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Pre-Conditioning with Neuronal Conditioned Medium Enhances the Induction of Dental Pulp Stem Cells into Dopaminergic Like Cells

Nareshwaran Gnanasegaran
Regenerative Dentistry Research Group (ReDReG), Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia, Departments of Restorative Dentistry, Faculty of Dentistry, University of Malaya, Kuala Lumpur Malaysia
Vijayendran Govindasamy
Persiaran Seri Perdana, Precinct 10, Federal Territory of Putrajaya, Malaysia
Sabri Musa
Regenerative Dentistry Research Group (ReDReG), Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia, Departments of Children's Dentistry and Orthodontics, Faculty of Dentistry, University of Malaya, Kuala Lumpur Malaysia
Noor Hayaty Abu Kasim
Regenerative Dentistry Research Group (ReDReG), Faculty of Dentistry, University of Malaya, Kuala Lumpur, Malaysia, Departments of Restorative Dentistry, Faculty of Dentistry, University of Malaya, Kuala Lumpur Malaysia

Abstract

Dental pulp stem cells (DPSCs) are one of emerging cell source in regenerative medicine especially in treating neuronal related diseases. In general cell transplantation set-up, undifferentiated cells were transfused into the recipient and being in an unclear state, these cells are failed to perform up to par. However, recent studies have shown that pre-conditioning of cells with artificial environment mimicking the target environment improves the efficacy of the cells upon transplantation. Here, we investigated whether pre-conditioning of DPSCs with neuronal cultured media enhances its' potential towards neurogenesis. Dental pulp stem cells from deciduous teeth (known as DPSCs) were isolated from 3 healthy donors. The cells were then pre-conditioned with culture media of ReNCell, a neuron progenitor cells, prior to neurogenesis induction by means of commercially-available media. The neuronal potential of DPSCs was checked in terms of their gene and protein expression. We found that cells pre-treated with conditioned medium were primed towards neuronal cells as compared to control cells. In conclusion, we suggest that cells should be primed to their respective environment prior to transplantation.

1. Introduction

Stem cells (SCs) are refer to a group of cells which are capable of self-renewal and able to differentiate into myriad of tissues. Traditionally, these cells are found in BM-MSCs and most characterized SCs up to date (Knight and Hankenson, 2013). However, due to the low yield upon ex vivo expansion coupled with invasive procedure has elicited the need to search for alternative cell source. As the consequences, SCs are now found nearly in all organs and tissues of a human body.

Among these, the tooth tissues namely the pulp offers an excellent reservoir of SCs. We have previously shown that these under appropriate cue can be differentiated into beta-like cells (Govindasamy et al., 2010a), adipocytes (Govindasamy et al., 2010b), chondrocytes and osteocytes (Govindasamy et al., 2010a). Likewise, others also have reported the ability of dental pulp stem cells (DPSCs) into cardiac-like cells and hepatic like cells. DPSCs now are associated as a very close cell source for neurodegenerative diseases. This is due to their inborn inclination towards ectodermal origin. The cells are also shown remarkable outcome in some pre-clinical studies and have been used in clinical trials as well.

Among the debilitating diseases, neurodegenerative possessed a great challenge to treat due to the inadequate knowledge about disease etiology and pathogenesis of neurodegenerative diseases such as Parkinson, Alzheimer`s, Huntington`s, stroke and anoxic brain injury. Although these poorly managed diseases are worthy targets for cell replacement therapy, many factors especially whether the SCs are able to differentiate into cells of interest of a neuronal region need to be taken into consideration. This is because arrays of neuronal subtypes are available such as motor neurons, glial cells (oligodendrocytes and astrocytes), dopaminergic, Schwann cells and etc. To ascertain this notion, we have selected dopaminergic as a study model and investigated whether or not DPSCs could be able to differentiate into this cell lines.

The differentiation into dopaminergic neuron can be mediated in different ways. The first studies used feeder layers of mice PA6 or MS5 cells. Others have a direct differentiation into dopaminergic by adding mid-brain growth factors such as fibroblasts growth factor 8 (FGF-8), glial derived neurotrophic factor (GDNF), and brain derived neurotrophic factor (BDNF). Here, we have chosen pre-conditioning method to enhance the differentiation potential of DPSCs into dopaminergic.

Media pre-conditioning refers to co-cultivating cells with culture solutions consisting of certain chemicals with the aim of providing fitting environment that facilitate intended changes (Wu et al., 2011). Studies have shown that pre-conditioning enables cells to react positively in various aspects namely bone regeneration (Lu et al., 2013), immunomodulatory potency (Plotnikov et al., 2013), ameliorating renal ischemic injury (Masoud et al., 2012), repairing infarcted myocardium (Choudhery et al., 2012) and many more. On top of this, choosing proper cues is another vital factor to consider in order to maximize the pre-conditioning effect.

ReNcell VM is an immortalized neuronal progenitor cell line which is being applied in various studies related to neuron development (Mußmann et al., 2014; Chaudhry and Ahmed, 2013; Hernández-Benítez et al., 2013; Jaeger et al., 2013). The media of this cell line is chosen to serve as mediator to deliver appropriate signals to enhance the induction process. Having said thus we initiated this study to understand the end result of DPSCs pre-condition with ReNcell VM media in the differentiation into dopaminergic neurons.

