Original Article

Immunophenotypic and Molecular Analysis of Human Dental Pulp Stem Cells Potential for Neurogenic Differentiation

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Abstract

Background: Growing evidence shows that dental pulp (DP) tissues could be a potential source of adult stem cells for the treatment of devastating neurological diseases and several other conditions. **Aims:** Exploration of the expression profile of several key molecular markers to evaluate the molecular dynamics in undifferentiated and differentiated DP-derived stem cells (DPSCs) *in vitro*. **Settings and Design:** The characteristics and multilineage differentiation ability of DPSCs were determined by cellular and molecular kinetics. DPSCs were further induced to form adherent (ADH) and non-ADH (NADH) neurospheres under serum-free condition which was further induced into neurogenic lineage cells and characterized for their molecular and cellular diversity at each stage. **Statistical Analysis Used:** Statistical analysis used one-way analysis of variance, Student's *t*-test, Livak method for relative quantification, and R programming. **Results:** Immunophenotypic analysis of DPSCs revealed >80% cells positive for mesenchymal markers CD90 and CD105, >70% positive for transferring receptor (CD71), and >30% for chemotactic factor (CXCR3). These cells showed mesodermal differentiation also and confirmed by specific staining and molecular analysis. Activation of neuronal lineage markers and neurogenic growth factors was observed during lineage differentiation of cells derived from NADH and ADH spheroids. Greater than 80% of cells were found to express β-tubulin III in both differentiation conditions. **Conclusions:** The present study reported a cascade of immunophenotypic and molecular markers to characterize neurogenic differentiation of DPSCs under serum-free condition. These findings trigger the future analyses for clinical applicability of DP-derived cells in regenerative applications.

Keywords: *Cellular and molecular diversity, dental pulp-derived stem cells, gene expression profile, neurogenic differentiation, serum-free medium*

Introduction

Stem cells derived from dental pulp (DP) tissues represent an area of particular interest for their potential applicability in the treatment of various devastating conditions. Since DP tissues originate from the cranial neural crest cells, they have been proposed as an ideal source for generating neurogenic cells. The existence of adult stem cells in DP tissues was explored more than a decade;[1] however, better understanding of wide cellular and molecular dynamics is needed to explore the exact realistic potential of these cells. Until now, the adherent (ADH) and non-ADH (NADH) cell fractions obtained from DP-derived stem cells (DPSCs) have never been studied, and neurogenic differentiation is poorly understood.

Several reports have showed neuronal differentiation ability of DPSCs *in vitro* under high concentration of serum followed by neurogenic condition supplements.[2,3] These induction procedures are different from those employed for the neurogenic differentiation of neural precursor cells (NPCs) derived from central nervous system. The present study was focused to discriminate cellular and molecular phenotypes of neural precursor nature of DPSCs during *in vitro* proliferation under serum-free microenvironment. Further, the neurogenic differentiation ability was identified using similar induction supplement used for the neurogenic differentiation of human NPCs. Most interestingly, the strategy was designed to explore the neurogenic differentiation after removal of neural mitogenic factors (epidermal growth factor [EGF] and fibroblast growth factor [FGF]) and supplementing with retinoic acid (RA) of ADH and NADH spheroid cells derived from DPSCs under serum-free condition.

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The expression profile of major molecular markers was explored to evaluate the molecular dynamics in undifferentiated and differentiated DPSCs *in vitro*.

Subjects and Methods

Sample collection

Fifteen DP tissues were collected from healthy individuals (18–40 years old) as per the standard guidelines and approval from the Institutional Ethics Committee of Deccan College of Medical Sciences, Hyderabad, India. Both the impacted and erupted human third molar teeth were collected having proper orthodontic reasons from dental clinics at Hyderabad.

Dental pulp stem cells isolation

DP tissues from freshly extracted human third molar were surgically removed and kept in Dulbecco's modified Eagles medium-F12 (DMEM- 12) containing 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml amphotericin-B to avoid bacterial and/or fungal contamination [Figure 1a]. Pulp tissues were washed twice with DMEM-12 with antibiotics and minced into 1–2 mm^2 pieces. Single cell suspension was obtained after filtering the digested tissues from 40 μm cell strainer (BD Biosciences).

Cell counting, viability testing, and *in vitro* culture

The isolated cells were subjected to cell counting using hemocytometer. Cell viability was calculated by trypan blue staining. Further, the enumeration of DPSCs was done by culturing 2 × 10^3 viable DPSCs in 12-well plastic plates in DMEM-F12 with 10% fetal calf serum (FCS), 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml amphotericin-B. Medium was changed every after 3rd day and monitored for 60 days.

