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RESEARCH ARTICLE

Isolation and characterization of neural precursor cells from different regions of human fetal brain: Assessment of *in vitro* proliferation and differentiation

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Abstract

Human neural precursor cells (hNPCs) are believed to have major potential for their clinical applications and basic neuroscience research. Identification of highly enriched stem cell source and i*n vitro* behavior of these cells offers a vast potential for analyzing the molecular, cellular, and developmental processes to model various neurodegenerative disease. Hence, major goal of NPCs study is to understand the nature of the neurogenic niche and identification of a significant marker in order to facilitate NPCs self-renewal and regeneration potential both *in vitro* and *in vivo*. The aim of the present study was to identify the most enriched source for the isolation of NPCs from 18-22 wk gestation aged human fetal brain further to facilitate their proliferation, differentiation and long term survival.

The present study has demonstrated that in 18-22 wk gestation aged human fetal brain tissues, serves are most prominent source for the resident of enriched NPCs and SVZ serves as the most abundant niche for the isolation and enrichment of NPCs. Selection of CD133+ve cells using MACS/FACS represents a fast and effective means to enrich the large number of NPCs. Immunophenotypic analysis of stem cells sub-populations in magnetically sorted and unsorted cells using CD133, CD34, CD45, CD56 and Nestin provide a wide range of different cell populations present in human brain tissues. This will provides a combination of markers to facilitate the identification of NPCs isolated from various sources. Gene expression and sequencing data analysis provided a significant evidence for the expression of NPCs specific genes in CD133+ve sorted cells. Nucleotide sequence alignment for Nestin, Sox-2 and Oct-4 genes showed a consequence of structural and evolutionary relationship of embryonic origin. *In vitro* proliferation capacity of CD133+ve cells showed high degree of self-renewal ability and lineage differentiation capability to generate high number of neurons, astrocytes and oligodendrocytes in different culture conditions.

Hence, identification of enriched NPCs enriched source using combination of markers by immunophenotyping, ICC, MACS, and molecular approaches together provide a significant role in the enrichment and *in vitro* study of their behavior and long term survival of high quantity of NPCs isolated from frontal lobe, temporal lobe, occipital lobe and SVZ of different gestation aged human fetal brain.

1. Introduction

Human neural precursor cells (hNPCs) are believed to have significant potential for their clinical application and basic neuroscience research. However, paucity of these cells from a defined source having large number of homogeneous NPCs fades their clinical relevance and needs to investigate abundant supply for getting the significant number of NPCS from single tissue source. In order to this, various tissue sources have been used for the isolation and *in vitro* propagation of NPCs to get maximum number of homogeneous NPCs to prove their potential for the treatment of several neurodegenerative diseases by generating the desired central nervous system (CNS) cell type (Vescovi, et al. 1999; Toma, et al. 2001; Joannides, et al. 2004; Valenzuela, et al. 2008; Erceg, et al. 2008; Fu, et al. 2008; Zhang, et al. 2009; Jang, et al. 2010).

Subventricular zone (SVZ) that borders the lateral ventricles of the forebrain represents a highly neurogenic area (Alvarez-Buylla, et al. 2002). In this system, NPCs populations permanently generate large number of neuronal and glial precursors. Therefore, the present study was focused to isolate the NPCs from SVZ of different gestation aged human fetal brain and evaluated the presence of highest number of NPCs using CD133 cell surface marker by immune-selection technique (Uchida, et al. 2000).

Many cell surface and intracellular markers have been identified for the characterization of NPCs isolated from various tissues of human brain. But still there is need to investigate for more precise, consistent and reliable marker to identify maximum number of NPCs. The glycoprotein CD133 is a known stem/progenitor cell marker in many tissues and has been used to isolate NSCs from human brain (Schwartz, et al. 2003). A characteristic feature of this protein is its rapid down-regulation during cell differentiation which makes it a unique cell surface marker for the identification and isolation of immature precursor cells. It defines a population of immature/primitive and committed progenitor cells that subsequently can be isolated by CD133 antibody using magnetic activated cell sorting or fluorescence activated cell sorting (MACS/FACS). In early 1970s neurobiologist kept their efforts to establish a battery of neural cell-specific markers which would serve to study the lineage and functional identification both *in vivo* and *in vitro*.

To assess the stem cell characteristics of self-renewal and multipotency, the neurosphere assay is widely used method to get maximum number of NPCs (Reynolds, et al. 1992). To test for self-renewal, clonally derived neurospheres are dissociated and then replated at clonal density, in order to determine the cells' capacity to form new spheres, so called secondary sphere formation. To test for multipotency, clonally derived neurospheres are cultured under differentiating conditions, in order to monitor the ability of these cells to generate the three main cell types of the CNS, i.e. neurons, astrocytes and oligodendrocytes (Reynolds, et al. 1996). Antibodies developed for intermediate filament proteins have been extensively used for cell identification such as neurons can be characterized by their associated neurofilament protein Tuj1 (b tubulin-III) (Hyndman, et al. 1987; Katsetos, et al. 2003), astrocytes by glial fibrillary acidic proteins (GFAP) (Bignami, et al. 1972) and oligodendrocytes by O4 (Sommer, et al. 1981). In brief, advances in understanding the structure and role of cell-specific markers have greatly increased their usefulness in that they will allow functional aspects of the brain to be studied in its developments, differentiation and diseased states.

The aim of present study is to develop an effective approach for the isolation and characterization of NPCs from various niches from human fetal brain of 18-22wk gestation age. Appropriate combination of markers will be identified to characterize the stem cell population from various sources to promote long-term survival and differentiation of NPCs *in vitro*. We assume to characterize the stem cells from frontal, temporal and occipital lobes and compared with the SVZ derived cells using a combination of markers and enrichment technologies. The most enriched cells will be cultured *in vitro* in different conditions and analyzed for expression of specific markers and survival rate. The *in vitro* cultured cells will be differentiated into NPCs specific lineages; neurons, astrocytes and oligodendrocytes in 1% FBS and Retinoic acid induced medium.