2. Materials and Methods

2.1. Sample Population

The study was conducted using samples isolated from paediatric donors. Prior to the commencement of subject recruitment, approval for the study was acquired from the Medical Ethics Committee, Faculty of Dentistry, University of Malaya. (Ethics approval number: DF CD1201/0013[P]).

2.2. Pulp Collection and Isolation of Cells

Stem cell cultures derived from healthy deciduous donors (n=3) were established as previously described by our group [Govindasamy et al., 2010]. Briefly, root surfaces were cleaned with Povidone-iodine (Sigma Aldrich, St. Louis, MO, USA; http://www.sigmaaldrich.com), and the pulp were extirpated within two hours post-extraction, and processed. The pulp tissue were minced into smaller fragments prior to digestion in a solution of three mg/mL collagenase type I (Gibco, Grand Island, NY, http://www.invitrogen.com) for 40 min at 37°C. After neutralization with 10% FBS, the cells were centrifuged and seeded in culture flasks.

Cells were cultured in identical culture condition, namely in T75 cm^2 culture flasks (BD Pharmingen, San Diego CA, USA; http://www.bdbiosciences.com) with culture medium containing 1X KO-DMEM, 200 units/mL and 200 µg/mL of penicillin/streptomycin (Invitrogen); 0.01X Glutamax (Invitrogen) and 10% FBS with humidified atmosphere of 95% of air and 5% of CO2 at

37°C, as well as cell seeding of 1000 cell/cm^2. Non-adherent cells were removed 48 h after initial plating. The medium was replaced every three days until the cells reached 80-90% confluency.

2.3. Growth Kinetics

The proliferation rate of DPSCs were determined by plating 5000 cells per cm^2 into separate T25 cm^2 culture flask (BD Pharmingen). There were three replicates for each passage. DPSCs were detached by trypsinization after reaching 90% confluency. Cells were counted and assessed for viability using trypan blue dye exclusion before the next sub-culture. Cells were re-plated for subsequent sub-culture, and a total of 5 sub-cultures were studied in this experiment. Growth kinetics was analyzed by calculating population doubling (PD) time. The PD time was obtained using:

PDT=tplg2/ (lgNH-lgNI)

NI: the inoculums cell number; NH is the cell harvest number and t is the time of the culture (in hours).

2.4. Senescence Assay

DPSCs were tested for senescence-associated β-galactosidase (SA-βgal) activity at sub-culture 3 (SC 3) with the SA-βgal staining kit (Sigma Aldrich) and used according to the manufacture's instruction. Briefly, DPSCs were washed twice with DPBS (-Ca^{2+}, -Mg^{2+}, Invitrogen) and incubated with 1X fixative solution for 15 min at room temperature. Subsequently, the fixed cells were re-washed using 2 mL of DPBS (-Ca^{2+}, -Mg^{2+}, Invitrogen) and stained with 1 mL of the staining solution mixture overnight at 37°C in a dry incubator. The development of the blue colour was observed under a phase-contrast microscope and the quantitative analyses of the SA-βgal staining was done by counting the percentage of blue-stained cells that represent senescence cells in the selected field of each sample.

2.5. Trilineage Differentiation

Mulitipotency of DPSCs was assessed by differentiating them into adipocytes, chondrocytes, and osteocytes as described previously (Yazid et al., 2014; Govindasamy et al., 2011). Briefly, adipogenic differentiation was initiated by inducing the cells with 200 mM indomethacin, 0.5 mM 3-isobutyl-1-methyxanthine (IMBX), 10 mg/mL insulin and 1 mM dexamethasone (all reagents from Sigma-Aldrich). Lipid droplets in the adipocytes generated were visualized by staining with Red Oil staining (Sigma-Aldrich). For chondrogenic differentiation, the cells were supplemented with ITS-1 (Sigma-Aldrich), 50 mM L-ascorbic acid-2 phosphate, 55 mM sodium pyruvate (Invitrogen), 25 mM L-proline (Sigma-Aldrich) and 10 ng/mL transforming growth factor-beta (TGF-β; Sigma-Aldrich). Assessment of proteoglycan accumulation was visualized by Alzarin Blue staining (Sigma-Aldrich). The osteogenic differentiation was stimulated in a 3-week culture with 10^{-7} M dexamethasone, 10 mM β-glycerol phosphate (Fluka, Buchs, Switzerland) and 100 mM L -ascorbic acid-2 phosphate. Assessment of calcium accumulation was visualized by Von Kossa staining (Sigma-Aldrich). Important genes related to fat, cartilage and bone development were also checked and the primers are listed in Table 1.

2.6. Neuronal Induction

DPSCs were subjected into neuronal induction at SC 3 by using chemically-defined media as described by Wang et al. (2010). Briefly, two groups of DPSCs were prepared; one to undergo pre-treatment by co-culturing with conditioned media (CM) of immortalized cell line (ReNCell VM) for 7 days while the other group with Neuronal Media A which consist of Neurobasal A, B27 supplement, 20ng/mL basic Fibroblast Growth Factor (bFGF), 20ng/mL Epidermal Growth Factor (EGF) for 9 days. Following this, these cells were then treated with second phase of neuronal differentiation (Neuronal Media B) which consist of Neurobasal A, 200ng/mL sonic hedgehog (SHH), 100ng/mL Fibroblast Growth Factor 8 (FGF8), 10ng/mL brain-derived neurotrophic factor (BDNF) and 10µM forskolin (Sigma-Aldrich) for 7 days. All chemical were purchased from Invitrogen unless stated otherwise.

