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RESEARCH ARTICLE

ESTABLISHMENT AND CHARACTERIZATION OF A NEURONAL CELL LINE DERIVED FROM A 2-CELL STAGE HUMAN EMBRYO: CLINICALLY TESTED CELL-BASED THERAPY FOR NEUROLOGICAL DISORDERS

Geeta Shroff

Director, Nutech Mediworld, H-8 Green Park Extension, 110016, New Delhi

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ABSTRACT

Regeneration of damaged neurons in neurodegenerative disorders like cerebral palsy and spinal cord injury is a major challenge. The possibility of replacing the non-functional neurons remains an attractive but an unflinching approach. We describes a novel human embryonic cell (hESC) line that has been developed in animal-free conditions during derivation and long-term culture. The absence of any xeno-product makes it suitable for clinical cell therapy. The cell line has been derived using a patented technology from a single, spare, throw-away fertilized ovum 24 to 48 hr after fertilization donated during a regular *in vitro* fertilization cycle. The cells thus obtained are very small cells (50 nm-2.5 µm) procured 24 hr after fertilization. They harbor all the properties of hESCs and blastocysts and express pluripotent stem cells markers like octamer-binding transcription factor 4, (sex determining region Y)-box 2, Nanog, stage-specific embryonic antigen-4, trophoectoderm marker; keratin 18, beta-human chorionic gonadotropin (negative), immune-regulatory marker; human leukocyte antigen G (negative), gene activating marker 5-methylcytosin, and other markers like telomerase and α fetoprotein. We have also explored the differentiation of neuronal cells by determining the lineage specific neuronal marker, Neu N. This is the first report of culturing and maintaining hESCs obtained from 2-celled stage without providing any culture feeder layer embryo. Further, these cell lines have proven to be effective for the treatment of neuronal disorders.

INTRODUCTION

Regenerative medicine helps improve the quality of life of patients by utilizing body's own cells, tissue and organs to restore or establish lost functions(Kulbatski *et al*., 2005). The technology targets to harness the intrinsic power of the body to heal, and then accelerate this healing in a clinically relevant manner. The regenerative potential of stem cells in neurological disorders or injuries is being investigated in various therapeutical aspects(Kulbatski *et al*., 2005). Stem cell therapy has shown tremendous potential in treating diseases like diabetes, traumatic brain injury, stroke, spinal cord injury (SCI) Parkinson's disease, Alzheimer's disease, heart failure and bone marrow failure in the past(Willerth, 2011, Cao *et al*., 2013, Balakumaran *et al*., 2014, Dantuma *et al*., 2010). Adult stem cells like mesenchymal stem cells (MSCs) which can give rise to cartilage, bone and adipose cells (Prockop, 1997), hematopoietic stem cells in bone marrow that produce all type of blood cells(Worton *et al*., 1969), neural stem cells in the brain that give rise to glial cells and neuronal cells (Reynolds *et al*., 1992) and broncho-alveolar stem cells in the lungs that produce alveolar epithelial cells and bronchus epithelial cells have been often used to treat these conditions (Kim *et al*., 2005). Embryonic stem (ES) cells have been used in neurodegenerative diseases as a source of neuronal progenitors which could potentially secrete regeneration promoting factors or as a source of ES cells derived neurons and oligodendrocytes to replace the lost neurons(Willerth, 2011, Shroff *et al*., 2014, Shroff *et al*., 2014). Human embryonic stem cells (hESCs) which have a remarkable property of pluripotency, self renewal and differentiation can be a breakthrough technology aimed at this direction. Due to its unmatched potential, FDA has also given approval for the use of hESC based therapy for treatment of SCI(Geron, 2009).

There is another category of small-sized stem cells (0.1 µm -5 µm) known as blastomeric like stem cells (BLSCs) and very small embryonic like stem cells (VSELSCs) studied intensively by different scientific groups(Bhartiya *et al*., 2014, HE). These VSELSCs express pluripotency genes and differentiate into cell types from germ layers *in vitro;* however they lack *in vivo* evidence. A study reported that human VSELSCs differentiate in to adipocytes, neurons, osteoblasts and chondrocytes. No teratoma formation was observed (Havens *et al*., 2014).

In this paper, we report VSELSCs of pre-blastomeric origin derived from 2-celled stage. These cells were taken from a fertilized ovum (50 nm-2.5 µ in size) discarded in a regular *in vitro* fertilization (IVF) cycle, with full consent from the donor.

Corresponding author:* **Geeta Shroff
Director, Nutech Mediworld, H-8 Green Park Extension, 110016, New Delhi

The safety and efficacy of the cell line has been established(Shroff *et al*., 2015 b).We have used these cells to treat over 1300 patients with diverse ailments including diabetes, myocardial infarction, cerebral palsy, SCI, Lyme disease, Spinocerebellar Ataxia, Friedrich's Ataxia, Alzheimer's disease, Parkinson's disease, autism and cerebral palsy(Shroff *et al*., 2014, Shroff *et al*., 2014 , Shroff, 2015 d, Shroff, 2015 e, Shroff *et al*., 2015 a, Shroff, 2015 c). No teratoma formation has been observed till date.

