (SVZ) and hippocampus, NSCs can be derived from virtually every part of the developing central nervous system in the first half of pregnancy [25-31]. Human fetal NSCs (hfNSCs) possess unique regional and temporal identities which are postulated to develop during early embryogenesis due to graded morphogen levels and the differential expression of regulatory genes found in the developing brain [32,33]. Subpopulations of NSCs, with a distinct expression of transcription factors, can also be found in the telencephalon [34]. Weiss et al. [35] Provided pioneering evidence of the different mitogenic requirements of adult spinal cord versus forebrain NSCs in culture. Mukhida et al. reported that telencephalon-derived hfNSCs exhibited significantly higher cell-fold expansion rates and larger neurosphere diameters than ventral mesencephalon (VM)-isolated fNSCs [36]. hfNSCs derived from more rostral regions of the CNS were shown to display faster proliferation rates [37]. Short and long-term cultures revealed that forebrain NSCs were consistently more neurogenic than midbrain and hindbrain NSCs [38]. Extended cultures generally exhibited reduced neurogenesis and increased astrocyte production except for cerebellar NSCs, which differentiated into significantly more neurons [38]. This suggests a developmental timing whereby a maturing brain generally produces more astrocytes [39], which is concordant with what is known about the timing at which neurogenesis and astrogliogenesis take place [40]. More recently, we have reported that the regionally derived NSCs from a fetus between 14 and 23 weeks exhibit different efficiencies in neurosphere initiation and neuronal/glial differentiation [31]. We have also shown pre-differentiated GABA neurons from hfNSCs are more efficacious in engraftment and bringing about functional recovery after transient ischemic stroke [41].

NSC differentiation has been shown to be regulated by intrinsic genetic programming. An inductive signal produced by floor plate cells, the amino-terminal product of Sonic hedgehog auto-proteolysis, Shh-N, can determine DA neuron differentiation in vitro and in vivo through a contact-dependent manner [42]. Exogenous factors present in the differentiation medium, such as interleukin-1, can similarly influence DA neuron induction [43]. We hypothesize that regionally-derived second-trimester primary hfNSCs have different neurogenic capacity for DA neuronal differentiation due to differences in intrinsic genetic programming. Our objectives are to define the optimal DA neuronal differentiation conditions for the different regionally-derived hfNSCs. Understanding these keys may facilitate the choice of hfNSCs for different clinical scenarios such as neurodegenerative or traumatic brain injuries.

Materials and Methods

All materials were obtained from Sigma-Aldrich unless otherwise stated.

Isolation and culture of human fetal neural stem cells

Human fetal brain tissue samples from clinically-indicated termination of pregnancies at 14-21 weeks of gestation were collected (n=8) with full ethical approval granted by Domain Specific Review Board (D06/0154) of National Healthcare Group, Singapore. The spinal cord, brain stem, cerebellum, thalamus, anterior, mid and posterior cerebra, SVZ and hippocampus were dissected and processed as detailed [31]. Briefly, they were mechanically dissected before under enzymatic dissociation with pre-warmed 1X Trypsin-EDTA (Invitrogen). The suspension was then strained through a 70 μm cell strainer (BD Falcon) and the cell pellet was collected after two rounds of density gradient centrifugation (30% sucrose and 4% BSA). The cell pellet was re-suspended and cultured in neurosphere medium comprising 1:1 DMEM/F12 (Gibco) supplemented with 1% B27 supplement (Gibco), 20 ng/ml hEGF and bFGF (Peprotech), 50 ng/ml of leukemia inhibitory factor and 1X antibiotic/antimycotic (Gibco). Neurosphere medium was replenished twice a week by a 25% replacement of medium. During subculture, neurospheres were collected and centrifuged. Pre-warmed, Tryple^{TM} (Invitrogen) was added to the cell pellet before incubation at 37°C for four mins. Tryple^{TM} was then inactivated by adding neurosphere medium prior to centrifugation. The cell pellet was re-suspended in neurosphere medium, followed by gentle trituration until all neurospheres broke up to form a cloudy solution. The trypan blue (Invitrogen) exclusion method was used to count the cells. Lastly, the cells were plated at an estimated density of 55×10^4 cells per ml.