2 Materials and methods

2.1 Isolation of CNS cells

Human fetuses' (gestation 18-22 week) were obtained from local maternity hospitals as a result of spontaneous abortion following all national guidelines. The brain was kept on ventral side and dissected in its four lobes. Tissues from frontal, temporal and occipital lobes and SVZ were removed. Tissues were minced by mechanical dissociation into 0.3mm tissue pieces and subjected to enzymatic dissociation by incubating the mechanically dissociated tissue at 37°C for 1 hour in Dulbecco's Modified Eagles Medium (DMEM-F12, Invitrogen) containing 0.1% Collagenase. The cell suspensions were filtered through a 40μm nylon cell strainer and subjected to further analysis. The study protocol was approved by the Institutional Ethics Committee, Deccan College of Medical Sciences, Hyderabad, India.

2.2 Immunophenotypic analysis of human fetal brain derived cells

To explore the presence of stem cell population, before MACS the cells from frontal lobe, temporal lobe, occipital lobe and SVZ were stained with fluorescent labeled secondary antibody specific prominin-1 (CD133-PE). Briefly, 5 x 10^5 cells from each source were incubated with CD133-PE antibodies for 30min at room temperature in dark and fixed with 4% paraformaldehyde (PFA) in Phosphate Buffer Saline (PBS). PE-conjugated mouse IgG1 ((BD Biosciences, SanJose, CA) was used to set the background fluorescence as isotype control. Both the isotype control as well as stained cells were applied to the FACS Calibur flow-cytometer (BD Biosciences) and analysed using CellQuest software.

2.3 Magnetic activated cell sorting (MACS)

The monoclonal antibody CD133 (Miltenyi Biotec) was used to recognize the cell surface antigen; CD133 in SVZ derived cells from human fetal brain. Mini MACS separation kit (Miltenyi Biotec) was used for the sorting of CD133+ve/CD133-ve cells followed by the manufacturer's instructions. Briefly, single cell suspensions of dissociated cells were first incubated with CD133 antibody for 30min at 4°C. Cells were washed twice and then treated with magnetic microbeads (Miltenyi Biotec) for 15min at 4°C. The magnetically labeled cells were applied on to MS column followed by washing. After the suspension had gone through the column, the cells were washed three times using DMEM-F12 and the total effluent was collected as unlabeled negative fraction (CD133-ve). The MS column was removed from the magnet and again the medium was applied on to the column. The retained magnetically labeled cells were gently flushed out by a plunger and collected as CD133+ve cell fraction.

2.4 Fluorescence activated cell sorting (FACS)

Further to analyze the neural stem cells and its pluripotency CD133+ve and CD133-ve sorted cells were immunostained with CD56, CD49f and Nestin. Hematopoietic components were also identified using CD34 and CD45 antibodies. Staining procedure was followed as described previously. PE-conjugated mouse IgG1 ((BD Biosciences, SanJose, CA) was used to set the background fluorescence as isotype control. Both the isotype control as well as labeled cells were applied to the FACS Calibur flow-cytometer (BD Biosciences) and analysed using CellQuest software.

2.5 Identification of NPCs using immunocytochemical (ICC) staining

Magnetically sorted CD133+ve and CD133-ve cells (20 wk gestation, SVZ) were fixed with 4% PFA in PBS for 30min at room temperature, and then treated with 0.3% Triton X-100 (v/v) followed by 4 hours incubation at 4 °C using mouse anti-nestin monoclonal antibody (1:1000; R&D Systems, Minneapolis, MN). Stained cells were identified by using the software Axiovision, Version: 4.7 in an inverted fluorescent microscope (Axiovert 40 CFL, CarlZeiss).

2.6 Gene expression analysis using RT-PCR and sequencing

Total RNA isolated from CD133+ve cells (20 wk gestation, SVZ) were quantified and reverse transcribed into complementary DNA (cDNA) using reverse transcriptase enzyme (Invitrogen) and oligo dT primers (Invitrogen). 4ng cDNA in each reaction was used for gene expression analysis by polymerase chain reaction (PCR) in S1000 Thermal cycler (BIORAD). Initial denaturation of cDNA was performed at 94°C for 10min. Further gene sequences were amplified for 35 cycles on 94°C for 30 sec, 45-60°C for 45 sec and 72°C for 45 sec. Final extension was performed at 72°C for 5 min. The amplicons were visualized on 2% agarose gel by ethidium bromide (EtBr) staining. Primer details are provided in table 1. The image was captured in Quantity One software using Gel documentation system (BIORAD).

Table 1 Primers used in the study to amplify the NPCs markers using RT-PCR

S. No.	Target Gene	Primer sequences (5'-3')	Tm (°C)	Amplicon size (bp)
1.	Nestin	F- CCTTCAATTTATTGTCCC	45	403
		R- CAACAAGAGTGAGTGCCA		
2.	Sox-2	F- AGGGCCGGCTCAGCCCGCTGTGGTTCC	59	317
		R- CATTTCCCTCGTTCATGTTGTTTCTTATT		
3.	Oct-4	F- CCGTCCTGCAGGCCGGAA	60	173
		R- CTCGCGCCGGTGACAGAA		
4.	Notch-2	F- ACATCATCACAGACTTGGTC	54	400
		R- CATTATTGACAGCAGCTGCC		

The amplified PCR products Nestin, Sox-2, and Oct-4 were purified from the agarose gel using QIAquick Gel Extraction Kit (Qiagen) followed by manufacturer's instructions. Purified PCR products were quantified and sequenced using ABI 3100/3130 Sequencer (Azco Biotech, Inc.). Nucleotides sequences were analyzed by the chromatogram reading and subjected to National Centre for Biotechnology Information (NCBI) Basic Alignment Search Tool (Blast). E value ≥1e-04 was considered significant and ≥1e-04 was assumed to have an error rate of less than 0.01%.

2.7 Cell viability assays

Cell viability and counting was done by Trypan-Blue Exclusion Assay before and after MACS. Cell membrane integrity and viability was further confirmed by immunocytochemical staining and immunophenotypic analysis using Fluorescien Di-Acetate (FDA).

2.8 MTT cell proliferation assay

MTT [3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, (Sigma)] was used to evaluate the proliferation and viability of cultured CD133+ve cells according to the manufacturer's instructions. Briefly, CD133+ve enriched cells were resuspended in complete proliferation medium at different cell densities (0, 5000, 10000, 15000, 20000, 25000 and 50000). Triplicate 100μL cell samples were distributed within a 96-well plate (BD Biosciences). After 24h of incubation, 10μL of MTT reagent was added to each well and then the cells were incubated for an additional 2h. After precipitate formation, 100μL of Di-methyl Sulfoxide DMSO (Sigma) was added to each well and the plate was then incubated for 4h at 37°C. Absorbance of each well was determined at 540nm using microplate reader (BIORAD). Average was calculated for each triplicate sample and blank. Average blank value was substracted from the average of each sample and absorbance vs. cell number/mL was plotted to establish the reference curve.