Further, the paper describes culture technique which produces hESC line free from animal products, feeder layers, growth factors, leukemia inhibitory factor (LIF), supplementary mineral combinations, amino acid supplements, fibroblast growth factor, membrane associated steel factor, soluble steel factor and conditioned media (United States Granted Patent No US 8592, 208, 52). The present study also discusses the characterization of hESC line for its pluripotent nature, its differentiation into neuronal lineages and its use for the treatment of neurological disorders. We have characterized the hESC at molecular, cellular and functional level using scanning electron microscopy (SEM), transmission electron microscopy (TEM), confocal microscopy, reverse transcriptase polymerase chain reaction (RT machines-PCR) and flow cytometry analysis. The cell line has also been characterized based on long-term proliferation and maintenance, karyotyping and *in vivo* differentiation as teratoma formation assay. It has been chromosomally stable since the year 2000 and for >4000 passages. The study also provides a composition of injectable stem cells in ready to inject form that is simple to prepare, safe, cost effective, efficient, easily transportable, scalable and has a shelf life of six months.

MATERIAL AND METHODS

Ethics Statement

The work done was approved by an Independent Ethics Committee (IEC). The institutional committee for stem cell research and therapy of Nutech Mediworld, New Delhi, India reported the clinical study to National Apex Committee for Stem Cell Research and Therapy (NAC-SCRT). The study was conducted in accordance to the Declaration of Helsinki in a good clinical practices (GCP) compliant condition (Reynolds *et al*., 1992). A verbal, written and video consent was provided by the patient. The ethics committee approved this procedure of obtaining the consent.

Good Practices in Stem Cell Culture Laboratory

The cells are cultured and maintained as per our in-house patented technology (United States Granted Patent No US 8592, 208, 52) in a good manufacturing practice (GMP), good laboratory practice (GLP) and good tissue practice (GTP) compliant certified laboratory.

Origin of Cell Line

Directed neuronal cell line was obtained from a single, spare, expendable, pre-implantation stage fertilized ovum taken during natural IVF process with due consent.

Derivation and Culture of Expendable Cells

For derivation of cell line, a spare embryo obtained from IVF procedure was suspended in a small amount of minimal essential medium (RMPI with 2.2 g/L sodium bicarbonate) and the hESCs were isolated from the embryo by mechanical means (e.g. shaking). Additional medium (RPMI and Dulbecco's Modified Eagle's Medium [DMEM; Himedia Labs, Mumbai, India]) was added in separate flasks containing isolated cells along with a β-hCG agonist (16-64 µl of 500 IU/ml, Serum Institute of India, Pune, India) and progesterone (16-64 µl of 250 mg/ml, Sun Pharma, Mumbai, India) in aerobic conditions. After 24 hr, 1 mL of the media containing embryonic cells were transferred to a 40 mL cell culture medium (RPMI and DMEM) containing progesterone and β-hCG in a 50 mL container. The culture medium along with stem cells was incubated in a horizontal position at ambient temperature in an environment containing carbon- dioxide (CO_2). After 24 hr, the product was divided into two different flasks and RPMI and DMEM were added separately in a ratio of 1:3.5 to 1:35 volume by volume. The cells thus obtained were re-incubated at 37^{o}C in a water jacketed incubator with an atmosphere of 3.5-6% CO_2 to obtain the desired cell density. During incubation, 0.1 µAmp to 1 mAmp current was given to the cell culture for a short duration. The application of microcurrent is crucial for maintaining the cells in their undifferentiated and pluripotent stage. After reaching the cell density of 10^7 to 10^8 cells per cubic centimeter, the cells were separated with centrifugation. The detailed cell culture and differentiation techniques have also been elaborated in our patent document (detailed compositions comprising human embryonic stem cells and their derivatives, methods of use, and methods of preparation are available at http://patentscope.wipo.int/search/en/WO2007141657). The cells were then divided in to three aliquots- one aliquot was reincubated in anaerobic condition with either DMEM or RPMI; second aliquot was stored at freezing temperature and the third aliquot was made ready to injection (RTI). The cells were stored in a deep freezer after adding dimethyl sulphoxide (DMSO, 0.2-2% w/v) to the culture medium. The level of DMSO used is typically less than 1:1600. DMSO is used for cryopreservation to avoid inducing differentiation or damage to the cells.

Sample collection for Marker Study

The RTI was obtained after centrifuging the cells for 5 min at 1000 rpm and the pellet was suspended in normal saline (Nirlife, Nirma Ltd. Ahmedabad, India). The frozen syringes of cells were thawed by placing the syringes in between palms of the hands till they attain the body temperature prior to marker study. Characterization of expression of different stem cell markers was done with different approaches including flow cytometry, RT-PCR and immunoflourescence.

Sterility tests

A quality check was performed on the stored cell batches and the cultured cells taken from GLP compliant laboratory which included integrity, viability and microbial contamination. The tests included those for HIV, HbsAg, conventional PCR for

Koch's test, chromosomal analysis by CG method of Giemsa banding. The culture condition was tested by the manual culture plate method and manual sensitivity. Identification with versatrek/AP1 was carried out for fungal infection.