Directed differentiation

Coverslips were washed in one molar HCl at 50°C to 60°C before coating with 0.01% poly-L-lysine for 30 mins followed by overnight incubation with 20 μg/ml laminin at 37°C. Neurospheres at passage one to four, seven days after subculture were re-suspended in either differentiation medium 1 or 2 (DM1 or DM2) at a density of 1×10^5 cells per ml of medium. DM1 comprises DMEM/ F12 (Gibco) containing 1% fetal bovine serum, 10 ng/ml ml interleukin-1β (IL-1β) and 1% antibiotic/antimycotic (Gemini Bio-Products), whilst DM2 comprises DMEM/F12 containing 50ng/ml recombinant human brain-derived neurotrophic factor (BDNF, Millipore), 10 μM dopamine, 10 μM forskolin, 0.5 μM retinoic acid, 1% N2 supplement (Invitrogen) and 1% antibiotic/antimycotic. DM2 was chosen on the basis that Riaz

et al. had used this to achieve DA differentiation in human fetal neuronal progenitors [44]. The cells were cultured for 17 to 21 days with a change of medium twice a week. Following differentiation, immunocytochemistry and RNA extraction was carried out. All differentiation experiments were done in biological and technical triplicates.

Immunocytochemistry

The cells were fixed and permeabilized in 1:1 acetone/ methanol for five mins at -20°C before washing with 1X PBS. Cells on each coverslip were blocked with protein blocking solution (Thermo Electron) for 30 mins at room temperature (RT) before incubation with primary antibodies for an hour. Primary antibodies used were mouse anti-nestin (1:100, MAB5326, Clone 10C2, Millipore), rabbit anti-GFAP (1:200, G9269, polyclonal), mouse anti-MAP2ab (1:100, 631102, Clone MT-08, Biolegend), mouse anti-TH (1:100, 818001, Clone 2/40/15, Biolegend), rabbit anti-TH (1:100, NB100-80063, Clone EP1533Y, Novus) and rabbit anti-PDGFRα. (1:100, 07-276, polyclonal, Upstate). Coverslips were then washed twice in PBS before incubation with secondary antibodies (1:400), 594 conjugated goat anti-mouse (A11005, Invitrogen) and 488 conjugated goat anti-rabbit (A11008, Invitrogen). Negative controls without primary antibodies were in place. Each coverslip was placed on a slide with a drop of hard set medium containing DAPI (Vector Labs) and dried in the dark for an hour at RT prior to confocal microscopy (Fluoview FV1000, Olympus). Laser wavelengths used were 25 mW Argon ion 488 nm and 1 mW HeNe Green 543 nm. The different neural phenotypes were quantified by counting immuno-labeled cells on coverslips. Five to fifteen randomly chosen separate fields were counted from images taken with a 60X. The percentage of each phenotype was generated over the total number of nuclei stained with DAPI.

Total RNA extraction

Cells were detached from coverslips by incubation with 1X Trypsin-EDTA for four mins at 37°C. Cells were collected and lysed by centrifugation for 10 mins at 10,000 rpm. 800 µl Trizol® (Invitrogen) was used to extract the RNA as per the manufacturer's manual. DNase I (Qiagen) was used in the process to eliminate DNA contamination. The reaction was terminated using 350 µl Trizol, followed by 100 µl of chloroform. The RNA concentration was measured from 1 µl of the RNA suspension using Nanodrop® (BioFrontier, Singapore).

Microarray analysis

Total RNA was extracted from neurospheres (n=3 fetal samples consisting of 8 regionally derived NSCs) and processed using microarray chips (Affymetrix GeneChip Human Genome U133 Plus2 Set). Data were analyzed using GeneSpring 11 (Agilent). The normalization was done by a percentile shift to 75%, without baseline transformation. The average was taken over replicates. For quality control, probe-sets were filtered for present and marginal flags, with absent flags omitted. Fold change analysis was done on the filtered entity list, with brain stem NSCs being the selected condition, against which gene expression levels of other regional NSCs were compared. Fold change cut-off was two. Results were interpreted by gene ontology (GO) analysis at a 0.1 p-value cut-off. Clustering of gene entities within a GO term was done by the hierarchical algorithm, with Euclidean distance metric and Centroid linkage rule. Ingenuity Pathway Analysis® software was utilized to construct pathways from genes that were two folds or more differentially expressed.