Using the above determined information, CD133+ cells were seeded at 1,500 cells per well on a 96-well plate and analyzed daily over a 7-day incubation period in complete neural proliferation medium. The protocol used for MTT analysis was similar to that used to establish the reference curve. Plates were maintained in 5% CO_2 at 37°C incubation prior to MTT analysis. Medium changes were conducted daily in order to maintain a constant cell proliferation. The experiment was replicated in order to verify the results.

2.9 In vitro proliferation

The sorted CD133+ve and CD133-ve cells from (20 wk gestation, SVZ) were cultured in serum free medium as suspension. In brief, the cells were washed with DMEM-F12 (Invitrogen) and resuspended in complete neural proliferation medium (Stem cell technologies, Japan) containing 20μg/mL epidermal growth factor (EGF; PeproTech), and 20μg/mL basic fibroblast growth factor (bFGF; PeproTech). Cells were plated at 5,000cells/200μL, 10,000 cells/200μL, 25,000 cells/200μL, and 50,000 cells/200μL per well in six well culture plates. Cultures were fed every 3rd day with 50% fresh complete neural proliferation medium. Neurospheres were maintained for 21 days in suspension and cultured for 4 passages. The number of neurospheres generated per well were counted respectively after 14 days of each passage.

2.10 Neurosphere growth rate calculation

During the second passage at 14^{th} day new spheres (>0.35mm diameter) were chopped into quarters as described previously (Svendsen, et al. 1998) and seeded at 1 quarter/200μL in complete neural proliferation medium per well in 96 well plates. The growth rate of each quarter was measured horizontally and vertically every day. The volume of spheres was calculated from $V=4/3\pi(xy/2)^{3/2}$, where x and y are the horizontal and vertical axis. These neurosphere quarters were allowed to grow for 10days.

2.11 Lineage differentiation

For the differentiation assay, neurospheres (after 14 days) were dissociated and plated on human forecast skin fibroblast (HFF; Invitrogen) coated coverslips in two different culture conditions: 1) medium supplemented with 1% fetal calf serum (FCS; Sigma) 2) medium supplemented with Retinoic acid (1μm in 0.01% dimethyl sulfoxide, Sigma). The cells were cultured for 15 days and stained with antibodies against neuronal (β-tubulin III-FITC, Millipore), astrocytic (GFAP-PE, R&D System), and oligodendrocytic (O4-PE, R&D System) specific markers. The percentage of differentiated lineage cells was compared in both the culture conditions.

2.12 Statistical data analysis

All data were expressed as mean ± SEM. An independent-sample T test (SPSS 10.0; SPSS Inc., USA) was used to compare the purity and viability of CD133+ve cells before and after MACS. The Microsoft Excel was used to generate the graphical representation of CD133+ve cells quantitative yield, viability, proliferation and differentiation potential. $P<0.05$ was considered to be significant.

3 Results

3.1 Isolation of stem cells from human fetal brain

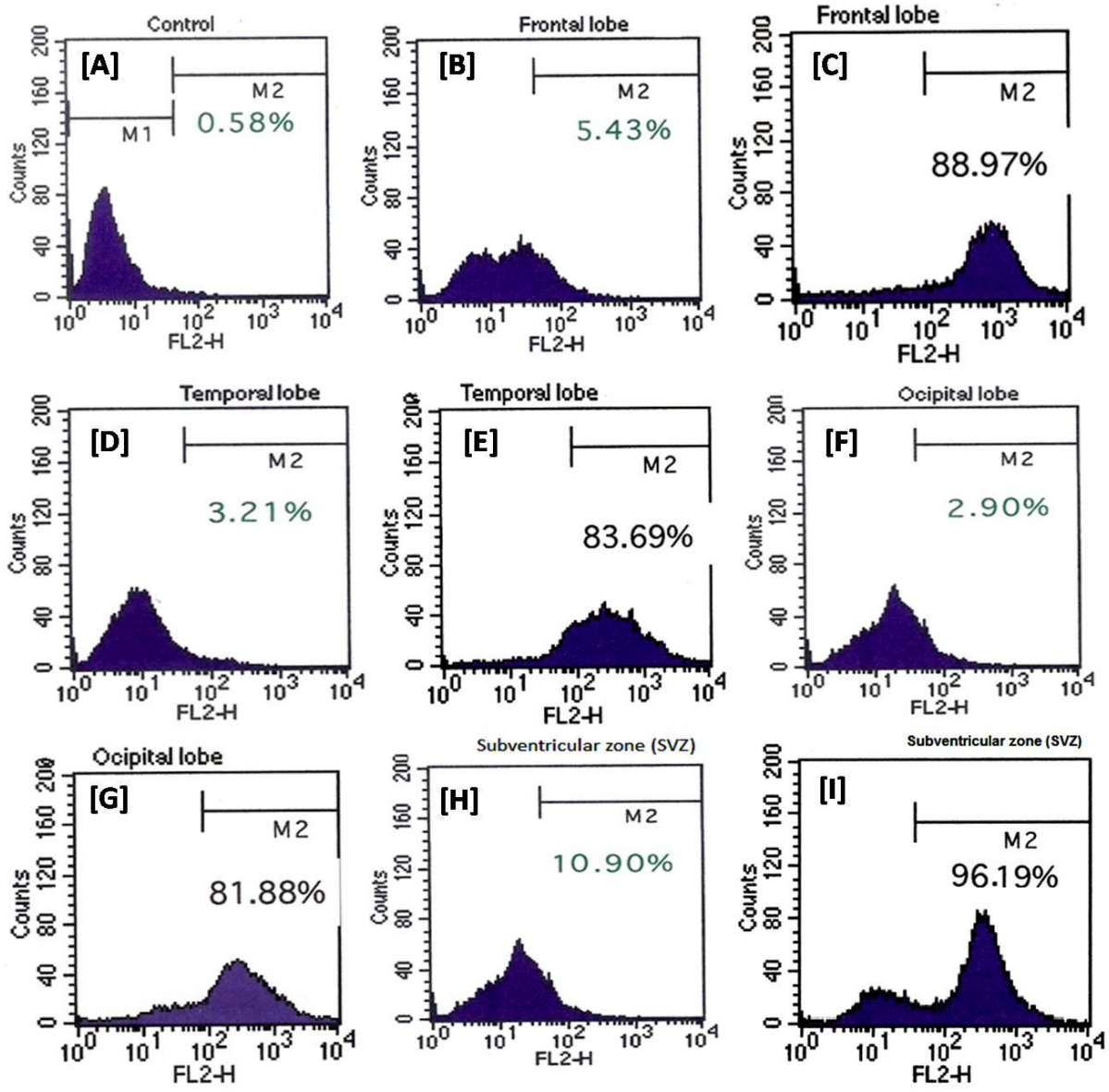
Tissue of frontal lobe, temporal lobe, occipetal lobe and SVZ was dissected from human fetal brain (gestation 18-22wk). Single cell suspension was prepared by mechanical and then collagenase dissociation that was further subjected to immunophenotypic analysis of stem cells using CD133-PE. Data from flow-cytometry revealed that CD133 expression was highest (approximately 7-10%) in SVZ cells derived from 18-20 wk gestation aged cells from different parts of the human fetal brain; whereas, percentage of CD133+ve cells before MACS (10.90%) and