Microscopy

Electron microscopy

Scanning Electron Microscopy

Scanning electron microscopy was done to obtain information about sample's surface topology and composition which was not possible in normal microscopic observation.

For SEM analysis, tissue was fixed in ½ Karnovsky's Fixative and OsO_4. Following the fixation, the tissue was washed in 0.1M Cacodylate buffer. Then, the dehydrated tissue was washed using a series of ethanol washes: 50% ethanol for 15 – 30 min, 70% ethanol for 15 – 30 min, 95% ethanol for 15 – 30 min, 100% ethanol for 15 – 30 min and finally, 100% ethanol for 15 – 30 min. The tissue was immersed in pure hexamethyldisilazane (HMDS) for 15 min, washed and again immersed in pure HMDS. Afterwards, the sample was covered with fresh HMDS and left under the hood until the HMDS evaporates off. Specimens were ready to mount onto stubs and sputter coated with Au/Pd in the morning or stored desiccated until coated.

Transmission Electron Microscope

A transmission electron microscope is an analytical tool allowing visualization and analysis of specimens in the order of mm to nm range, which is thousands of times smaller than the smallest resolvable object in a light microscope. For TEM analysis, hESCs were centrifuged and suspended in phosphate buffer saline (PBS; pH 7.3). The cells were fixed in 4% glutaraldehyde for 2 hr and stored at 4º C for further use. Afterwards, 200 µl of the sample was put onto formvar-coated copper grids and allowed to settle for 20 min at room temperature (RT). Excess PBS was removed by wicking with filter paper before fixation using a 2% paraformaldehyde, 2% glutaraldehyde, and 0.05 M phosphate solution for 2 min. Grids were washed with distilled water followed by counter staining with 1% phosphotungstic acid (PTA) for 1 min. Samples were allowed to dry overnight at RT. Grids were analyzed using a Technai G220 TEM (FEI, Hillsboro, OR) at Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University, New Delhi, India.

Phase Contrast Microscopy

Phase contrast microscopy helps view the transparent and non-light absorbing specimens. A specialized type of phase-contrast objective is added in the microscope. The light causes destructive interference pattern in the image, making the details in the image appear dark as opposed to the lighter background. Prior to harvesting of the cells, they were kept in presence of emecolcine for 2 hr. Subsequently, the cells were incubated at 37°C in 0.56% potassium chloride solution for 5 min. Then the cells were fixed at 4°C for 15 min with methanol-acetic acid fixative. This cell suspension was added onto semi-dry cold glass slides and kept for 1 hr at RT to dry. The cells were then stained with giemsa for 10 min before viewing neuronal cells using phase contrast microscopy.

RNA Extraction and RT-PCR

A total of 2 x 10^6 to 3 x 10^6 cells were taken for PCR analysis. Total RNA was isolated from hESCs using RNA easy mini kit as per manufacturer kit protocol (RNA easy kit, Qiagen Inc., Valencia, CA, USA Cat No. 74104). The concentration and purity of RNA was determined at 260/280 O.D. in a spectrophotometer. RNA was converted into cDNA using oligo (dT) primers with the help of SuperScript III Cells Direct cDNA Synthesis System (Invitrogen, Cat. No.18080-051). The cDNA synthesis was done by heating sample at 50ºC for 50 min followed by termination of reaction at 85ºC for 5 min. Afterwards, the reaction mixture was cooled at 4ºC for 5 min. Synthesized cDNA was treated with RNAse H for 30 min at 37ºC. PCR reaction was performed using cDNA as a template with Paq5000 DNA polymerase (Agilent technologies, Cat No. 600680). Human β-actin gene was used as a house keeping control. The amplified PCR products of specific genes using specific primers were analyzed by electrophoresis on 1% agarose gels.

Table 1 Primer Sequence, size and reaction conditions for PCR cycles

Genes	Primers	Sequence	Size	Annealing temperature
β-actin	Forward	ATCTGGCACCACACCTTCTACAATGAGCTGCG	800 bp	60ºC
	Reverse	CGTCATACTCCTGCTTGCTGATCCACATCTGC		
SSEA 4	Forward	TGGACGGGCACAACTTCATC	118 bp	58ºC
	Reverse	GGGCAGGTTCTTGGCACTCT		
Sox 2	Forward	CCCCCGGCGGCAATAGCA	48 bp	58ºC
	Reverse	TCGGCGCCGGGGAGATACAT		
Nestin	Forward	GAAACAGCCATAGAGGGCAAA	167 bp	58ºC
	Reverse	TGGTTTTCCAGAGTCTTCAGTGA		
Keratin 18	Forward	CACA GTCTGCTGAGGTTGGA	164 bp	58ºC
	Reverse	GAGCTGCTCCATCTGTAGGG		
Oct 4	Forward	CGACCATCTGCCGCTTTGAG	577 bp	58ºC
	Reverse	CCCCCTGTCCCCCATTCCTA		
Keratin 18	Forward	CACA GTCTGCTGAGGTTGGA	164 bp	58ºC
	Reverse	GAGCTGCTCCATCTGTAGGG		
β-HCG	Forward	ATGTGCAGGATTGCCCAGAA	510 bp	58ºC
	Reverse	CCCCCATTACTGTGACCCTG		
β-HLAG	Forward	ATCATACTGACCTGGCAGCG	158 bp	58ºC
	Reverse	CTGCACATGGCACGTGTATC		

For all reactions, 35 cycles of PCR were performed.