Flow cytometry analysis

After 21 days, cells were harvested from the culture by trypsinization. The expression of immunophenotypic and molecular markers was characterized using CD90, CD105, CD71, CXCR3, CD34, and CD45. The fluorescent intensity of each sample was measured using FACS Calibur (BD). The primary gating was performed to exclude the cellular debris and dead cells.

Long-term analysis of population doubling for dental pulp-derived stem cells

After 21 days of enrichment, trypsinized and subcultured for twenty passages. Passage 1, 10 and 20 population doubling was analyzed to identify their growth kinetics. Changes in cell number and cumulative population doubling (CPD) were calculated and plotted for passage 1, 10, and 20.

Lineage differentiation

Lineage differentiation of DPSCs into osteogenic, adipogenic, and chondrogenic lineage was identified by stimulating with respective medium. Osteogenic differentiation of DPSCs at day 21 was evaluated by staining the cells with alizarin red. Whereas adipogenic and chondrogenic differentiation was evaluated by staining with oil red O and Alcian blue, respectively. Molecular characterization of these three lineages was further confirmed by quantitative gene expression analysis of RUNX 2, osteocalcin (OCN), osteopontin (OPN), and dentin matrix protein 1 (DMP1) for osteocytes, leptin, and adipsin for adipocytes and COL2a1 and Sox-9 for chondrocytes.

Neurogenic stimulation of dental pulp-derived stem cells

DPSCs from passage 3 to 4 were further induced with serum-free human neural proliferation medium (Stem Cell Technologies, Canada). Mitogenic factors such as EGF (20 μg/ml) and basic FGF (10 μg/ml) were used with 1X antibiotic solution to stimulate neurogenic cells at 37°C and 5% CO_2 atmosphere. Fresh medium was replenished every after 3rd day, and cells were maintained for 21 days.

Adherent and nonadherent cell population

At each time point of medium change, the floating cells were collected up to 21 days and referred as NADH cell population. The proliferation efficiency and spheroid development from NADH cells were evaluated by their neurospheres forming ability and gene expression analysis. While the adhered spheroids (ADH) were trypsinized and analyzed similarly for their neurosphere development potential and gene expression analysis.

Neurosphere development

One of the exceptional characteristics of neural stem cells to produce neurospheres under influence of mitogenic factors was evaluated poststimulation of ADH DPSCs. Morphological analysis was performed by optical microscopy imaging. The neurosphere development per well was calculated and further analyzed for the expression of nestin and neural cell adhesion molecule (NCAM) using immunofluorescence analysis.

Neurogenic lineage differentiation

Neurogenic lineage differentiation ability of ADH and NADH spheroid cells was triggered by removing mitogenic factors and supplementing 0.05 μM RA and 2% fetal bovine serum (FBS) in human neural differentiation medium (Stem Cell Technologies, Canada). Cells were allowed to differentiate for 21 days under continuous stimulus and evaluated by morphological, immunofluorescence, scanning electron microscopy (SEM), and molecular characterization.

Scanning electron microscopy analysis of neurogenic differentiation

At day 21, post-stimuli of RA and FBS, cells were harvested from the differentiation culture plate and fixed in 2.5% glutaraldehyde. Cells were processed further for SEM analysis using standard protocol described elsewhere.[4] The images were documented using SEM (JOEL-JSM 5600) at Ruska Labs, College of Veterinary Science, SVVU, Hyderabad, India.

Immunofluorescence staining

For immunofluorescence staining, neurosphere-derived cells as well as DPSCs differentiated cells into neurogenic lineages were fixed in 4% paraformaldehyde. After 20 min of fixation, cells were washed with 1X phosphate-buffered saline and blocked using anti-goat serum. Cells were stained directly with antibodies conjugated with fluorophores to identify the cell surface markers. While for intracellular staining, cells were permeabilized with 0.01% Triton-X-100 and washed before staining. Poststaining, cells were washed to remove unbound antibodies and visualized under inverted fluorescence microscope using Axiovert software (Carl Zeiss, Germany).

Quantitative real-time polymerase chain reaction assay

Total RNA was isolated from cells derived from undifferentiated and differentiated DPSCs and NADH and ADH spheroid cultures. RNA was quantified and converted into complementary DNA strand using Oligo (dT) primer. Quantitative real-time polymerase chain reaction (RT-PCR) was carried out using gene-specific oligonucleotide primers using SYBR Green-based assay. Glyceraldehyde-3-phosphate dehydrogenase primers were used as endogenous control to normalize the expression of each transcript.