after MACS (96.19%) was highest in 20 gestation week human fetal SVZ cells (Fig.1, H-I). The percentage of CD133+ve cells from frontal, occipital and temporal lobes before enrichment varied and found not as much of SVZ (Table 2).

Table 2 Percentage of CD133+ve cells before enrichment in 18-22wk gestation aged human fetal brain derived cells from frontal lobe, temporal lobe, occipital lobe and SVZ regions

S. No.	Brain tissue	Percentage of CD133+ve cells (%) before MACS				
		18wk	19wk	20wk	21wk	22wk
1.	Frontal lobe	4.87	5.12	5.43	5.10	4.22
2.	Temporal lobe	3.01	2.98	3.21	2.34	2.21
3.	Occipital lobe	2.80	2.16	2.90	2.10	1.94
4.	SVZ	8.16	9.14	10.90	8.21	7.90

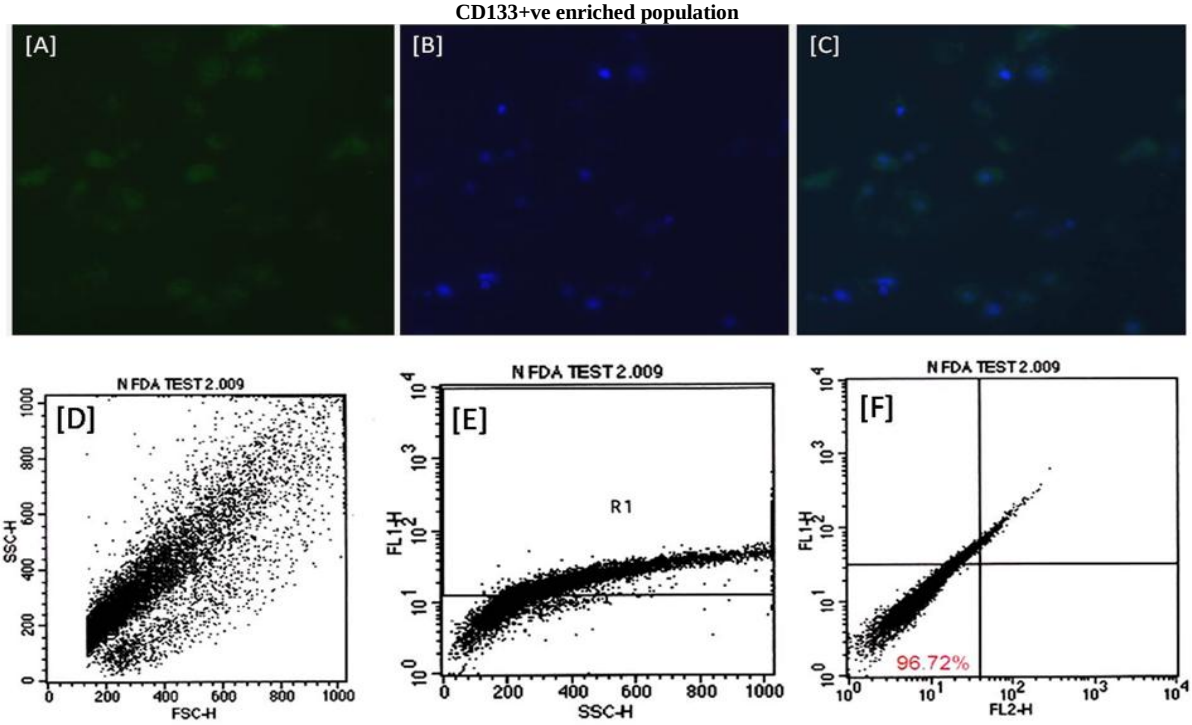
Fig. 1 Immunophenotypic analysis of CD133+ve cells before and after MACS in 20 wk gestation aged human fetal brain derived cells. Histographs representing the percentage of CD133+ve cells before and after MACS [A] control [B-C] frontal lobe (before MACS 5.43% and after MACS 88.97%) [D-E] temporal lobe (before MACS 3.21% and after MACS 83.69%) [F-G] occipital lobe (before MACS 2.90% and after MACS 81.88%) [H-I] SVZ (before MACS 10.90% and after MACS 96.19%)



3.2 Cell viability assays

Trypan blue exclusion assay showed no significant change in cell viability ($p < 0.05$) before and after MACS in 20wk gestation aged SVZ tissue (data not shown). Further analysis using FDA immunocytochemical staining showed that most of the cells were viable before and after MACS retaining their intact membrane (Fig. 2A-2C); whereas, FACS analysis showed >96% cell were viable after MACS (Fig. 2D-2F). Taking together, these results suggested that by using MACS system, ICC and FACS represents that a large amount of relatively pure population of CD133+ve cells could be obtained without impairing the cell viability.