Statistical analysis

Processing of cell count data and graph plotting were done using Prism 5 (GraphPad). All data were expressed as means ± SEM (standard error mean). To compare between differentiation mediums, a paired T-test was done, assuming Gaussian distribution. For comparisons between different CNS regions, a non-parametric Kruskal Wallis test, followed by Dunn's posthoc test was run. p-value cut-off was 0.05.

Results

Regionally derived neurospheres respond differently to different stimuli in the medium

Regionally derived neurospheres from eight distinct CNS regions were derived after suspension culture over a week where they were subcultured after reaching more than 100 µm in diameter with the ability to form secondary neurospheres as previously reported [31] (Figure 1A). The neurospheres grown were morphologically similar as previously reported [31]. Multi-lineage differentiation capacity was observed after dissociation of the neurospheres and withdrawal of growth factors, with the appearance of GFAP-expressing astrocytes and MAP2ab-expressing neurons (Figure 1B), in different proportions across the different regions. The regional identity of these neurospheres were cross-checked for regional specificity by means of qPCR for elevated expression of EMX1 (forebrain), En1 (for midbrain) and HoxB6 and 8 (for hindbrain) (data not shown).

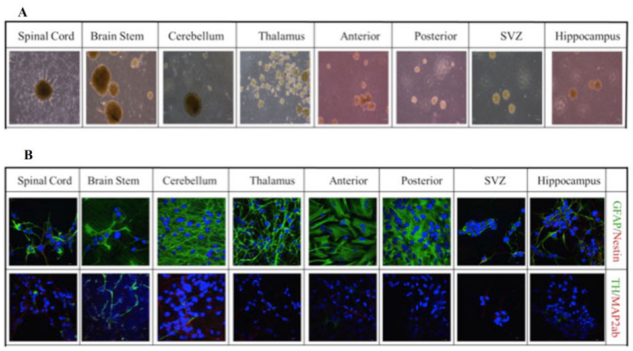

Figure 1: Characteristics of NSC. Neurospheres seen after a week in initiating cultures from different CNS regions (A) demonstrating multipotent differentiating capacity with nestin-ir (white arrows in first row of B) stem/progenitor cells and differentiated astrocytes (GFAP-ir), neurons (MAP2ab-ir, white arrows in 2nd row of B)) and TH-ir DA neurons. Scale bar: 100 µm (A), 50 µm (B).

We initiated DA differentiation of the regionally-derived neurospheres using two different medium; DM1 containing interleukin 1β (IL-1β) and FBS, which is largely a media used to induce non-specific tri-lineage differences [31,45], and DM2 containing BDNF, dopamine, forskolin and retinoic acid, which has been previously used for DA neuronal differentiation in hfNSCs [44]. After three weeks of differentiation, there was only minimal tyrosine hydroxylase (TH) positive cells found in the cultures which ranged from 0% in the thalamus-fNSCs to 9.6% ± 5.9% in the cerebellum-fNSCs [46] with DM1 (Figure 2A). In fact, only cerebellar and

hippocampal hfNSCs were found to be responsive to DM1 for dopaminergic differentiation. DM2 induced more efficient dopaminergic differentiation than DM1 in spinal cord (SC) (37.5% TH +), brain stem (BS) (17.6%) and SVZ-hfNSCs (11.4%), with increases of 33.4%, 17.3%, 10.3% over the use of DM1 respectively, although statistical significance was reached only for SC (p=0.02), (Table 1 and Figure 2A). DM2 induced more efficient neuronal differentiation than DM1 in SC (59.3% MAP2ab+), posterior cerebrum (32.1%), SVZ (33.7%), thalamus (19.8%) and BS NSCs (18.2%), with increases of 48.5%, 18.2%, 16.5%, 15.4% and 15.3% over the use of DM1 respectively, with statistical significance reached only for SC-NSCs (p=0.03), (Table 2 and Figure 2B). Collectively for all eight regional NSCs, TH and MAP2ab positive neuronal differentiation with DM2 was higher than DM1 (10.4 vs 4.6%, p=0.01 and 27.6 vs 11.6%, p=0.01 respectively).