Statistical analysis

The data of the present analysis were represented as mean ± standard deviation. Statistical analyses of means were calculated using one-way and two-way analysis of variance using GraphPad Prism software (Version V). RT-quantitative PCR (RT-qPCR) efficiency was calculated using StepOne (Version 2.2) software. RT-qPCR for all the genes was performed in triplicates. The relative quantification of gene expression was performed by Livak method[5] which was further validated using Pfaffl method.[6] A regression value (R^2) ≥0.99 was considered to be significant with 100% PCR efficiency. $P < 0.05$ was considered statistically significant. Online R programming was used on a UNIX platform (https://discover.nci.nih.gov/cimminer/cimMinerUpdate.do) for statistical computing of messenger RNA (mRNA) transcripts.

Results

Enrichment and characterization of dental pulp-derived stem cells in dental pulp tissue

The enumeration of DPSCs in DP tissues was confirmed by their ability to adhere on plastic plate and further to change their morphology into spindle shaped from day 7 to 21 [Figure 1b-d]. Greater than 80% confluency was observed at day 21 with thin long body [Figure 1e]. NADH cells were unable to survive and removed from the culture at every 3rd day of culture. During long-term *in vitro* culture, DPSCs derived showed longer time for cell division with increasing the culture period [Figure 1f]. As shown in Figure 1g, immunophenotypic analysis of DPSCs at day 21 revealed that >80% cells are positive for mesenchymal stem cells (MSCs)-specific markers CD90 and CD105, >70% were positive for transferring receptor (CD71), and >30% of the total population of cells

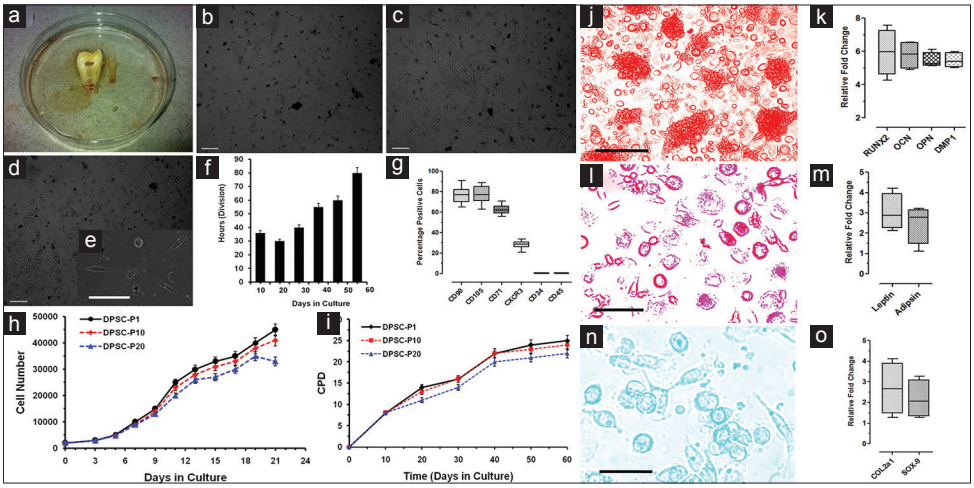

Figure 1: (a) Surgically dissected and extracted dental pulp tissue. *In vitro* cultured dental pulp-derived stem cells on plastic plates for (b) day 7, (c) day 14, and (d) day 21 (SB: 50 μm, ×10). (e) Magnified dental pulp-derived stem cells at ×40 (SB: 50 μm). (f) Cell division time after *in vitro* culture (g) immunophenotypic expression of markers at day 21. (h) Changes in relative cell number and (i) cumulative population doubling during *in vitro* culture from day 0 to day 21 at passage 1, 10, and 20. (j) Alizarin red staining (l) oil red staining (n) Alcian blue staining and molecular analysis for (k) osteocyte, (m) adipocyte, and (o) chondrogenic markers (SB: 100 μm, ×20)

were expressed the chemotactic factor (CXCR3). Whereas these ADH MSCs did not express markers specific for hematopoietic stem cells such as CD34 and CD45. Long-term culture of DPSCs derived from DP tissue for 20 population doubling showed continuous increase in cell number from day 0 to day 21 at each passage (P1, P10, and P20). However, no significant change was observed in cell number among each population [Figure 1h]. Similarly, the CPD for 60 days of DPSCs culture for twenty passages revealed no significant change at different passages (P1, P10, and P20) [Figure 1i].