2.7. Semi-Quantitative and Quantitative Gene Expression Via Polymerase Chain Reaction (PCR)

PCR was performed in 0.2 mL eppendorf tubes (Axygen) with a final volume of 12.5 µL. cDNA amplification was performed in a thermocycler using Taq polymerase supplied with KCl buffer and 1.5mM $MgCl_2$ (Invitrogen) at 94°C for 1 min, 58°C for 30 s and 72°C for 1 min. PCR products were resolved on 1.5% agarose (Invitrogen) gel in 1x Tris borate–EDTA buffer. The expression levels of the genes via real-time PCR were quantified in duplicates, using SYBR Green Master Mix (Applied Biosystems). PCR reactions were carried out on an ABI 7900HT RT–PCR system (Applied Biosystems), and the results were analyzed using a software called SDS v 2.1. Gene expressions were analyzed via comparative CT Method (ΔΔCT) and were normalized to 18s rRNA. The primer sequences are listed in Table 1.

2.8. Immunofluorescent Analysis

Differentiated cells were fixed for 20 min in 4% ice cold paraformaldehyde, treated with 0.1% Triton-X for optimal penetration of cell membranes, and incubated at room temperature (RT) in a blocking solution (0.5% BSA; Sigma Aldrich) for 30 min. Primary antibodies [Oligodendrocyte marker (mouse, Abcam), Musashi 1 (rabbit, Abcam), beta-tubulin III (mouse, Millipore), neurofilament (mouse, Upstate), Microtubule-associated protein 2 (MAP2) (rabbit, Abcam), tyrosine hydroxylase (TH) (rabbit, Abcam) with dilution of 1: 400 for all] were incubated overnight at 4°C, washed with Dulbecco's Phosphate Buffer Saline (DPBS; Invitrogen), and then incubated with secondary antibodies (either fluorescein isothiocyanate [FITC]-conjugated IgG or rhodamine-conjugated IgG) at RT for 90 min. Slides were counterstained with 4',6'-diamidino- 2-phenylindole dihydrochloride (DAPI, Chemicon, Temecula, CA, USA) for 5 min. Fluorescent images were captured by means of a Nikon-Eclipse-90i microscope (Nikon, Tokyo, Japan, http:// www.nikon.com).

3. Results

3.1. Basic Characterization of DPSCs

The cells were shown to have a fibroblastic feature throughout the culture period. In terms of growth analysis, the accumulative cell shown to increase by almost 4-fold at SC5 as compared to SC1. This reflected in the PDT analysis whereby two-fold changes in terms of hours was recorded at SC5 as compared to SC1 (Fig. 1B). Senescence assay (Fig. 1C) further showed little percentage of beta-galactosidase activity depicted by blue-coloured region with the percentage of 2.7% indicating less aging process in DPSCs. The cells were also subjected to some mesoderm differentiations in which it were able to produce oil droplets (adipogenesis), forming networks of proteoglycan (chondrogenesis) as well as accumulation of black calcium precipitate (osteogenesis) upon chemical stimulation (Fig. 1D). The differentiation into these lineages were further confirmed with qRT-PCR and it was found that the expression of PPAR-gamma-2 and LPL for adipogenesis, Aggrecan and Collagen 2A1 for chondrogenesis, RUNX and OSTEOCALCIN for osteogenesis were present.

3.2. Induction of DPSCs into Neuronal-like Cells

Upon co-cultivation of CM with DPSCs for 7 days, significant changes in terms of morphology of the cells were noted. (Fig.2). Cells were shown to be more elongated and extensively branched as compared to control sample. As the induction period progresses, more extensions and cell branches were observed. Towards the end of induction (day 16), bipolar-like cells were witnessed with nucleus in the middle while the axon-like structures were found to be stretched away from nucleus. Conversely without the pre-treatment with CM, DPSCs were seen to differentiate a little slow with less cell number (Fig. 3). During the induction period, DPSCs grew in colonies and formed cell cluster prior to day 10, surprisingly, at day 11 onwards the cells begin to grow away from one another and started to elongate. Even though the cell number was reduced, the final morphology was still similar as pre-treated DPSCs whereby bipolar-like features were observed.

3.3. Gene Expression Profile of DPSCs Transformed into Neuronal-like Cells

DPSCs from both conditions showed similar gene profile as described in Figures 4 and 5. Pluripotent markers like OCT4, SOX2, NANOG, REX1, and ABCG2 were lightly present as compared to undifferentiated cells. Furthermore early and mid-neuronal genes like NESTIN, NURR1,

PAX6, and MUSASHI1 were present in both conditions with more expression was observed in pre-treated DPSCs. Likewise, mature neuronal markers namely glial-fibrillary acidic protein (GFAP), beta-tubulin, tyrosine hydroxylase (TH), dopamine active transporter (DAT), and neural cell adhesion molecule (NCAM) were expressed slightly higher in pre-treated DPSCs as compared to non-treated.