The primer sequence, annealing temperature, number of PCR cycles and fragment size are provided in Table 1.

Flow Cytometry

Expression of different intracellular molecules and surface markers were determined through fluorescence-activated cell sorting (FACS). Single cell suspension at a concentration of $1x10^6$ to 2 $x10^6$ was fixed in 4% paraformaldehyde for 20 min at RT. After washing of cells with saline, permeabilization of cells was done with 0.25% Triton X-100 for 20 min at RT. Cells were washed twice and blocked with 5% bovine serum albumin (BSA) (or 10% serum/Fc block receptors) for an hour at RT. Cells were washed again and labeled with primary antibodies Oct-3/4 (1:100, Santa Cruz), Nestin (1:100, Neuromics), stage-specific embryonic antigen-4 (SSEA-4; 1:150, Abcam), keratin-18 (Krt-18;1:100, Abcam), SRY-related HMG-box gene 2 (Sox2; 1/10, Abcam) and human leukocyte antigen G (HLA-G; 1:100, Santa Cruz). Washed cells were further labeled with secondary antibody- Alexa fluor©488 donkey anti-mouse IgG (Invitrogen Life technology, Cat No. R37114) or Alexa fluor©647 donkey anti-Mouse IgG (1:200) (Invitrogen Life technology, Cat No. A-31571) for 30 min at RT. Cells were sorted on BD AccuriC6 flow cytometer and analyzed with BD Accuri ™ C6 Software (BD, Biosciences).

Immunofluorescence/Confocal Microscopy

For immunostaining, cells at a concentration of 1 x 10^6 were adhered (or cytospin or smear preparation) on to a polylysine coated cover/ slip slide. After brief washing in PBS, samples were fixed in 4% paraformaldehyde (PFA) for 10-20 min at RT. The PFA-fixed cells were permeabilized in 0.25% Triton-X at RT for 20 min. PBS washed cells were blocked with 2% BSA and 5 % donkey serum for 2 hr. Cells were incubated with primary antibodies Oct-3/4(1:100, Santa Cruz), Nestin (1:100, Neuromics), SSEA-4 (1:150, Abcam), Krt-18 (1:100, Abcam) for an hour at 37°C.

Washed cells were further labeled with secondary antibody-Alexa fluor©488 donkey anti-mouse IgG (Invitrogen, Life technology, Cat No. R37114) or Alexa fluor©647 donkey anti-Mouse IgG (1:200) (Invitrogen, Life technology, Cat No. A-31571) for 30 min at 37°C. After staining with secondary antibody, cells were counterstained with 0.1-1 µg/ml 4', 6-diamidino-2-phenylindole (DAPI) for 5 min. The immunostained cells were mounted in ProlongR Gold antifade (Life technologies, OR, USA) and images were evaluated first under immunofluorescence microscope and then confocal microscope.

RESULTS

Integrity, viability and microbial contamination

The viability testing of the cells is done by staining with tryphan blue and propidium iodide and viewing under FACS. Subsequent to the viability testing, these cells are frozen that helps in maintaining the shelf life of these cells.

Scanning electron microscopy

SEM shows the clear morphology of hESCs (**Figure 1)**. The cell surfaces seem to be embossed and few cells exhibited bulging relief.

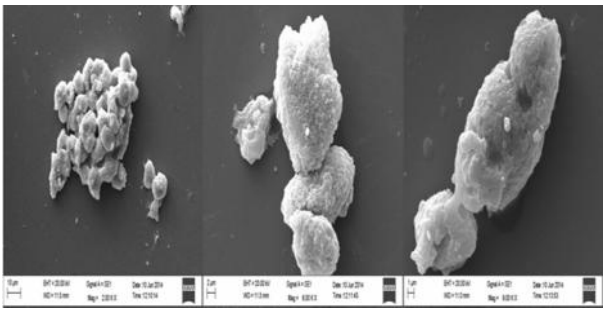

Figure 1 Determination of Size and Surface Morphology of Cells by SEM (100000x)

Transmission electron microscope

Figure 2 is the image obtained through TEM for the cells. Multi-layered colonies were observed. Numbers of cell layers were highest in the center and flattened towards the periphery. Small projections from the surface of the cells were also observed.