Identification of mesodermal differentiation capacity of dental pulp-derived stem cells

The mesodermal differentiation of DPSCs into osteogenic, adipogenic, and chondrogenic was identified under respective condition. After 3 weeks of culture, DPSCs were found to differentiate into above three major mesodermal lineage cells [Figure 2]. The osteogenic differentiation of DPSCs was confirmed by the accumulation of mineralized matrix and confirmed by Alizarin red S staining [Figure 1j], and quantitative mRNA expression analysis using osteogenic lineage-specific markers such as RUNX2, OCN, OPN, and DMP1 [Figure 1k]. All these genes showed approximately 6-fold upregulation as compared to undifferentiated cells. Adipogenic induction was evident by the presence of lipid droplets which was apparent through oil red O staining [Figure 1l] and approximately 3-fold upregulation in adipocyte-specific markers, i.e., leptin and adipsin [Figure 1m]. The chondrogenesis was further confirmed by Alcian blue staining [Figure 1n] and >2-fold upregulation in COL2a1 and SOX-9 markers specific to chondrocytes [Figure 1o].

Neurogenic induction of dental pulp-derived stem cells under mitogenic microenvironment

Under neural mitogenic microenvironment, DPSCs started to develop ADH and NADH spheroids at day 3 and enlarged in size >100 μm in diameter at day 15 [Figure 2a-d] and termed neurospheres. Few spheroids remained adhered on the surface and mostly found floating in the medium. The number of ADH spheroids was quite less, whereas the NADH spheroids were quite large in number which was continuously increased with increasing the time in culture [Figure 2e]. The NADH neurospheres were dissociated at different time points in culture which showed continuous increase in cell density and number with increasing the time [Figure 2f], whereas in ADH neurospheres, the change in relative cell number was nonsignificant (data not shown). Further, the neurogenic induction of DPSCs under mitogenic environment was confirmed by immunocytochemical (ICC) staining of neurosphere-derived cells using nestin and NCAM neural stem cell-specific markers. ICC staining of cells showed that >80% cells were positive for both the markers confirming the induction of DPSCs into neural precursor nature in cells present within the neurospheres [Figure 2g-l].

Molecular diversity within nonadherent and adherent spheroids

The molecular diversity in cells derived from NADH and ADH spheroids of all 15 patients' pulp tissue was identified by relative quantification of neural stem cell markers such as microtubule-associated protein 2, intermediate filament protein VI (nestin), and NCAM, MSCs-specific markers

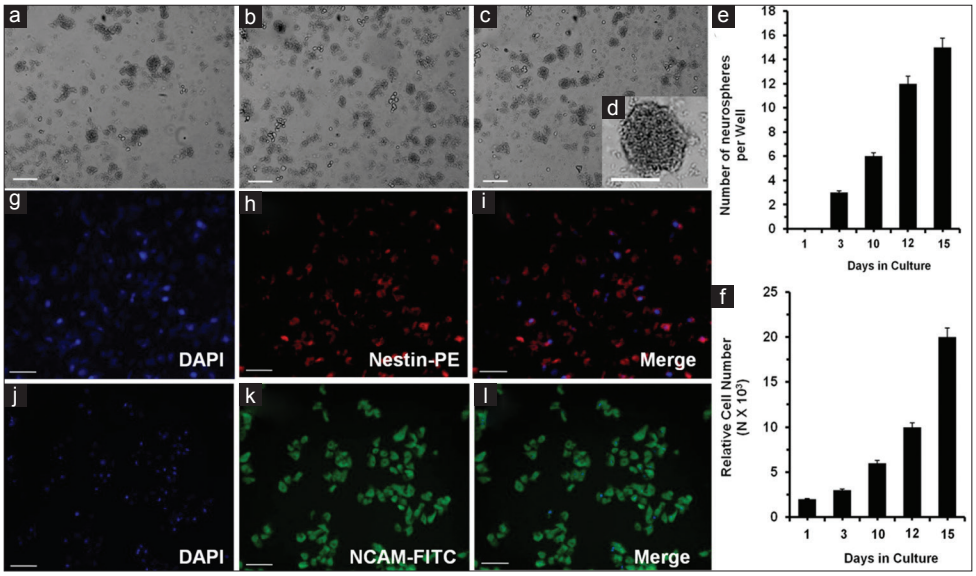

Figure 2: Morphological changes and spheroid development during neurogenic induction. Dental pulp-derived stem cells under mitogenic stimulation at (a) day 3, (b) day 7, and (c) day 15 (d). At day 15, neurosphere was approximately 100 μm in size (SB: 100 μm, ×40). (e) Changes in the number of neurospheres developing from dental pulp-derived stem cells at day 1, day 3, day 10, day 12, and day 15. (f) Changes in the cell number during neurospheres development at different time points under mitogenic stimulation. Immunofluorescence imaging of neurosphere-derived cells for neural precursor cell marker (g-i) nestin and (j-l) neural cell adhesion molecule (SB: 100 μm, ×20)