Regions	DM1	DM2	T test p-value
Cerebellum	9.55 ± 5.92	6.42 ± 4.36	0.67
Brain Stem	0.33 ± 0.40	17.62 ± 6.60	0.09
Spinal Cord	4.13 ± 5.06	37.53 ± 10.09	0.02
Thalamus	0 ± 0	2.01 ± 0.91	0.11
Ant. Cerebrum	0.85 ± 0.67	1.02 ± 0.87	0.42
Post. Cerebrum	2.13 ± 1.64	3.04 ± 1.55	0.68
SVZ	0.44 ± 0.54	11.39 ± 9.68	0.28
Hip	6.06 ± 0.16	4.49 ± 5.50	0.77

Table 1: Table showing the percentage of TH -expressing cells in the different regions, when subjected to the 2 mediums.

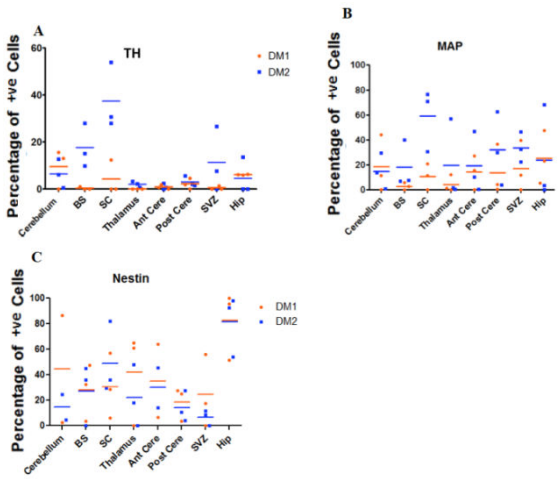

Figure 2: Percentage of staining for tyrosine hydroxylase (A): MAP2ab; (B): and nestin; (C): on the differentiated cells of regionally derived neurospheres in two different medium.

Regions	DM1	DM2	T test P-value
Cerebellum	18.63 ± 16.17	14.72 ± 10.18	0.70
Brain Stem	2.82 ± 2.01	18.16 ± 13.35	0.30
Spinal Cord	10.78 ± 7.35	59.31 ± 17.66	0.03
Thalamus	4.44 ± 4.77	19.83 ± 22.80	0.41
Ant. Cerebrum	14.33 ± 9.69	19.24 ± 17.18	0.76
Post. Cerebrum	13.86 ± 14.09	32.09 ± 20.71	0.49
SVZ	17.18 ± 14.45	33.69 ± 8.49	0.32
Hip	25.37 ± 14.98	23.90 ± 27.15	0.91

Table 2: Table showing the percentage of MAP2ab-expressing cells in the different regions, when subjected to the 2 mediums.

Expression of nestin, a neural stem/progenitor cell marker ranged from 18.7% ± 7.6% in the cerebrum to a high of 82.5% ± 15.5% in the hippocampus in DM1, and from a low of 6.8% ± 3.5% in the SVZ to a high of 81.5% ± 13.8% in the hippocampus when cultured in DM2 (Figure 2C). Paired Student's t-test between DM1 and DM2 proved non-significant across the regions while ANOVA showed statistical significance between the regions cultured in DM2 (p-value:0.01, the p-value for DM1:0.4).

The differentiated TH+ neurons displayed a variety of neuronal morphologies (Figure 3). BS NSCs specifically differentiated into TH+ neurons with complex morphologies and exhibited stronger/darker TH staining. In comparison, hfNSCs isolated from all other CNS regions typically differentiated into bipolar and unipolar TH+ neurons, with the occasional multipolar phenotype.