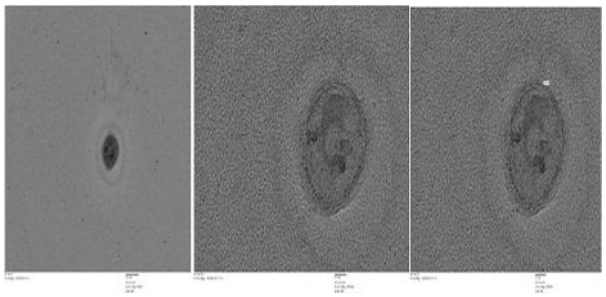

Figure 2 Determination of Size and Surface Morphology of Cells by TEM (500000x)

Phase Contrast Microscopy

Phase contrast images of neuronal cells were obtained at 10 x and 20 x. The images presented clearly show a neuron with a nucleus. The image also shows the presence of a cellular extension from the cell body referred to as axon. (Figure 3)

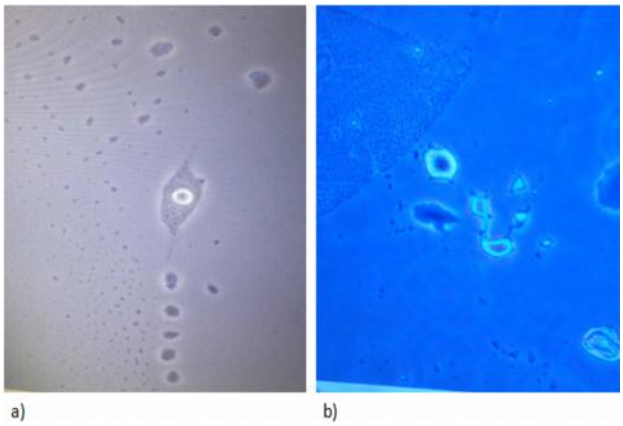

Figure 3 Image of the Neuron Obtained With Phase-Contrast Microscopy

Gene Expression Profile of Different Intracellular and Surface Markers of hESCs Determined by RT-PCR

Distinction of different cell population can be done by morphological and developmental criteria along with temporal and spatial expression of marker gene (Noisa *et al*., 2012). To determine the human origin of cells, we amplified human β-actin gene from cDNA prepared from RNA of hESC. Amplicon generated by RT-PCR (Figure 4) confirmed the presence of human β-actin gene in this very small population of cells and supported its human origin. After confirming their origin, we checked the expression of various pluripotent stem cell markers in hESCs including, OCT4, NANOG, Sox2, SSEA-4, trophoectodermal marker Krt18, α-fetoprotein, neuronal lineage specific markers like nestin (neuronal progenital cells, NPCs) and NeuN (neuronal marker in differentiated cells) and other important markers including GFAP (astrocytes and glial marker), HLA-G (-ve) (a major histocompatible factor), 5-methyl cytosine(-ve) (gene activation marker), telomerase(+ve) (maintenance of genomic integrity and pluripotency of stem cells) and β- human chorionic gonadotropin (β-hCG) (immune modulator) by RT-PCR. We found the amplification of these markers which indicates the presence of all these genes in hESCs at mRNA level (Figure 4).

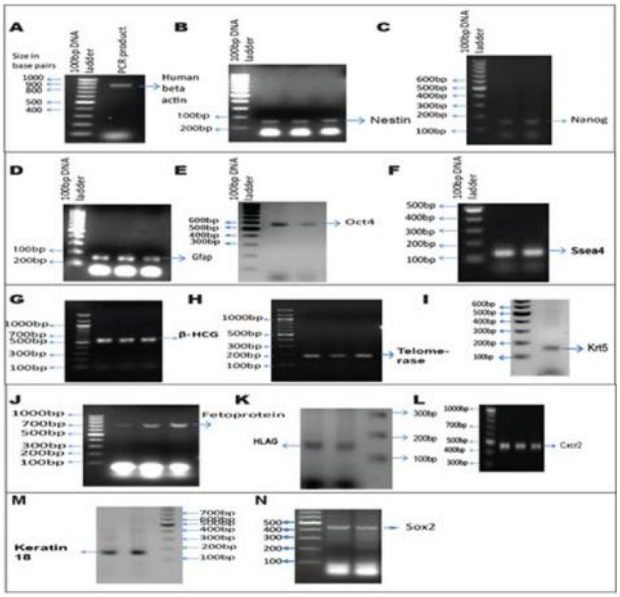

Figure 4 Determination of Expression of Different Pluripotent Markers in hESC by RT-PCR

Expression Profile of Different Intracellular and Surface Markers of hESCs Determined by Confocal Microscopy:

To characterize the pluripotent properties of hESCs, the hESCs were stained with markers specific antibodies namely Oct4, Sox2 and SSEA-4. The results showed labeling of transcription factors; Oct4, Sox2 intracellular marker, Nestin, surface marker; SSEA-4 and neuronal specific markers; Nestin and-NeuN (Figure 5). Since cells are <2 µm, 100 x magnification gave clear discrete images. These cells mostly appeared in clumps because of their inherent property of clumping. They appeared as small chunks of green and red stain in suspension after staining. This pattern of staining in clumps corresponds to immunofluorescence studies in many published articles of hESCs (Lowell *et al*., 2006, Yu *et al*., 2008). To further clarify that the staining is specific to cells, colocalized images were obtained with DAPI and DIC (Figure 5). The staining of HLA-G was found to be negative. The negative control did not show any staining and appeared as a black image. These results indicated the pluripotent nature of hESCs and lineage specific differentiation into neuronal cells without any immune response. The result showed label of transposition factor; Oct4, neuronal marker; Nestin and surface marker; SSEA-4. Culture also showed staining with Neu N which indicated the presence of neuronal differentiating cell.