3.4. Protein Expression Analysis of DPSCs Transformed into Neuronal-like Cells

Further analysis by means of immunofluorescence technique revealed that pre-conditioned DPSCs expressed all markers namely beta-tubulin, neurofilament (NF), microtubule-associated-protein 2 (MAP2), oligodendrocyte (OLIG), Musashi (MSI1) and TH slightly more in comparison with those without pre-conditioning (Figures 6 and 7).

4. Discussion

In this study, our results were concurrent with previous reports whereby cells were of fibroblastic phenotype and can be expanded in culture flasks (Yazid et al., 2014; Abu Kasim et al., 2012; Govindasamy et al., 2010a). On top of that DPSCs were shown to have decent outcome in terms of cell count, PDT as well as senescence assay. This specify that cells at optimal state and hence suitable for down-stream works (Sethe et al., 2006). Further, despite being a cell of neural crest origin (Kanafi et al., 2014), the ability of DPSCs to differentiate into three types of cell lineages indicates plasticity nature (Hass et al., 2011).

Moving on to the neurogenesis induction, both culture condition revealed apparent phenotypic structure of neurons as previously reported (Kanafi et al., 2014; Govindsamy et al., 2010a; Wang et al., 2010). DPSCs pre-treated with CM were shown to have better morphology as compared to without pre-treatment. It has been reported previously that pre-conditioning improves cell differentiation to a greater extent (Wu et al. 2012) and this is due to the presence of numerous cytokines and neurotrophic factors within the microenvironment (Plotnikov et al., 2013). These factors could serve as internal signaling molecules to guide DPSCs to differentiate into dopaminergic neurons accordingly.

In addition similar pattern was observed in gene expression whereby pre-conditioned DPSCs were seen to have better molecular signature profiles as compared to those without pre-conditioning. Overall, the expression of pluripotent indicators such as OCT4, SOX2, NANOG, REX1, and ABCG2 were decreased as compared to undifferentiated cells indicating a commitment of DPSCs towards neuronal-lineage (Chen and Dent, 2014). Pre-conditioned DPSCs have shown lower expression of the aforesaid genes as compared to those without pre-conditioning. This perhaps could be due to more proportion of cells have fully differentiated into dopaminergic neurons thus having low number of stem cells expressing pluripotent indicators.

As distinctive neurogenesis takes place, early and mid-neuronal genes like NESTIN MUSASHI1, NURR1 and PAX6 begin to show up in both conditions with more expression being observed in pre-treated DPSCs. It was reported previously that NESTIN is well known for its role in inducing neurogenesis (Lagace et al., 2007). Furthermore MUSASHI1 is involved in stem-cell self-renewal and asymmetric cell division which is highly correlated with proliferation, survival and neurogenesis (Pozniak and Pleasure, 2006). Additionally NURR1 was reported as one of major key regulators in dopaminergic neuron formation (Hong et al., 2014). PAX6 gene moreover is responsible in survival of immature neurons via CREB-signalling (Faigle and Song, 2013). Collectively these molecular cues have systematically coax DPSCs from their pluripotent stage to differentiate into mid neuronal phase.

As the neurogenesis process begin to develop, mature neuronal markers namely GFAP, beta-tubulin, TH, DAT, and NCAM were also seen to be obviously present with higher expression being observed in pre-conditioned DPSCs in comparison to those without pre-conditioning. GFAP has been reported as the major indicator related to intermediate filament of mature astrocytes in the mammalian central nervous system (Hagemann et al., 2013). Furthermore both NCAM and beta tubulin were shown to detect the incidence of mature neurons especially those of granule cell layer and external plexiform layer which are typical in olfactory bulb granule cells (Lledo et al., 2006). TH and DAT are mutually vital key indicators for fully functional dopaminergic neurons (Agoston et al., 2014; Kim et al., 2007). These genes altogether have shown the presence of mature neuronal markers which evolved from their previous mid-neuronal phase. Similarly protein expression profile by means of

immunofluorescence revealed that pre-conditioned DPSCs having more expression as compared to those without pre-conditioning. On top of indicating the presence of the said protein in different stages of neurogenesis, another notion whereby CM has influenced all these features in positive ways can be said as well.

Besides, we have shown in this study the potentiality of DPSCs to be induced into dopaminergic neurons. These neurons are very important predominantly in Parkinson's disease (Wang et al., 2010). Perhaps CM can be taken to our advantage provided that we have understood enough about it in order to find answer for this neurodegenerative problem. It is believed that the cytokines and small molecules which are present in CM played vital role in enhancing neurogenesis throughout the study period. A study has showed that glial conditioned medium (GCM) has neuroprotective effect onto immortalized striatal neuronal progenitor cell lines. Not just that, the GCM was also able to reduce cell death, increase cell survival, and reduce caspases fragmentation as well as accumulation of reactive oxygen species in addition to polyubiquitinated proteins. The neurotrophic factors that were present in GCM were believed to be responsible for such findings. Contrastingly, Horn et al (2011) have reported that the CM from MSC was apparently toxic to cell culture originated from hippocampal and this was due to the side effects of the small molecules present in the said CM. Further work revealed that the secreted cytokines from MSC triggered reactive species generation and neuroinflammation in organotypic cultures of hippocampus.