such as Stro-1, CD90, CD105, and CD73, neural lineage markers such as neurofilament protein (NF-M) and tubulin protein (β–tubulin III) specific to neurons, glial fibrillary acidic protein (GFAP) to astrocytes and O4 and PLP1 to oligodendrocytes, osteogenic marker such as OCN, and dental marker such as dentin sialophosphoprotein. Gene expression analysis revealed upregulated expression for neural stem cell-specific markers in few samples of both ADH and NADH spheroid cells [Figure 3a-e]. However, PLP1, β–tubulin III, and NF-M were found highly upregulated in all the samples in both types of spheroids. The nestin expression was more prominent in ADH spheroids as compared to NADH one. However, both nestin and NCAM had almost similar expression pattern in NADH spheroids. The MSCs, osteogenic, and dental markers were not expressed similar to nestin, NCAM, β–tubulin III, PLP1, and NF-M in both types of spheroids. Clustering analysis based on the expression category demonstrated distinct characteristic gene expression which does not differ much based on the individuals [Figure 3c and e]. Elucidation distance provided similarity in the upregulated expression of PLP1, β–tubulin III, and NF-M in case of NADH, whereas additionally nestin in ADH spheroids with larger distance. MSCs-specific markers were commonly had the same elucidation distance and expression pattern in both types of spheroids.

Neurogenic lineage differentiation of adherent and nonadherent cells

Morphological changes

After 7 days of culture, most of the NADH spheroid cells showed morphological change to long and thin body. The cytoplasm was found to be contracted toward the nucleus and acquired multipolar shape. Cells displayed spherical and smaller contracted bodies with conical cytoplasm and several branches resembling with the neuronal perikaryon, dendrites, and axons. The formation of axons and dendrites in the absence of serum and neuromitogenic growth factors revealed neurogenic lineage differentiation due to the condition provided but not spontaneous [Figure 4a-c]. Greater than 80% of cells were stained positive for β–tubulin III and proved to be neurons [Figures 5d and 4e]. Greater than 15% cells did not pass the long axons, instead had several dendrites which were further characterized for astrocytes after staining with GFAP [Figure 4d and e] and >5%–10% of total population of cells stained positive for O4 marker specific to oligodendrocytes [Figure 4d and f]. The phenotype of developing neurons was similar to adult neurons as depicted in Figure 5g and further explored for the development of neurons under high-resolution SEM [Figure 4h]. Cells derived from ADH spheroids tend to differentiate into neurogenic lineage cells in similar