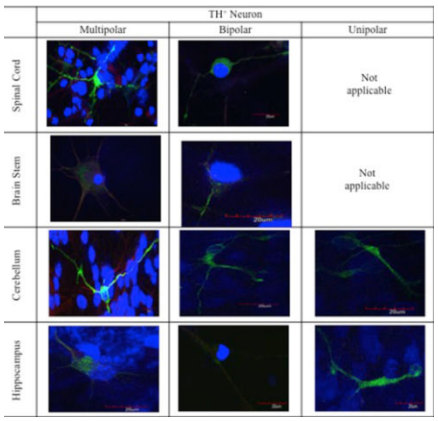

Figure 3: Spinal cord, brain stem, cerebellum, and hippocampus NSC all differentiated into both multipolar and bipolar TH+ (green) neurons. Unipolar TH+ neurons were observed in the cerebellum and hippocampal NSC differentiation cultures. Scale bar: 20 μm

Gestational age and neurogenic potential

In order to explore the effect on gestational age on neuronal differentiation capacity, we subjected different NSC samples harvested from 15, 18 (2 biological samples) and 21 gestational weeks to directed differentiation in DM2. Neurogenic differentiation (MAP2ab+) increases with increasing gestational age in most regional-NSCs (Figure 4A), while this effect was not seen with dopaminergic differentiation (TH+, Figure 4B).

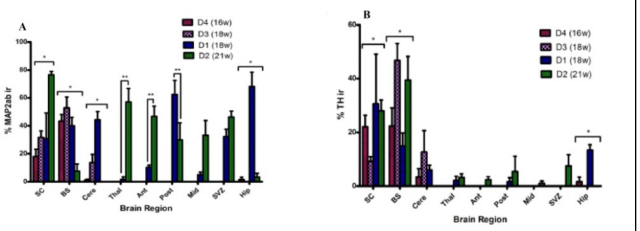

Figure 4: Gestational differences in DM2. Graph presenting percentage of MAP2ab+ (A) and TH+(B) neurons for 16, 18 and 21 week illustrate an uptrend of neuronal differentiation by the regionally derived hfNSCs, with increasing gestations.

Microarray analysis of gene expression

We performed a whole genome expression array on undifferentiated regional-hfNSCs in order to understand the differential differentiation capacity of the regional hfNSCs in response to the different priming media. For example, both BS and SC-NSCs exhibited a substantially higher degree of TH differentiation in DM2 versus DM1, which was not seen in other regional NSCs such as the cerebrum and hippocampus-hfNSCs. As BS-NSCs are the bona fide location of DA, we compared other regional NSCs to them. We found that BS and SC-NSCs are transcriptionally most similar, while the SVZ and cerebellum-derived NSCs have the largest differences in differentially regulated genes compared to the BS-NSCs (Table 3). SVZ has more than 10,000 probesets that belonged to 165 different gene ontology (GO) value that that is more than 2 fold different in level of expression as compared to BS. While the posterior cerebra have approximately 8,800 probesets that are more than 2 fold difference in the expression, these probesets were most varied in the GO involved, at 319 (Table 3). Compared to the BS, SC has only 6,646 probesets involving 94 GO that are more than 2 fold differentially expressed. Next, we performed a Gene Ontology (GO) analysis based on growth factor binding, cytokine binding, and neurogenesis, which would most likely govern responsiveness to differentiation cues found in the differentiation media (Table 4). Compared to BS-NSCs, anterior cerebrum and hippocampal NSCs exhibited differences in all three GO interrogated, while SC, cerebellum, and thalamus only exhibited significant differences in neurogenesis pathway compared to BS-NSCs (Table 4). The cerebra have the most entities in the growth factor binding that's significantly different compared to the BS. Only hippocampus and the anterior cerebra have significantly differential expression in terms of cytokine binding, as compared to the BS. Interestingly although not unexpectedly, neurogenesis is the GO with the biggest differences across all the regions, with at least 178 entities that are significantly different in the case of SC and as high as 277 in the case of SVZ (Table 4). The finding of most differences in the neurogenesis GO is expected as reported in Silbereis et al, where neurogenesis was to occur between 4 and 27 weeks post conception. In our samples of mid-trimester tissues, it is exactly the timing of neurogenesis and our microarray experiments analysis do capture neurogenesis at the stipulated moment [40].