Despite these distinctions further understanding regarding CM is paramount since the mechanism behind this phenomenon is highly related to the small molecules. Secretome profiling of CM is required so that the components of CM can be fully described which will enhance our appreciation towards CM. This will further help us to translate this critical information in clinical settings which involves regenerative medicine especially those concerning cellular transplantation. There are reports showing that *in vivo* studies which have showed positive outcome in animal models could not be replicated in humans (Ankrum et al., 2014; Skuk, 2013).

Thus we speculate that cells could possibly be pre-conditioned according to their respective microenvironment prior to transplantation. Perhaps pre-conditioning would help prime the cells so that they could home themselves in target organ plus differentiate accordingly in the event of cellular transplantation. However other dynamics like procurement of CM, length of pre-conditioning time, immune-rejection and cell management approaches should be properly revised and tested for various times in various models to effectively establish this method for future purposes.

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6. Conflict of Interest

Authors declared no conflict of interest.

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Table-1. List of genes with primer sequence and their product size

Gene name	Forward sequence (5'-3')	Reverse sequence (5'-3')	Base pair size
RUNX2	GTCACTGTGCTGAAGAGGCT	GTCACTGTGCTGAAGAGGCT	119
OSTEOCALC	CAGAGGTGCAGCCTTTGTGTC	TCACAGTCCGGATTGAGCTCA	150
PPARγ2	ACAGCAAACCCCTATTCCATGCT GT	TCCCAAAGTTGGTGGGCCAGAA	159
LPL	TGGACTGGCTGTCACGGGCT	GCCAGCAGCATGGGCTCCAA	167
AGGRECAN	AGGGCGAGTGGAATGATGTT	GGTGGCTGTGCCCTTTTTAC	68
COLLAGEN 2A1	CTGCAAAATAAAATCTCGGTGTT CT	GGGCATTTGACTCACACCAGT	101
18s rRNA	CGGCTACCATCCAAGGAA	GCTGGAATTACCGCGGCT	186
Oct 4	CGACCATCTGCCGCTTTGAG	CCCCCTGTCCCCCATTCCTA	573
Sox 2	CCCCCGGCGGCAATAGCA	TCGGCGCCGGGGAGATACAT	448
Rex 1	TCGCTGAGCTGAAACAAATG	CCCTTCTTGAAGGTTTACAC	170
ABCG 2	GTTTATCCGTGGTGTGTCTGG	CTGAGCTATAGAGGCCTGGG	652
Nestin	CAGCGTTGGAACAGAGGTTGG	TGGCACAGGTGTCTCAAGGGTA G	389
Nanog	ATGCCTCACACGGAGACTGT	AGGGCTGTCCTGAATAAGCA	66
Nurr 1	CGGACAGCAGTCCTCCATTAAG GT	CTGAAATCGGCAGTACTGACAG GC	712
Pax 6	ATGAACAGTCAGCCAATGGG	CACACCAGGGGAAATGAGTC	63
Musashi	CAGCCAAAGGAGGTGATGTC	CGCTGATGTAACTGCTGACC	451
GFAP	GGCCCGCCACTTGCAGGAGTAC	CTTCTGCTCGGGCCCCTCATGAG	328
Beta-tubulin	GCGAGATGTACGAAGACGAC	TTTAGACACTGCTGGCTTCG	115
TH	TCATCACCTGGTCACCAAGTT	GGTCGCCGTGCCTGTACT	125
DAT	CTGGTGTCTGGAAGATCTGC	AGCTGTCTCCACTGGAGTCA	219
NCAM	CAGTCCGTCACCCTGGTGTGCGA TGC	CAGAGTCTGGGGTCACCTCCAGA TAGC	727

Figure-1. 1A and 1B: Accumulated cell count and population doubling time (PDT) over five sub-cultures to understand the growth kinetics of DPSCs. 1C: Senescence assay to assess the activity of beta-galactosidase in DPSC at SC3. 1D: Trilineage differentiation of DPCS at SC3. Adipogenesis was assessed via Red Oil staining and oil droplets formation was observed. Similarly, chondrogenesis was evaluated using Alzarin Blue staining with the formation of proteoglycan networks. Osteogenesis was also measured using Von Kossa staining and black precipitate was observed. Micrographs were taken at 10x magnification. 1E: Real-time PCR was performed to detect presence of representative genes related to adipogenesis, chondrgenesis and osteogenesis.