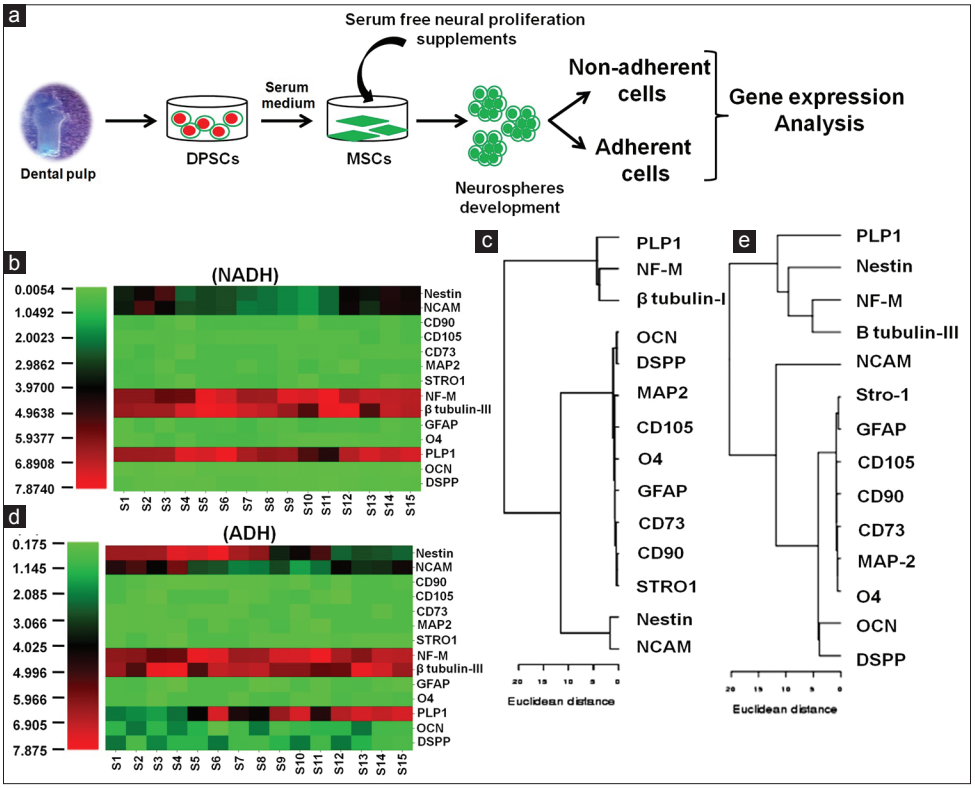

Figure 3: (a) Schematic representation of dental pulp-derived stem cells culture under mitogenic stimulation and development of nonadherent and adherent spheroids. Heat map showing changes in gene expression levels for (b) nonadherent and (d) adherent cells. Phylogenetic association and elucidation distance for expression of different molecules in (c) nonadherent and (e) adherent cells

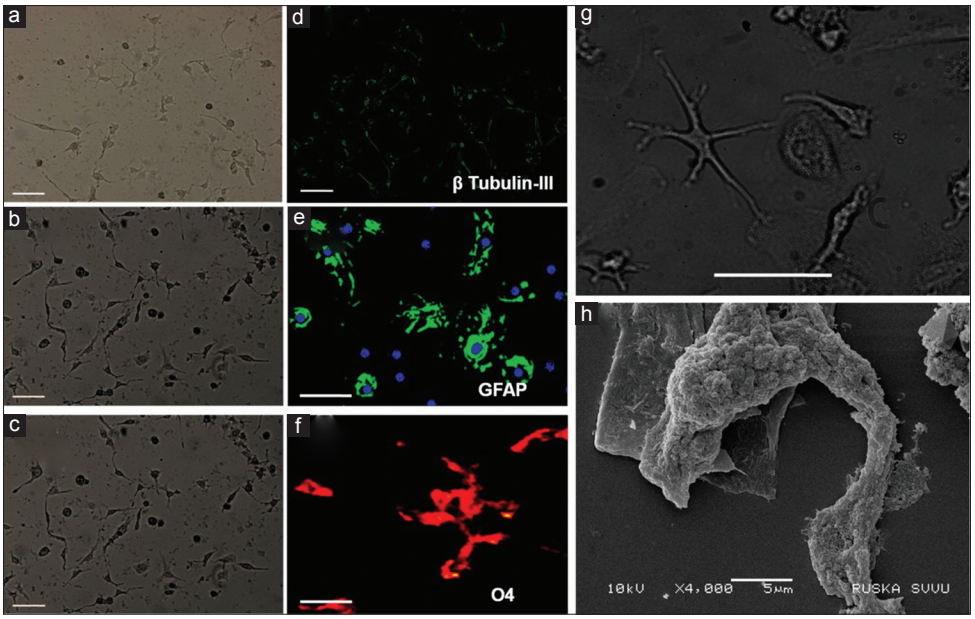

Figure 4: Morphological changes noticed for dental pulp-derived stem cells differentiation into neurogenic lineage cells at (a) day 7, (b) day 14, and (c) day 21. During stimulation using neurogenic differentiation supplements, spindle-shaped dental pulp-derived stem cells changed into neuron-like cells. Immunofluorescence staining of neurogenic cells using (d) β–tubulin III for neurons, (e) glial fibrillary acidic protein for astrocytes, and (f) O4 for oligodendrocytes. (g) High-resolution phase contrast image of well-differentiated mature neuron (SB: 100 μm, ×40). (h) Scanning electron microscopy image of a neuron differentiated from dental pulp-derived stem cells (SB: 5 μm, ×4000)

condition, but the differentiation capacity was quite less as compared to NADH spheroid cells [Figure 5g-i].

Activation of neurogenic lineage markers and growth factors

Activation of neuronal lineage markers and supply of continuous neurogenic growth factors was observed at day 21 during lineage differentiation of cells derived from NADH and ADH spheroids. Greater than 80% of cells were found to express β-tubulin III [Figure 5b] which was similarly reported after RT-qPCR analysis for several markers involved in neural lineage differentiation [Figure 5]. β-tubulin- III was found to be the highest upregulated marker, whereas nestin, NCAM, and O4 were the least expressed markers in both conditions, i.e., NADH and ADH spheroid cells. Continuous upregulated expression of neural growth factors (brain-derived neurotrophic factor [BDNF], glial cell-derived neurotrophic factor [GDNF], and nerve growth factor [NGF]) showed the higher cell survival and differentiation in both NADH and ADH cells [Figure 5e and j]. Clustering analysis based on the expression category demonstrated highest similarity between the expression of NF-M and PLP1, NCAM, nestin and O4, and BDNF, GDNF, NGF, and GFAP. Elucidation distance also showed β-tubulin III as the most solitary marker to be expressed in the highest quantity distinct from other markers.