Comparisons	No. of probesets with two or more fold difference in expression	No. of GO values
BS vs Spinal cord	6,646 out of 32,232	94
BS vs Thalamus	6,783 out of 34,844	166
BS vs Hippocampus	7,585 out of 34,637	275
BS vs Posterior	8,850 out of 34,774	319
BS vs Anterior	8,888 out of 35,256	220
BS vs Cerebellum	9,477 out of 32,719	150
BS vs SVZ	10,403 out of 35,335	165

Table 3: Pair-wise comparisons of the regionally derived NSCs (against brain stem).

Comparison	Growth Factor Binding	Cytokine Binding	Neurogenesis
(no. of entities)			
BS vs. Spinal cord	nil	nil	178
BS vs. Thalamus	nil	nil	204
BS vs. Hippocampus	77	51	221
BS vs. Posterior	84	nil	266
BS vs. Anterior	86	55	253
BS vs. Cerebellum	nil	nil	260
BS vs. SVZ	78	nil	277

Table 4: Selected GO terms, Number of entities with two or more fold difference in expression.

Ingenuity Pathway Analysis of the regional NSCs along the chosen GO of interest revealed ten differentially regulated genes (Table 5). Among the eight regional-NSCs, Leukemia Inhibitory Factor Receptor Alpha (LIFR) was found to be most highly expressed in hippocampal, anterior and posterior cerebrum-derived NSCs. Compared to NSCs derived from the Hippocampus, BS-NSCs had higher expression of BDNF associated factors such as TrkB/NTRK2 (3.1x), dopamine receptor D1 (DRD1) (5.0x), and nerve growth factor receptor (NGFR) (164.7x). DRD1 and NGFR were confirmed to be most highly expressed in the brain stem as compared to the other brain regions by qPCR in two other biological samples, corroborating the microarray data (Supplementary Table 1). In addition, the transforming growth factor β pathway genes were also differentially up-regulated (TGFβ): TGFβ receptor II (2.9x) and III (3.0x), and TGFβ3 (2.0x) (Table 5) in BS-NSCs over hippocampal NSCs. However, BS-NSCs had a lower gene expression among the genes in the cytokine binding pathway, specifically PDFGRA (4.6x), LIFR (2.4x) and AGT (2.2x) was noted (Table 5).

Comparison (against BS)	Growth Factor Binding					Cytokine Binding			Neurogenesis	
	TrkB	NGFR	TGFβR2	TGFβR3	TGFβ3	PDGFRA	LIFR	AGT	DRD1	BDNF
Anterior	-6.5	-24.2	-2.8	-6.3	-2.1	+4.0	+3.0	N.S.	-3.2	+3.2
Posterior	-10.8	-45.5	-2.3	-3.0	-2.7	+3.2	+2.7	N.S.	-3.6	+2.2
Cerebellum	-5.9	-9.6	N.S.	N.S.	N.S.	-2.4	N.S.	N.S.	-16.2	N.S.
Hippocampus	-3.1	-164.7	-2.9	-3.0	-2.0	+4.6	+2.4	+2.2	-5.0	N.S.
Thalamus	-3.2	-15.3	N.S.	-2.5	N.S.	N.S.	N.S.	N.S.	-7.0	N.S.
Spinal cord	-3.5	N.S.	N.S.	-2.2	N.S.	+3.3	N.S.	+2.1	N.S.	+2.0
SVZ	-3.4	-105.8	-4.3	-2.8	-2.2	+3.1	N.S.	N.S.	N.S.	N.S.
N.S.: no difference in gene expression										

Table 5: Genes of interest, Fold change up/down-regulated with respect to the brain stem NSCs.

Discussion

We have previously shown that regionally-derived human second-trimester NSCs have different intrinsic neurogenic potential as a result of divergent programming [31]. Here, we extended our findings to the potential of regional NSCs to undergo DA differentiation through extrinsic stimulation with two different culture media. We find that BS, SC AND SVZ-NSCs had the greatest potential for DA differentiation, and were preferentially stimulated by DM2, while cerebellar and hippocampal NSCs responded to DM1. This demonstrates intrinsic differences in neurogenic programming as alluded to in whole genome expression studies.