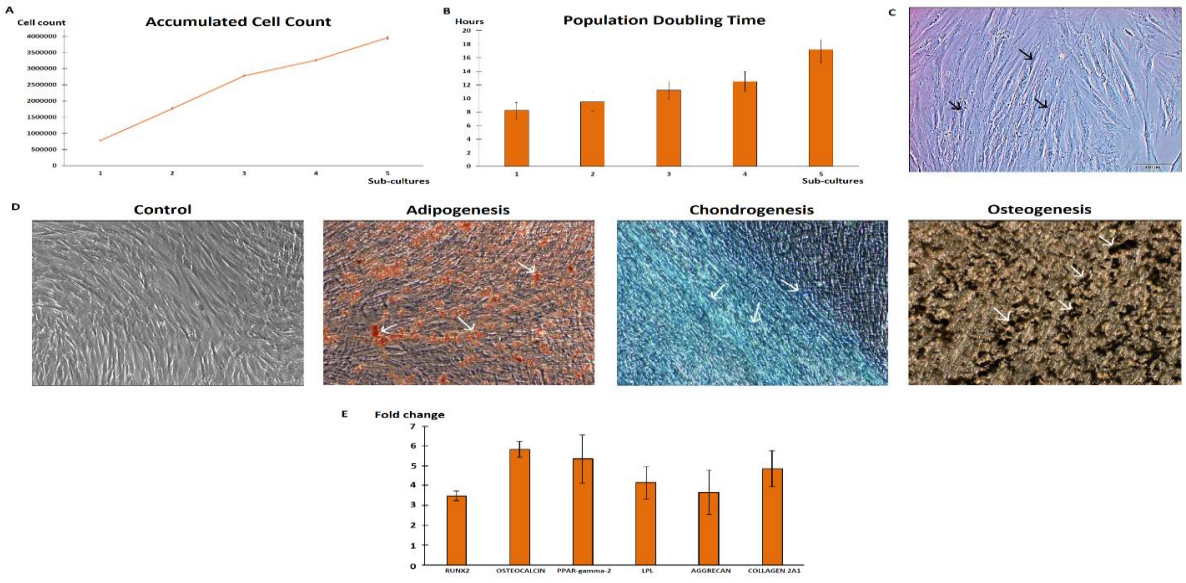

Figure-2. Induction of pre-conditioned DPSCs into dopaminergic neurons. Cells rapidly begin to elongate and form branches while some even form star-shaped cells up to Day 10. Following Day 10, cells were found to grow near each other and form 'whirlpool' shaped colonies up to Day 16. Bipolar phenotypes were also observed in some of the cells. Micrographs were taken at 10x magnification.

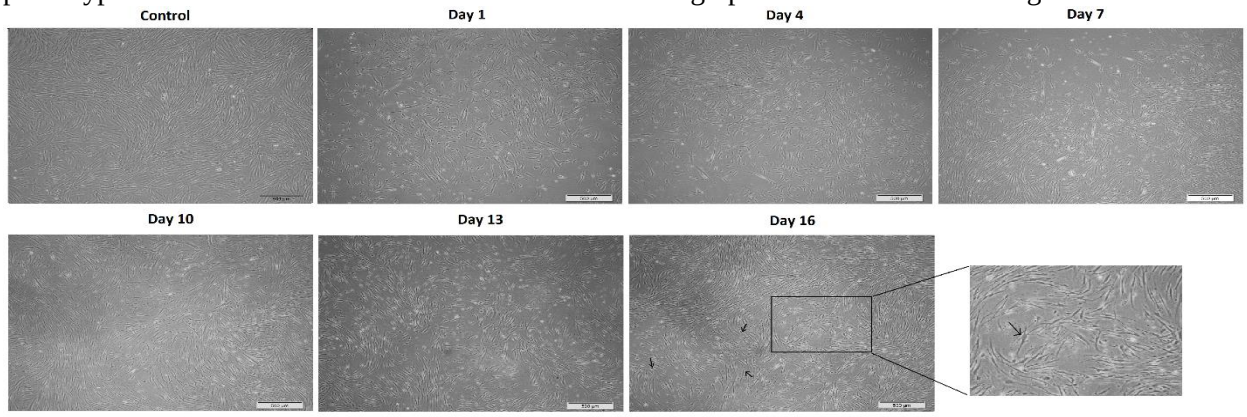

Figure-3. Induction into dopaminergic neurons in DPSCs without pre-conditioning. Cells were growing in clusters in elongated form till Day 7. From Day 10 onwards, the cells begin to thin with branch formation till Day 16. Cell number was found to have been reduced throughout the study period. Micrographs were taken at 10x magnification.