Discussion

The present study aimed to identify cellular and molecular diversity of DPSCs (derived from the DP tissues collected from different individuals) *in vitro* proliferation and neurogenic lineage differentiation. Currently, the functional involvement of other source of cells in neurotransmission is not clearly defined. We hypothesized that DPSCs which originate from the neural crest cells might be a valuable candidate to repair neurological damage and treat several devastating neurological disorders. However, these cells were previously expected to be utilized for repairing the damaged dental nerve tissues. Various other studies have demonstrated the DPSCs characteristics and their potential to make neurogenic cells *in vitro* under various microenvironments.[7-9] However, for clinical translation of these cells requires a significant quantity of cells with similar growth potential and molecular profile and can be maintained long term without any potential risk. In addition, the differentiation protocols should be easy and acceptable which could provide desired cell type for different applications.

The current study showed a detailed analysis of phenotypic and molecular variations during long-term enrichment and differentiation of DPSCs into various lineages. A simple method was followed for enriching DPSCs using DMEM with 10% FCS. Interestingly, >60% of cells were found to express the transferring receptor protein (CD71) which is essential for proliferation and several other metabolic activities of neurons and astrocytes in adult rat brain.[10] Another important finding of our study was the expression of CXCR3 by >30% of DPSCs. CXCR3 is an important cytokine which is associated with the inflammatory response and has proposed to be involved in guiding NPCs at the site of brain injury/damage.[11] The DPSCs expressing CXCR3 may find its potential role to migrate these cells at