Different priming agents

Several compounds have been reported to enhance the generation of TH+ neuron. They can be generally grouped into cytokines (IL-1β, IL-11, LIF), growth factors (BDNF, GDNF, FGF-8) and other agents (dopamine, forskolin, retinoic acid, ascorbic acid) [44,46-52]. While current research efforts have focused on the synergistic effects of compounds in directing DA neuron differentiation, few had studied the influence of NSCs' regional specifications. Several groups have compared differentiation potential between first-trimester VM and forebrain NSCs in a chosen priming media [47,49]. In our study, we show that the regional and temporal aspects of NSCs, along with the retained ability to respond to extracellular cues, can be harnessed to maximize DA differentiation. SC, BS, and SVZ-NSCs were found to possess significant DA neuron differentiation potential in vitro. These three regional NSCs were specifically primed by BDNF, dopamine, forskolin and retinoic acid. We also found an interaction between the gestational age and DA differentiation capacity of the regional NSCs (temporal specifications). We found that specific receptors were required to be highly expressed for NSCs to respond to particular stimuli. For example, SC NSCs had high expressions of NGFR and DRD1, which responded to BDNF and dopamine found in DM2. Therefore, elucidating the level of expression of receptors and other endogenous molecules would allow the selection of the appropriate factors to optimize DA neuron differentiation.

Regional specification and plasticity

DA neurons are generally not found in the spinal cord and brain stem during normal development, however the appropriate external cues stimulated TH+ neuron differentiation in these hfNSCs, demonstrating neuronal-lineage plasticity. This is in agreement with the findings of Hitoshi and colleagues, who concluded that the fate of murine regional fetal NSC lineages, are not committed to specific compartments and some phenotypic plasticity is inherent to all NSCs lineages [53]. However, differentiation into MAP2ab+ neurons was higher in SC-NSC (59.3%) than cortical NSCs (32.1%), which contrasted with some other reports [47,54]. Maciaczyk et al observed that cortex-derived NSCs (27.2% ± 2.2%) were more efficiently differentiated into a neuronal lineage as compared to VM (6.0% ± 1.2%) and SC NSCs (0.6% ± 0.1%)[47], while Ostenfeld et al noted that only mesencephalic NSCs produced neurons with DA markers [54].

IL-1β, the active cytokine in DM1, had been implicated in the induction of the key fate-determining transcription factors for DA differentiation in embryonic mesencephalon-derived NSC [55]. The reported efficacy of IL-1β directed differentiation of NSCs into TH+ neurons had been controversial, with Riaz and colleagues claiming a two-fold increase in DA neurons [56], although this was not observed by Ling and colleagues [56]. However, this dichotomous observation could be due to experimental variations in tissue source, brain We found that the SC, BS and SVZ NSCs were more efficiently differentiated in DM2, which contains the neurotrophin, BDNF in addition to dopamine, forskolin and retinoic acid. BDNF has been reported to enhance the differentiation of neural precursors and the survival of cultured DA neurons [57,58] while BDNF was found to have proliferative effects on NSCs through truncated-TrkB in the presence of EGF [59]. While EGF removal alone causes the cessation of proliferation and astroglial differentiation, it is required for neurotrophin-mediated neuronal and oligodendroglial differentiation [60]. BDNF is able to increase both neuronal and astroglial cells differentiation [61].

Gestational effect of DA differentiation in DM2

While neurogenic differentiation in DM2 was more efficient with increasing gestation from 16 to 21 weeks, we did not see a similar pattern with DA differentiation capacity. During development, the predominant source of TH+ neurons arises from the mesencephalic region of the CNS, while SC-NSCs primarily generate cholinergic neurons and GABAergic or glutamatergic interneurons in vivo. Therefore from our study, fNSCs populations possess differentiation plasticity and neuronal lineage choices are amenable to in vitro manipulation. The observation that some cells stained for TH but not the more mature neuronal marker MAP2ab, and the co-expression with GFAP and nestin suggested an immature phenotype. This suggests that further optimization or a longer duration of differentiation is required for full DA neuron phenotypic maturation [62].