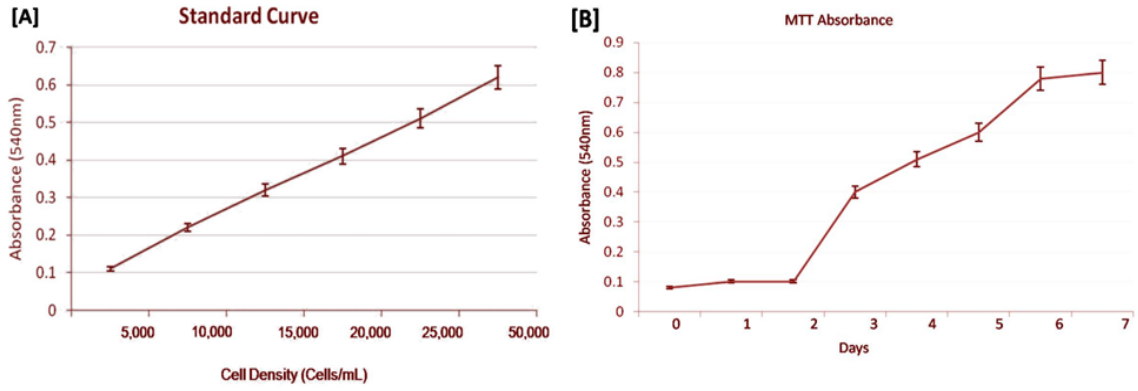
Fig. 2 Percentage of viable cells in SVZ derived cells after MACS [A-C] ICC staining with FDA showed mostly viable cells with intact membrane (Green) and cell nucleus (Blue) (Magnification: 10X) [D-F] Flow cytometric analysis showed 96.72% of viable cells in CD133+ve enriched population



3.3 MTT cell proliferation assay

Cell metabolic activity and proliferation was measured by MTT assay. The linear regression equation (R^2=0.99857) derived from the best-fit line shows a direct correlation between absorbance and the cell number. This linear relationship therefore was used to determine the proliferation rate of cultured cells. A high absorbance value corresponds to greater number of cells and an increased rate of proliferation. A steady increase in absorbance was observed after two days of initial culture (Fig. 3A-3B).

Fig. 3 MTT cell proliferation assay in CD133+ve enriched NPCs [A] Standard curve in relation to cell density and absorbance after 24h [B] A steady increase in metabolic activity was observed after 2 days of initial culture in corresponds to the absorbance at 540nm till 7 days

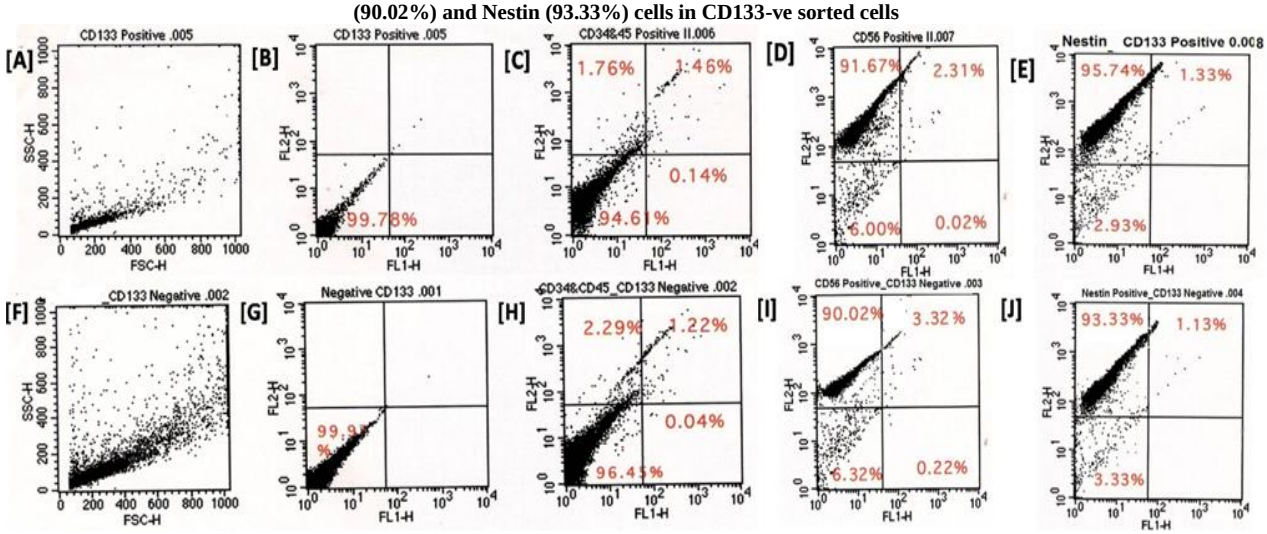


3.4 Characterization of NPCs in CD133+ve and CD133-ve sorted cells

3.4.1 Immunophenotyping

To further confirm the expression of hematopoietic lineage cells and NPCs in 20wk gestation aged SVZ derived cells, CD133+ve and CD133-ve sorted cells were stained with specific markers CD34 and CD45 (hematopoietic lineage markers), CD56 (NCAM-Neural cell adhesion molecule) and nestin-PE (neural precursor cell specific marker). Immunophenotypic analysis of stained cells showed very less percentage of CD34 (1.76%) and CD45 (1.46%) in CD133+ve enriched cells (Fig. 4C) and no significant difference was observed when compared to CD34 (2.29%) and CD45 (1.22%) percentage in CD133-ve cells (Fig. 4H). Expression pattern for NPCs specific markers CD56 and Nestin showed high percentage in CD133+ve and CD133-ve cell fractions both. In CD133+ve cells CD56 expression was measured as 91.67% (Fig. 4D) whereas in CD133-ve sorted cells it was slightly less 90.02% (Fig. 4I). Most commonly used marker for the identification of NPCs, Nestin when analyzed in CD133+ve enriched cells was found 95.74% cells expressing the nestin (Fig. 4E) whereas 93.33% of cells were found positive for nestin in CD133-ve sorted cells (Fig. 4J).