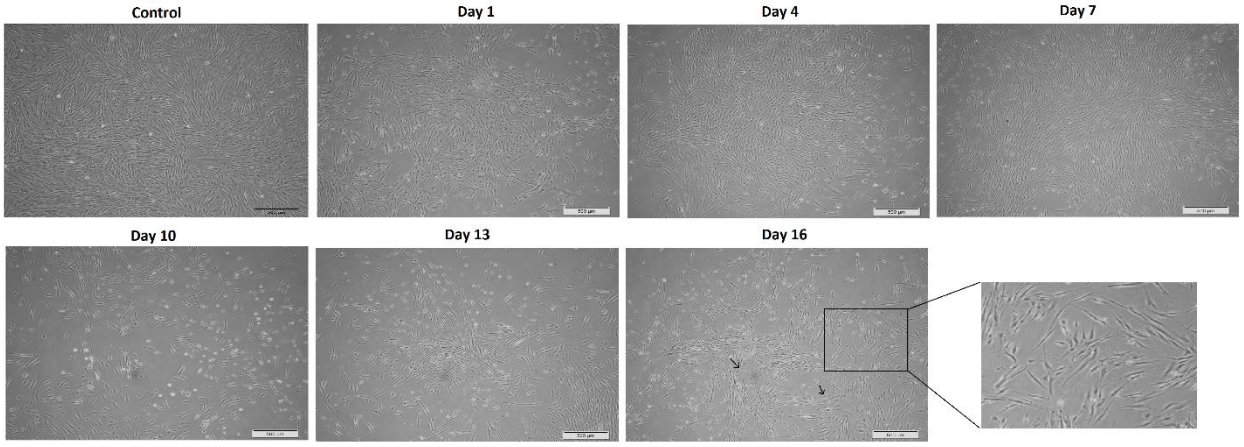

Figure-4. Detection of pluripotent indicators as well as neuronal markers via semi-quantitative PCR. Lane A refers to undifferentiated DPSCs; Lane B denotes expression using total RNA from Brain; Lane C shows the expression of pre-conditioned DPSCs and Lane D describes the gene expression of DPSCs without pre-conditioning. Pluripotent markers (OCT4, SOX2, REX1, and ABCG2) were highly expressed in undifferentiated cells (Lane A) whereas reduced expression was observed in Total RNA from brain (Lane B), pre-conditioned DPSCs (Lane C) and in DPSCs without pre-conditioning too (Lane D). Early neuronal markers (Nestin, Nanog, Nurr1, Pax6, and Musashi) were seen to present in both Lane C and Lane D with the former having more expression. Similarly, mature neuronal markers (GFAP, beta-Tub, TH, DAT, and NCAM) were shown to present in both Lane C and Lane D with the former having more expression.

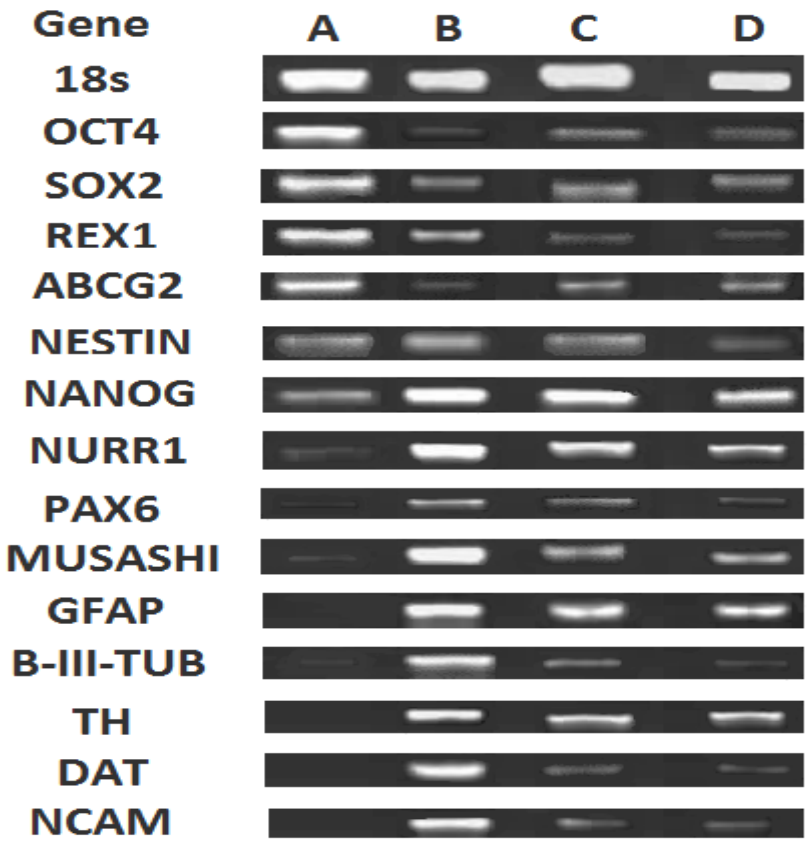

Figure-5. Detection of pluripotent indicators as well as neuronal markers. The Ct value of genes were analyzed in the study using SYBR green-based qRT–PCR for DPSCs. Generally the higher a fold change value, the more copies are present in the specific sample. Total RNA from brain was used as positive control. Values are presented after normalization to 18s mRNA levels.