Microarray data reveals differences in regionally derived hfNSCs

Various strategies have been employed to generate DA neurons, with varying efficiencies of TH+ neuron being reported [44,46-52]. At present, the molecular mechanisms underlying DA neuronal differentiation are still poorly understood. As each regional NSCs population responded differently to priming agents, we attempted to parse out possible mechanisms through pathway analysis into GO analyses based on growth factor binding, cytokine binding, and neurogenesis which picked up on ten relevant genes. This may explain the differential responsiveness towards the different stimuli found in the two differentiation media. In general, BS-NSCs had higher expression of TrkB, NGFR and TGFβR2/3 receptors, as well as DRD1 than other regional NSCs, with SC and SVZ-NSCs being most similar to BS-NSCs. The elevated TrkB/NTRK2 and nerve growth factor receptor (NGFR) in the BS and SC-NSCs is in broad agreement with the finding where NGF was shown to induce TH expression in 15% to 20% of striatum NSCs [60]. DRD1 signaling up-regulates BDNF transcription through a functional cAMP-Response Element (CRE) in the BDNF gene promoter forming a positive feedback loop [63], working synergistically in promoting DA neuron differentiation. Dopamine was demonstrated to activate TrkB via DRD1, leading to TrkB phosphorylation in the absence of neurotrophins [64]. This signaling pathway also enhanced cell surface expression of TrkB by increasing intracellular Ca^{2+} levels [64]. Pathway analysis alludes to the essential functions of neurotrophins and their receptors in nervous system development (Supplementary Figure 1). Expression of TrkB and DRD1 has been reported to be reduced in PD [65,66]. This reduction may be ameliorated by the introduction of BS-NSCs which possesses the highest expression for these two markers. Transforming growth factor β receptor II, III (TGFβR2/3) which were more highly expressed in brain stem NSCs could be potentially useful TH+ neuronal priming agents. The lower expressions of TrkB, NGFR and DRD1 mRNA levels in most of the regions could have contributed to the non-responsiveness to BDNF and dopamine TH+ neuron priming. The decreased expression of TGFβR2/3 in SVZ, hippocampus and cerebra NSCs suggest that TGFβ2/3 might not be efficient TH+ neuron priming agents for them, explaining their non-responsiveness to DM2.

Astrocytes are known to play an important role in maintaining homeostasis in the CNS environment. They are intimately coupled to neurons, oligodendrocytes and other astrocytes by both contact-dependent and non-contact-dependent mechanisms [67]. John et al. reported that 24 hours after adding 10 ng/ml IL-1β, expression of IL-11, HIF-1 and LIF were increased by 7.6, 3.3 and 2.4 folds respectively, with LIF up-regulation possibly peaking at 2.9 folds six hours after cytokine induction. The same concentration of IL-1β was utilized in DM1. Having established that LIFR was more highly expressed in hippocampal NSCs, IL-1β might have induced TH+ neurons indirectly through astrocytes. NSCs differentiate into different neural lineages through radial glial cells. Under IL-1β stimulation, HIF-1 and LIF produced by radial glial cells could also direct themselves towards the DA neuron lineage. Therefore, the participation of glial cells in neuronal lineage determination would provide multiple avenues for the induction and regulation of DA differentiation.

Conclusion

Current treatment modalities for PD have been hampered by limited efficacy and the eventual exhaustion of DA neurons in the nigra-striatal tract. While first-trimester neural tissues have been studied as a source for TH+ neurons, they are limited by the small cell numbers. Here we provide evidence for the differential neurogenic potential of regionally-derived NSCs and their putative genetic programming in the developing second trimester CNS. In turn, this may have implications for their utility as neural cell replacement sources for PD and other neurodegenerative disorders.

Acknowledgement

JCKY and MC received salary support from Singapore's Ministry of Health's National Medical Research Council (NMRC/CSA-SI/0008/2016 and NMRC/CSA/0059/2014 respectively.).

We acknowledge the kind assistance of Mr Edwin Shepherdson, from Department of Reproductive Medicine, KKH with the cell cultures.

Conflicts of Interest

The authors indicated no potential conflicts of interest.

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