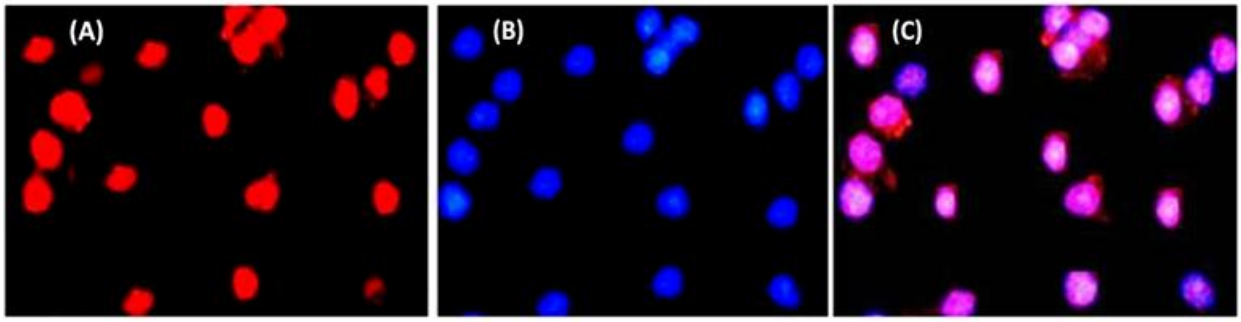
Fig. 4 Immunophenotyping of CD133+ve and CD133-ve sorted 20wk gestation aged SVZ cells [A-E] percentage of CD34 (1.76%), CD45 (1.46%), CD56 (91.67%) and Nestin (95.74%) cells in CD133+ve enriched cells [F-J] percentage of CD34 (2.29%), CD45 (1.22%), CD56 (90.02%) and Nestin (93.33%) cells in CD133-ve sorted cells



3.4.2 Immunocytochemistry (ICC)

Immunocytochemical staining of CD133+ve enriched cells when stained with mouse anti-nestin-PE, almost all the cells showed the cytoplamic expression for nestin (Red). DAPI (Blue) was used as a counter stain to visualize the cell nuclei (Fig. 5).

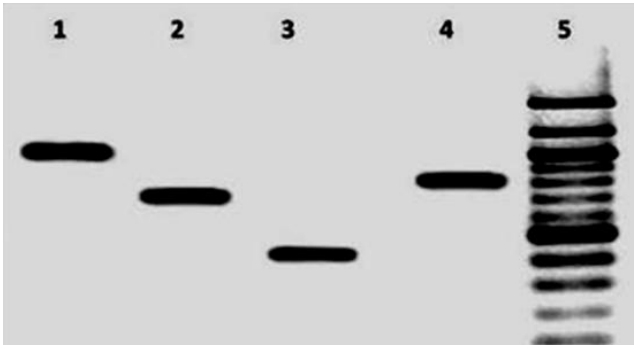
Fig. 5 ICC staining of CD133+ve cells using Nestin-PE secondary antibody to stain the intermediate VI filament expressed in cytoplasm of NPCs [A] Nestin staining of cytoplamic protein (Red) [B] DAPI staining of cell nuclei (Blue) [C] Merged ICC image showing the morphology and location of nestin protein (Red) and cell nuclei (Blue). Magnification: 20X



3.4.3 RT-PCR analysis

RT-PCR analysis of CD133+ve cells showed positive expression for pluripotent genes Nestin, Sox-2, Oct-4, and Notch-2 representing the presence of NPCs (Fig. 6). Further, sequencing and Blast results for widely used NPCs specific markers Nestin, Sox-2 and Oct-4 confirmed the sequence similarity with the NCBI human nucleotide database (data not shown).

Fig. 6 RT-PCR analyses of CD133+ve cells derived from 20 wk gestation aged SVZ of human fetal brain. Lane: 1 Shows expression of Nestin (403bp), Lane: 2 Shows expression of Sox-2 (317bp), Lane: 3 Shows expression of Oct-4 (173bp), Lane: 4 Shows expression of Notch-2 (400bp), Lane: 5 50bp DNA Ladder

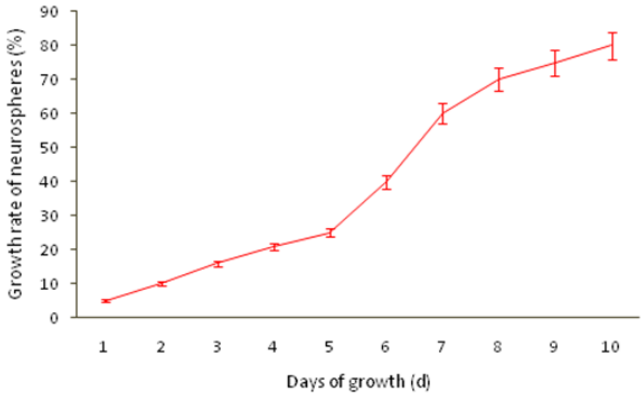


3.5 In vitro proliferation

3.5.1 Neurosphere growth rate analysis

During second passage growth curve of expanded CD133+ve cells as neurospheres after a period of relatively slow growth, the cells quickly expanded and volume of the spheres increased significantly (Fig. 7). Each neurosphere quarter was able to grow for 8-10 days and developed into mature neurosphere (~100µm in diameter).

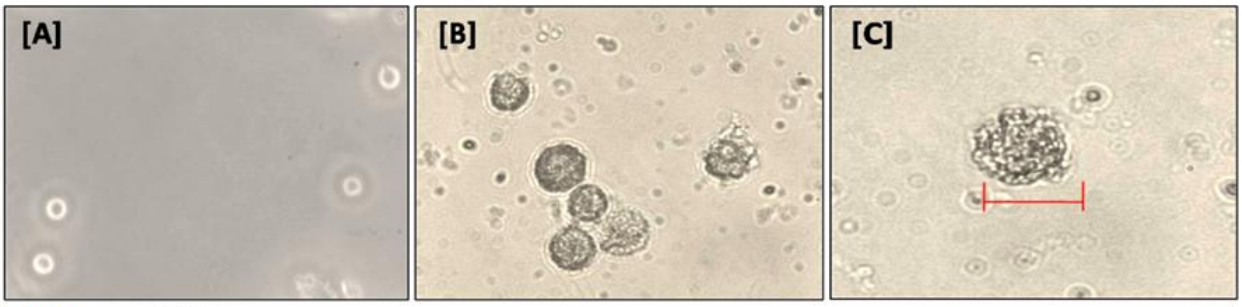
Fig. 7 *In vitro* growth curve analysis of human CD133+ve cells. After a period of slow growth, the cells quickly expanded and volume of neurospheres increased significantly (Displays error bars with 5% value)