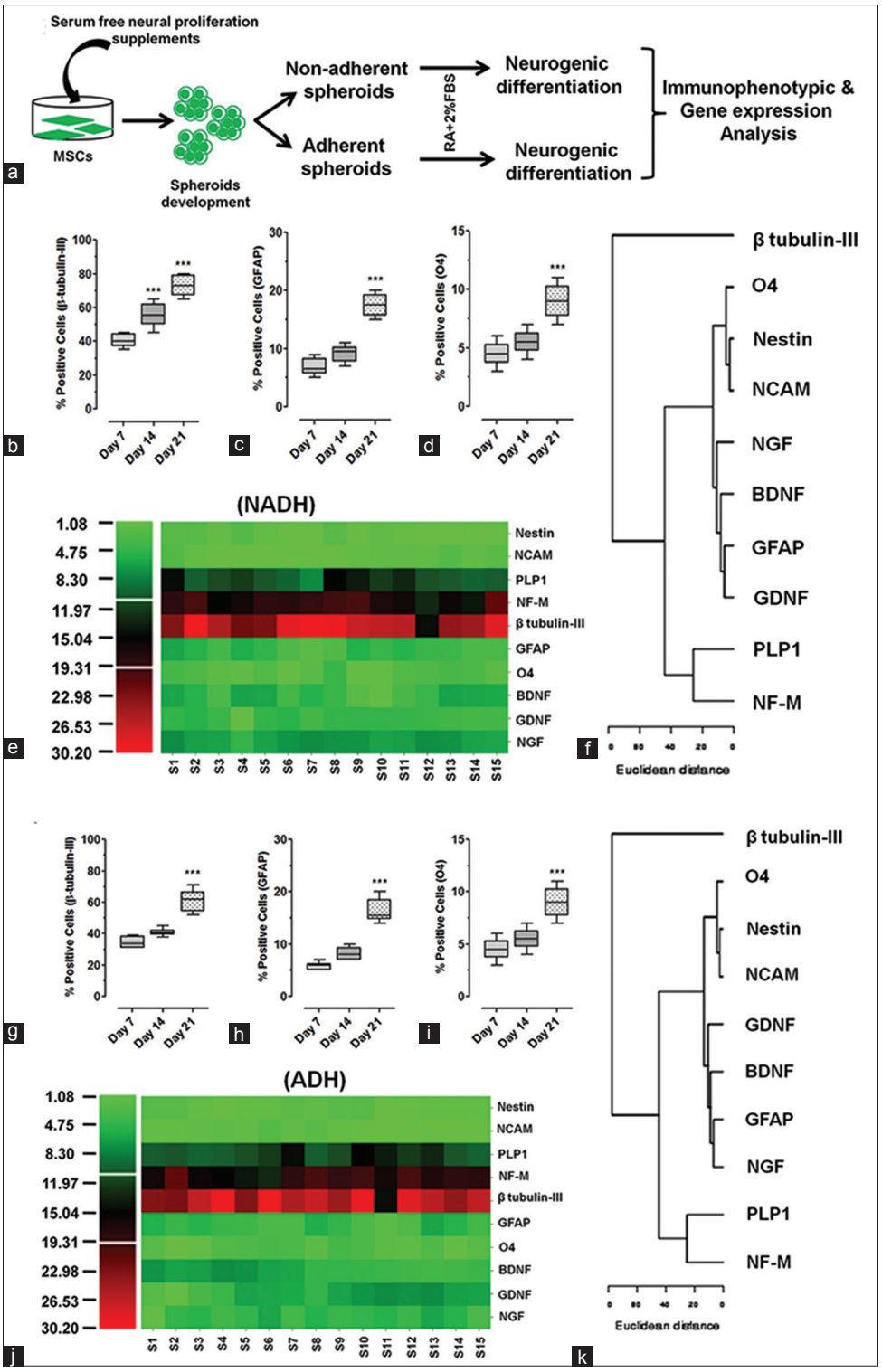

Figure 5: (a) Schematic representation of characterization of neurogenic differentiation from MSCs using RA with 2% FBS. Percentage cells showing highly induced expression from NADH cells at day 21 for neuronal marker (a) β-tubulin-III (b) astrocyte marker-GFAP and (c) oligodendrocyte marker-O4. Heat map showing changes in gene expression levels (e) and their relation (f) NADH in neurogenic differentiated lineages (at day 21). Percentage cells differentiated from ADH cell population showing highly induced expression for neuronal marker (g) β-tubulin-III (h) astrocyte marker-GFAP and (i) oligodendrocyte marker-O4. (J) Heat map showing relative changes in gene expression levels of neurogenic lineages derived from ADH cell population. (k) The pattern of relationship among the expression levels of different genes after differentiation of ADH cells into neurogenic lineages at day 21

the injury site requiring repair. This study also explored that DPSCs pass peculiar characteristics of MSCs derived from bone marrow or other sources to differentiate into default mesodermal lineages such as osteogenic, chondrogenic, and adipogenic [Figure 1j-o]. These essential features provide value to these cells for wider applicability.

Serum-free culture of stem cells is always desirable for their clinical applications.[12-14] In this study, a new strategy to develop neurogenic cells from DPSCs in serum-free condition has been reported. Cells formed neurosphere-like morphology at day 21 [Figure 2] containing a heterogeneous population of cells which was further evaluated for the variation in their molecular profiles [Figure 3] similar to other studies.[3,12] In this study, we showed that both ADH and NADH spheroids contain cells having potential to produce neurogenic lineage cells specifically neurons. This mode of differentiation could produce a large number (>80%) of neurons required for spinal cord injuries to improve the motor function.

Both the ADH and NADH spheroid cells triggered to differentiate into neurogenic lineage after withdrawal of neuronal mitogenic factors such as EGF and FGF. This reveals that neurosphere-derived cells do not require the mitogenic support for generating neurogenic lineage cells. The addition of RA was done based on the postulation that RA activates the pathways related with neurogenic lineage specialties in neural stem cells.[15] The NADH spheroid cells produced higher potential to generate neurons as compared to the ADH cells [Figure 5]. These neurons had well-developed axons and dendrites [Figure 4]. In addition, the expression of β–tubulin III was much higher in differentiated NADH cells as compared to ADH cells [Figure 5].

Exogenous neurotrophic factors have been proposed as a crucial requirement for *in vitro* survival of peripheral and central nervous tissues.[16] Because we did not supply any neurotrophic factor to the differentiating cells, we sought to find continuous upregulation in the expression of GDNF, BDNF, and NGF required for the long-term survival and function of neurogenic cells. Although glial cells have been shown to produce some amount of these neurotrophic factors in response to injury *in vitro* or *in vivo*,[17] they fail to provide enough support to the neurons in case of chronic injuries. Our study revealed that spheroid cells have potential to express such neurotrophic factors and could be an essential component in regenerative processes. To some extent, similar studies have reported the neurogenic differentiation potential of DPSCs;[3,18] however, further studies are required to define the true clinical nature with wide applicability. Further, the involvement of only small number of tissue samples may raise power issues and also for a more time-efficient method is needed for the safer applicability of serum-free neurogenic differentiation medium. Hence, future studies are required for elucidating more precise characterization and identification of neurogenic differentiation of DPSCs suitable for the therapeutic functions over the long term.

Conclusions

The present study reported a cascade of immunophenotypic and molecular markers to characterize neurogenic differentiation of DPSCs under serum-free condition. These findings trigger the future analyses for deep investigation of the hypothesis of existence for more than a single stem cell population within the DP which may provide better flexibility for clinical applicability of DP-derived cells in regenerative applications.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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