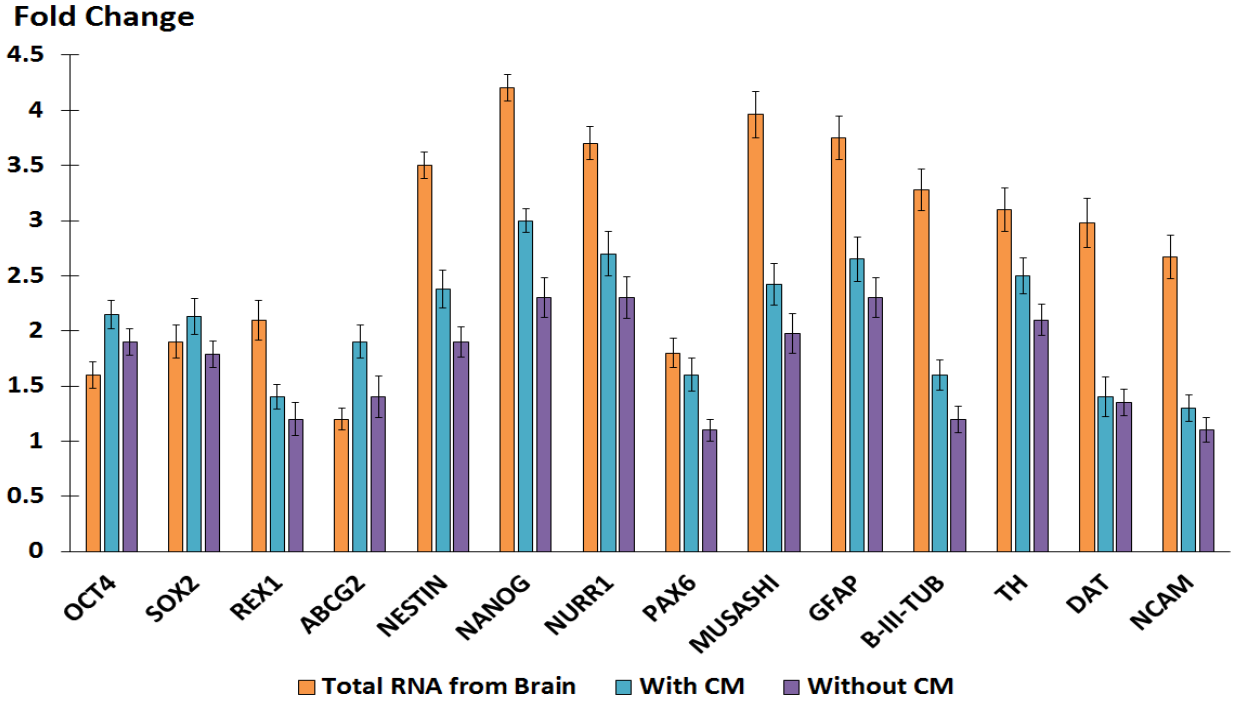

Figure-6. Immunofluorescence analysis revealing the presence of neuronal marker namely Oligodendrocyte transcription factor (OLIG), RNA-binding protein Musashi homolog 1 (MSI1), beta—tubulin III (b-TUB), neurofilament (NF), microtubule-associated protein 2 (MAP2), and tyrosine hydroxylase (TH) in pre-conditioned DPSCs. Micrographs were taken at 10x magnification.

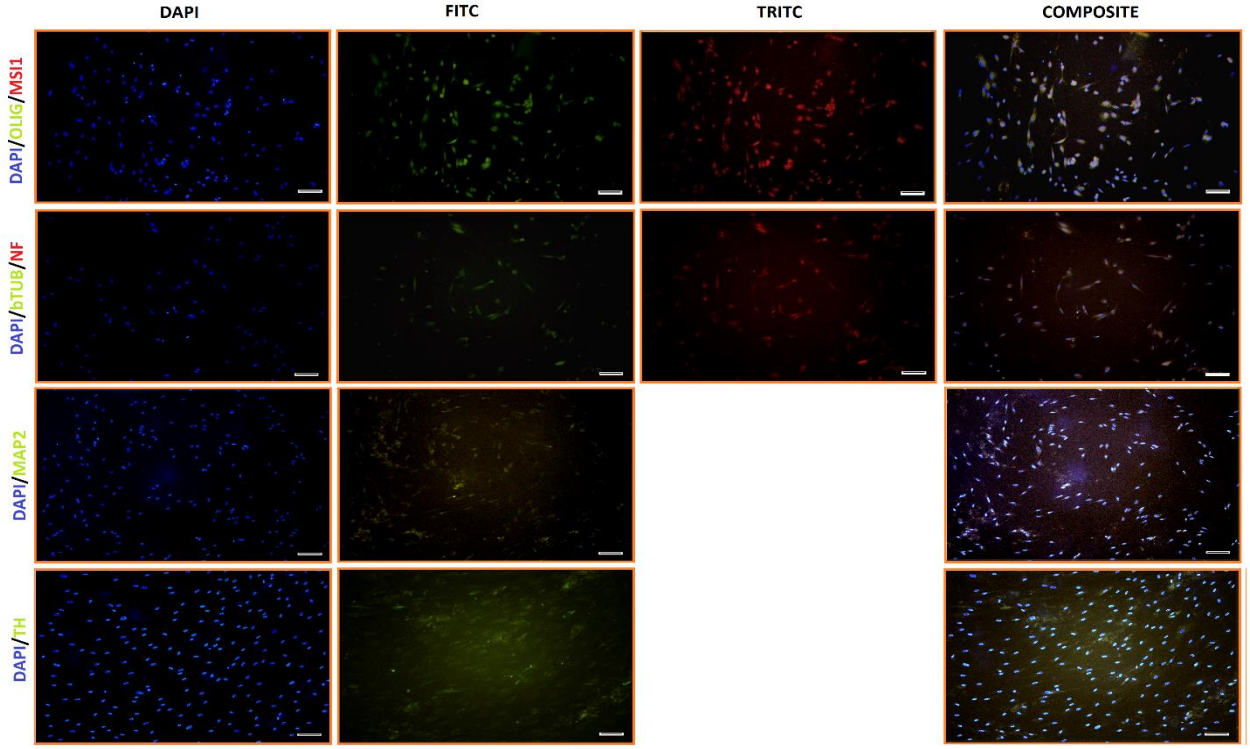

Figure-7. Immunofluorescence analysis revealing the presence of neuronal markers namely Oligodendrocyte transcription factor (OLIG), RNA-binding protein Musashi homolog 1 (MSI1), beta—tubulin III (b-TUB), neurofilament (NF), microtubule-associated protein 2 (MAP2), and tyrosine hydroxylase (TH) in DPSCs without pre-conditioning. Micrographs were taken at 10x magnification.