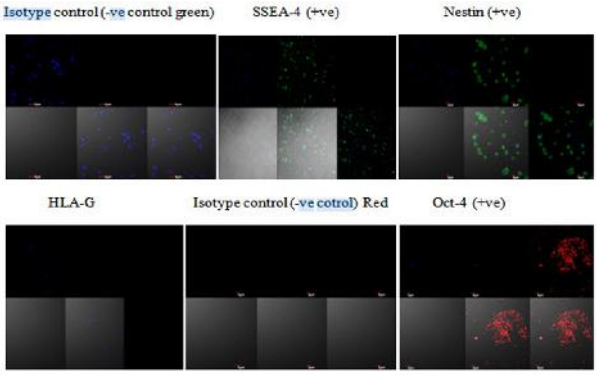

Figure 5 Determination of Expression of Different Pluripotent Markers in hESC by Confocal Microscope (100x)

Cell Size *versus* Cell Debris

The cell size of our hESCs is variable and small (< 2 µm). The minimum detectable particle size for BD Accuri C6 Flow cytometry is 0.5 µm which acts as a limitation for the analysis of the whole population of these cells. Threshold was used to remove the debris and noise. In flow cytometry, the cells having a cell size range from 0.5-2 µm have been read.

Expression Profile of Different Intracellular and Surface Markers of hESCs Determined by Flow Cytometry

Undifferentiated hESCs express ES cell specific markers similar to the cells in the inner cell mass (ICM). To determine the expression of different progenitor markers in our hESCs, flow cytometry was performed. The culture contains cluster of many progenitor cells such as hematopoietic stem cell progenitors, neuronal stem cell progenitors, MSC progenitors, insulin producing stem cell progenitors, hepatocyte stem cell progenitors, cardiac stem cell progenitors and epithelial stem cell progenitors which can be seen by the presence of respective progenitor markers. We focused our study on identification of different pluripotency transcription markers like Oct4, Sox2 and embryonic cell specific surface antigen; including stage specific embryonic antigens like SSEA-4 and Tra-1-81. The results showed that these hESCs express high level of embryonic cell specific surface antigens SSEA-4 (Figure 6) but relatively no or very low expression of TRA-1-81 (Figure 6) therefore, these hESCs can be proposed to be the initial Tra-1-81(-) and SSEA-4(+) population representing the cells of early neural differentiation. Another study has shown Tra-1-81(-) and SSEA-4(+) cell population at early stage neural differentiation of hESCs which further strengthened our findings (Noisa *et al*., 2012). The expression of pluripotent marker gene, Oct4 seems to be downregulated as not found in

FACS analysis (Figure 6). However, Sox2, another known pluripotent marker, was consistently expressed but the expression remained low (Figure 6). These hESCs expressed neuronal progenitor nestin but the expression and number of cells positivity for nestin remained low (Figure 6). Trophodermal marker krt-18 was expressed by a small population and the level of expression per cell remained high. We also found the negative staining of cells with immunomodulatory marker, HLA-G. These results show that our hESCs are capable of generating both neural and non-neural lineage cell types.

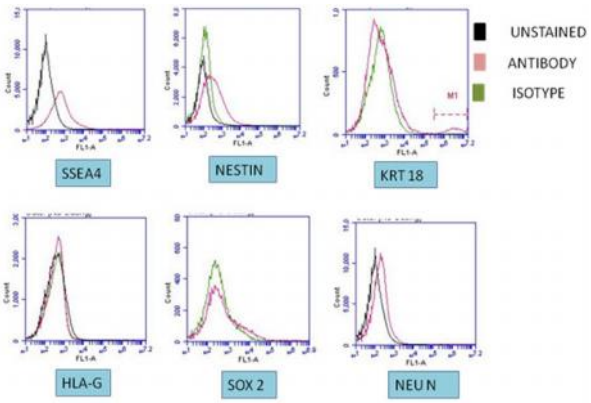

Figure 6 Determination of Expression of Different Pluripotent Markers in hESC by Flow Cytometry