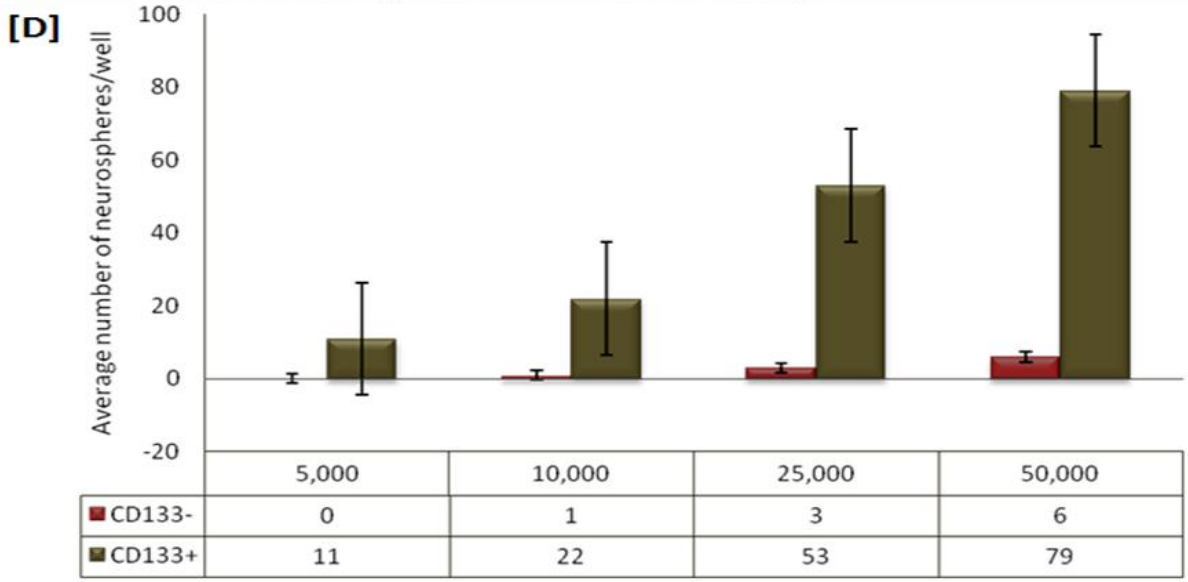


3.5.2 Difference in neurosphere forming capacity

Neural precursor cells could selectively proliferate to form heterogeneous mixture of cell aggregates called neurospheres in serum free culture system having the mitotic factors such as EGF and bFGF. To evaluate this characteristic, CD133+ve and CD133-ve sorted cells were compared in their neurosphere generation capacity. CD133-ve sorted cells developed only few neurospheres and most of the cells were separated (data not shown). At day 14 CD133+ve cells developed ~50µm diameter neurospheres that was further increased in size and observed ~100µm on day 21 (Fig. 8A-8C). CD133+ve cells generated more neurospheres as compared to the CD133-ve cells at different seeding densities (Fig. 8D).

Fig. 8 CD133+ve and CD133-ve sorted cells develop into spherical neurospheres in suspension culture. Phase contrast image of growing spheres at (A) Day 1 (B) Day 14 and (C) day 21(Magnification: 10X; Scale bar: 100mm) [D] Average number of neurospheres cultured at different densities under serum free conditions. Spheres were counted on day 14 and average was calculated from four individual samples (Error bars are represented in standard error)





	5,000	10,000	25,000	50,000
CD133-	0	1	3	6
CD133+	11	22	53	79

The CD133+ve sorted cells generated 31.25±1.65 spheres per well, whereas, CD133-ve sorted cells generated only 4.1±1.01 spheres per well at starting density of 5,000cells/mL. The neurosphere-forming rate was enhanced while increasing the plating cell density. However, striking difference was always present between CD133+ve and CD133-ve cells.

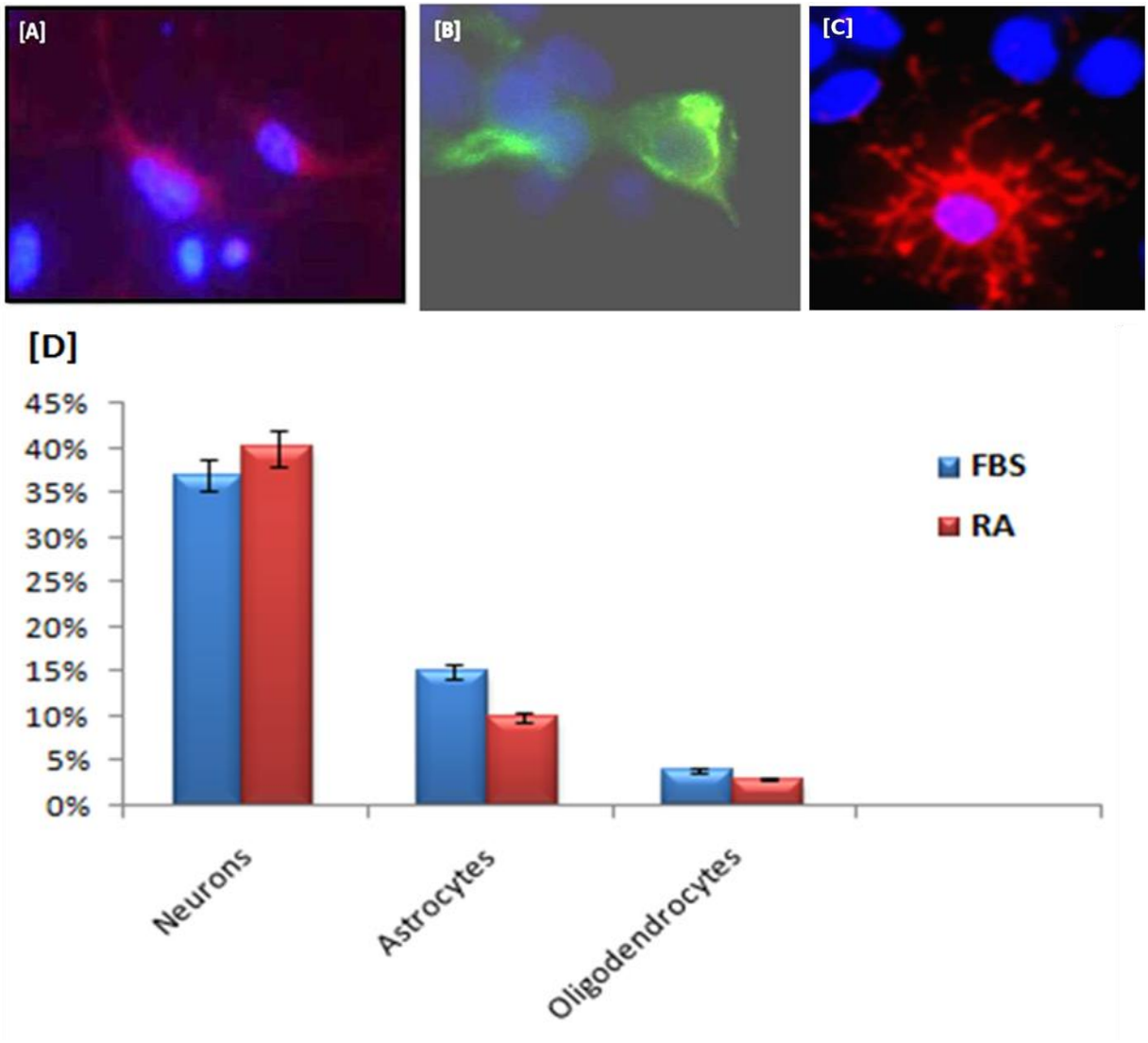
3.5.3 Self renewal capacity of CD133+ve cells