DISCUSSION

The fertilization of mammalian oocyte by sperm takes place in the ampulla of the oviduct(Coy *et al*., 2012). Post fertilization, the embryo undergoes a number of events such as fusion of paternal and maternal pronuclei, global demethylation, cleavage of cells, embryonic genome activation (EGA) and epigenetic reprogramming(Morgan *et al*., 2005). As the pronuclei fuse, maternal proteins inherited from oocyte cytoplasm causes the first cleavage of the cell to produce a 2-cell stage. The cleavage of the embryo neither occurs exponentially from 2-cell to 4-cell to 8-cell at regular time intervals nor is monitored by time, but by the maternal genes and activation of genome of the embryo as the cleavage progresses. By day 3, (72 hrs) embryo reaches to 16-cell morula stage which marks a major event of compaction wherein cells closely arrange themselves within zona pallucida, associate tightly and establish tight junctions with each other(Trounson *et al*., 1982). This event is considered as the first step towards differentiation. Before this event, each cell in an embryo is considered totipotent which is capable of producing a whole organism (Mitalipov *et al*., 2009). By the morula stage, major epigenetic modifications and EGA has already taken place(Chavez *et al*., 2014). Blastomeric cells derived from ICM of blastocyst are pluripotent in nature and are termed as hESCs, and maintain their stemness through an intricate array of regulatory proteins and surface markers like Oct4, Sox2, Nanog and surface markers SSEA-4 and TRA1-81 also serve as markers of pluripotency(Cauffman *et al*., 2009, Rizzino, 2009). Maintaining an undifferentiated state during *in vitro* culture and differentiating into all kinds of cells in human body has opened the opportunity to use hESCs in clinical transplantation. However, the use of hESCs in clinical applications has limitations of its distinction from the other cell types, particularly tumor cells, to avoid potential risk of teratoma formation.

Although, hESCs have been cultured in well-defined populations *in vitro* but majority of hESCs methodology of derivation and/or propagation includes indirect or direct exposure to the animal material. Transplanting of such contaminated hESCs to patients will be associated with risks of graft rejection and transfer of non-human pathogens which is inappropriate for clinical use. However, a wide group of researchers are working to establish a xeno-free system but have failed to fully eliminate the animal products. Fewer studies have further progressed by maintaining and expanding undifferentiated hESC on human feeder free layers(Klimanskaya I, 2005, Ludwig TE, 2006, Xu C, 2001 , Richards *et al*., 2002, Richards *et al*., 2003, Amit *et al*., 2003, Bergström *et al*., 2011). Till date, majority of the methods adopted for culturing hESCs have resulted in a loss of genomic integrity as evidenced by numerous conventional karyotyping studies, SNPs and CGHs arrays(Josephson *et al*., 2006 , Catalina *et al*., 2009). hESCs are mostly derived from ICM of blastocyst embryos(Thomson *et al*., 1998) but attempts have been made to derive these cells from single blastomeres(Klimanskaya *et al*., 2006, Geens *et al*., 2009) and 8-celled stage morula embryos(Strelchenko *et al*., 2004). Till date, no stable cell line has been derived from 2-cell stage of embryos. Though attempts have been made but most of the cell lines which are derived from pre-implantation embryos have been derived from 5 to 8-cell stage embryos. The usual cell lines are derived from a blastocyst which is 64 cells to 128 cells. The hESCs cultured at our facility hence provide a novel cell line. It has been derived from 2-cell stage embryo, post pronuclear fertilization and first cell division, grown in a culture medium that does not contain supplements such as basic fibroblast growth factor, leukemia inhibitory factor, membrane associated steel factor, soluble steel factor, serum, albumins or albumin supplements, amino acid supplements, vitamin supplements, transferrins or transferring supplements, antioxidants, insulin or insulin substitutes, collagen precursors or collagen precursor substitutes, trace elements, residues or "conditioned media", animal products, feeder cells and growth factors. During its derivation from 2-cell stage embryo, the cells were found fragmented and on mechanical shaking the cells along with small fragments dissociate in the media. It has been hypothesized that some fragments retained some part of nuclear DNA and are capable of growing in culture. Fragments (with fragmentation > 35%) if transplanted are reported to grow into a baby during IVF . But some fragments without DNA have also been detected by negative DAPI, thiozole orange or propidium iodide staining. Also, TEM images showcase some fragments with no nucleus. These fragments have been considered vesicles due to their small size and lack of nucleus. Detailed study of these fragments and their growth characteristics is beyond the scope of this paper. The dissociated blastomers and fragments were cultured on media. This day is called as Day "0" of hESC cell line. This cell line was further differentiated and the cells being used for this paper have been grown till day 2 of cell line. Analysis of further days of cell line has shown the presence of more differentiated markers.

In this study, we have characterized very small cells (even less than 1-2µm) that are human in origin and behave as stem cells. The advantage of using these cells for therapeutic purpose is their size and very high multiplication rate. Due to their very small size, these cells are able to cross the barriers of the body and thus access the sites of malfunction easily. With a few exceptions, these cells behaved like previously reported blastocyst derived like VSEL's(HE).

Human origin of our cell line has been confirmed by the amplification of human β-actin gene. In this study, we found the expression of Oct4, Sox2, NANOG and SSEA-4 which are pluripotent embryonic stem cell marker, Krt-18, trophoactodermal marker are expressed in these cells at RNA level. Apart from this, we have also found the expression of 5-methylcytosine -negative, telomerase. Expression of alpha-fetoprotein which is involved in the maintenance of pluripotency of embryonic cells was confirmed by RT-PCR. Oct4 is a POU domain containing transcription factor and is found to be the earliest expressed transcription factor which plays a crucial role in the murine pre-implantation development(Willerth, 2011, Cao *et al*., 2013). Previously, it has been shown that Oct4 expressed in ICM in murine blastomere(Balakumaran *et al*., 2014, Dantuma *et al*., 2010) acts as a transcription factor for many genes expressed in pluripotent cells and thus associated with totipotency(Prockop, 1997, Worton *et al*., 1969, Reynolds *et al*., 1992). We found that cell surface marker for ES cells; SSEA-4 is also expressed in these cells (Figure 6) which are pluripotent in nature. Sox2 is a transcription factor playing crucial role in pre-implantation in mammalian development. Sox2 forms a complex with Oct4 and NANOG and plays a critical role in maintaining self renewal state of the pluripotent ICM in embryo and ES cells *via* Oct4 expression (Kim *et al*., 2005, Bhartiya *et al*., 2014, HE). Previous studies have shown that the expression of Sox2 is developmentally regulated and present in ICM of blastomere, primitive ectoderm, extra embryonic ectoderm and developing nervous system(Cauffman *et al*., 2009, Rizzino, 2009, Klimanskaya I, 2005). Sox2 is considered as the earliest marker of inner cell prior to ICM formation(Ludwig TE, 2006).

Furthermore, we explored the expression of lineage specific marker. We found the mRNA expression of neuronal specific marker Neu N and Nestin; and GFAP that was confirmed by RT-PCR in our cells (Figure 4). Nestin is considered as the marker of multipotent neural stem cells in embryonic as well as adult tissue(Xu C, 2001 , Richards *et al*., 2002). Nestin is found in all parts of brain during embryogenesis. Nestin expressing cells are present and proliferate in injured brain(Richards *et al*., 2003). GFAP, which is a marker for the astrocytes and various neurofilament proteins, was also detected by RT-PCR in our cells. The expression of different immunomodulatory markers like HLA-G, β-HCG, CXCR2 and α- fetoprotein responsible for immune tolerance in pregnancy and required for the proper development of genetically different fetus in maternal body was observed to be expresses at mRNA level. We have also found the expression of genomic integrity marker telomerase at mRNA level.

After confirmation with RT-PCR, we investigated the expression of these markers through immunostaining involving FACS and immunofluorescence/confocal microscope. In FACS analysis, we observed staining of Oct4, Sox2, and SSEA-4 pluripotent stem cells markers where as other markers were not detected. Furthermore, confocal analysis results indicated the expression of Oct4, Sox2, Nanog, SSEA-4, NeuN and Nestin whereas other markers were not found to be expressed. Expression of these ES cells markers further strengthens the pluripotent nature of our cells. Expression of Neu N and nestin indicated the neuronal differentiation of these cells. Those markers which are present at RT-PCR level but not present at immunostaining level indicates that either these markers are not expressed at protein level or are not needed for maintenance of its pluripotency during *in vitro* culture. For example, HLA-G involved in immune tolerance during pregnancy and proper development of genetically different fetus in mother body was expressed at RT-PCR level but not present at protein expression level. 5-methylcytosine which is highly methylated at zygote level undergoes demethylation till blastocyst stage which further undergoes methylation stage. Methylation leads to activation of gene while demethylation leads to inactivation of gene. In our study, we were not able to get methylation of cytosine which indicates that genes are in inactive state and maintained in pluripotent state without going in to differentiation mode.

CONCLUSION

This study revealed a novel cell population which is human in origin and behaves like ES cells and can be used for regeneration. The cell line which is cultured from 2-celled staged discarded embryo obtained during IVF procedure expresses different pluripotent stem cells markers and can be directed towards neuronal lineage. It has no immunogenicity, making it an ideal candidate for allogeneic implantation. These cells have been used as therapy for the treatment of patients with neurodegenerative diseases or damage of brain or SCI and many neurological conditions(Shroff *et al*., 2014, Shroff *et al*., 2014 , Shroff *et al*., 2015 a, Shroff, 2015 c, Shroff, 2015 d, Shroff, 2015 e).

Abbreviations

ESC, Embryonic stem cells; hESCs, Human embryonic stem cells; IEC, Independent Ethics Committee; GMP, Good manufacturing practice; GLP, Good laboratory practice; GTP, IVF, In vitro fertilization; Oct4 + ve, Octamer-binding transcription factor 4 positive; SSEA-4, Stage-specific embryonic antigen-4; β –HCG, β-human chorionic gonadotropin; TRA, Transfer gene; FACS, Fluorescence-activated cell sorting; PCR, Polymerase chain reaction; MSCs, mesenchymal stem cells; SEM, Scanning electron microscopy; TEM, Transmission electron microscopy; BLSCs, blastomeric like stem cells; EGA, Embryonic genome activation; DMEM, Dulbecco's modified eagle's medium; DMSO, Dimethyl sulphoxide; HMDS, Hexamethyldisilazane; PBS, Phosphate buffer saline; PFA; Paraformaldehyde ; ICM, Inner cell mass; BSA, bovine serum albumin

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