To examine the self renewal capacity of CD133+ve enriched cells, primary neurospheres after 14 days of initial culture were dissociated into single cell suspension and re-plated into complete neural proliferation medium. Small growing neurospheres were observed within 2 days. The number and volume of secondary neurospheres increased with the time. These secondary spheres were again dissociated and sub-cultured for two more passages and observed their neurosphere forming capability.

3.5.4 Differentiation potential of CD133+ve enriched NPCs

The differentiation capability of cultured CD133+ve cells was examined in FBS and RA induced medium using neuron and glia cell specific markers. After 3-4 days of plating cells were flattened and started to differentiate into their respective lineages. After 15 days of palting on adhesive substrate cells were stained with lineage specific markers. The results showed that the differentiation system contained all of three neuronal lineage cells (Fig. 9A-9C) indicating that neurospheres derived from CD133+ve cells were multipotent. Further analysis in two different culture conditions showed different percentage of lineage cells (Fig. 9D).

Fig. 9 Immunocytochemical staining of differentiated neural precursor cells (NPCs) by inverted fluorescent microscopy (Magnification: 100X) [A] Neurons were positive immunostained with β III-tubulin-PE [B] Astrocytes were positive immunostained for GFAP-FITC and [C] Oligodendrocytes were positive immunostained for O4-PE [D] Average percentage of cell phenotypes differentiated from neurospheres developed by CD133+ve enriched cells in 1% FBS and RA induced culture medium (Error bars are represented with standard error)



4 Discussion

Large numbers of cells are needed to study the biological characteristics of neural precursor cells. As the source is limited and getting the large number of homogeneous precursor cells is quite difficult; identification of enriched source and *in vitro* expansion of NPCs provides an abundant supply to evaluate the *in vitro/in vivo* behavior of such rare cell population. However, after long-term culture under mitotic stimulation *in vitro* expanded cells may become phenotypically and genotypically unrepresentative of their founder cells but contains large potential for further assessment.

Here we report that CD133 can be used to isolate utmost number of NPCs from different niches of human fetal brain of 18-22wk gestation age. In gestation wk 20 highest percentages of CD133+ve cells were observed as compared to 18-22wk old human fetal SVZ and other regions before and after enrichment. The self-renewal and lineage differentiation potential of these NPCs were also employed to characterize the CD133+ve and CD133-ve cells. CD133+ve cells developed more number of neurospheres as compared to CD133-ve cells and maintained the more capacity to generate secondary neurospheres having more *in vitro* proliferative and self renewal capacity.

Immunophenotypic analysis of CD133+ve and CD133-ve cells using CD56, represented that they may also be used in combination with Nestin to identify NPCs from various sources as they showed high degree of expression along with Nestin. As very few cells showed expression for CD34 and CD45 markers they may be considered as negative markers for NPCs.

On the other viewpoint, we showed that SVZ derived CD133+ve sorted cells can differentiate into neurons, astrocytes and oligodendrocytes *in vitro*, showing their multi-potential differentiation. Further analysisof enriched

CD133+ve cells using Nestin ICC staining, Immunophenotyping and molecular analysis showed that they are the true candidate of immature NPCs.

Variable differentiation potential of these cells has been observed in different culture conditions. We compared the differentiation potential of NPCs in two different culture conditions in 1% FBS and retinoic acid. The highest percentage of neurons were generated in retinoic acid induced medium whereas, highest percentage of astrocytes were formed in FBS induced medium and no significant difference was observed for oligodendrocyte development in any of the induction medium. This particular observation proved the high differentiation potential for CD133+ve cells derived from 20wk gestation aged human fetal SVZ.

RT-PCR analysis of CD133+ve cells showed the positive expression for NPCs specific markers that defines typical molecular characteristics for the presence of NPCs population in enriched cells. Nucleotide sequencing results and nucleotide Blast analysis showed similarity to human NPCs specific markers; Nestin, Sox-2 and Oct-4 which provide more convenient and considerable identity for the presence of NPCs in CD133+ve cells isolated from SVZ of human fetal brain.

5 Conclusion

In summary, the present study demonstrated that in 18-22 wk gestation aged human fetaus's, 20 wk gestation aged SVZ serves as the most abundant source for the isolation of NPCs. CD133 defines the selection of desirable and abundant source of stem cells having high percentage of NPCs committed to neurogenic differentiation pathway which may serve as better marker for the isolation and characterization of immature cell phenotypes. Not only CD133+ve enriched cells but CD133-ve cells also contains NPCs that may be identified using a cocktail of markers CD56+ve/Nestin+ve/CD34-ve/CD45-ve which will represent a fast, effective and more appropriate combination to enrich the highest number of NPCs isolated from different sources. RT-PCR, sequencing and Blast results confirmed the close sequence similarity and high degree of identity for human NPCs specific markers Nestin, Sox-2 and Oct-4. *In vitro* proliferation capacity of CD133+ve cells showed highest self-renewal ability and lineage differentiation capability to generate neurons, astrocytes and oligodendrocytes as comparted to CD1333-ve cells. In conclusion, the present study provides SVZ as the highest reminent of NPCs as compared to Frontal, temporal and occipital lobes to further assess the *in vitro* biology of NPCs. Identification of suitable combination of markers will facilitate further enrichment of NPCs from various sources and provide putative clinical applications of NPCs.

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