Basal Expression of Pluripotency-Associated Genes Can Contribute to Stemness Property and Differentiation Potential

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Pluripotency and stemness is believed to be associated with high *Oct-3/4*, *Nanog*, and *Sox-2* (ONS) expression. Similar to embryonic stem cells (ESCs), high ONS expression eventually became the measure of pluripotency in any cell. The threshold expression of ONS genes that underscores pluripotency, stemness, and differentiation potential is still unclear. Therefore, we raised a question as to whether pluripotency and stemness is a function of basal ONS gene expression. To prove this, we carried out a comparative study between basal ONS expressing NIH3T3 cells with pluripotent mouse bone marrow mesenchymal stem cells (mBMSC) and mouse ESC. Our studies on cellular, molecular, and immunological biomarkers between NIH3T3 and mBMSC demonstrated stemness property of undifferentiated NIH3T3 cells that was similar to mBMSC and somewhat close to ESC as well. In vivo teratoma formation with all three germ layer derivatives strengthen the fact that these cells in spite of basal ONS gene expression can differentiate into cells of multiple lineages without any genetic modification. Conclusively, our novel findings suggested that the phenomenon of pluripotency which imparts ability for multilineage cell differentiation is not necessarily a function of high ONS gene expression.

Introduction

ABILITY TO ISOLATE AND establish pluripotent or multipotent stem/precursor population is a major objective for the biotechnological industry and clinical translation of regenerative medicine. A major impediment for using stem cells in a clinical setup is poor availability of cells, especially those obtained with noninvasive procedures without raising much ethical issues. These limitations greatly restrict the usage of stem cells in clinics, disabling treatments of many degenerative diseases. This lacuna can be filled if any tissue-specific cells can be verifiably demonstrated to possess pluripotent or multipotent capacity. This may elevate the hope to find a well-suited stem cell-like cell line that can serve as an autologous, noncontroversial, and renewable source for cell therapy without ethical and immunological concerns, which are usually associated with embryonic stem cells (ESCs).

Numerous gene and protein expression criteria have been set for recognizing a cell as pluripotent. Microarray analyses have demonstrated a set of various transcripts that are associated with stemness as in the case of ESCs [1]. Notably, it

has been demonstrated by Yamanaka and colleague that the combinations of four major transcription factors, *Oct-3/4*, *Nanog*, *Sox-2*, and *Klf-4*, is indispensable for the cells to maintain pluripotency as in the case of ESCs [2,3]. However, there are some other reports which indicate that expressions of not all the four genes are essential to maintain the stemness. According to NIH and ISSCR guidelines, teratoma formation is one of the major criteria for classifying a cell to be pluripotent [4]. Apart from this, the presence of alkaline phosphatase (ALP) is another reliable property shown for pluripotent cells such as ESCs [1,5].

In principle, a cell that is able to differentiate into cell types of all three germinal layers is considered pluripotent. On the other hand, a multipotent cell can give rise to cells originating from the same germ layer [6]. A classic example of a stem cell with pluripotent and multipotent potential is mesenchymal stem cells (MSC). MSCs can differentiate into various cell types. Depending on isolation procedure and tissue source, both pluripotent and multipotent type of stem cells can be isolated [7]. Mesenchymal cells display fibroblastic cell morphology and express vimentin protein as a

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characteristic marker along with high *Oct-3/4*, *Nanog*, and *Sox-2* (ONS) expression [8]. Bone marrow MSC (BMSC) isolated from crude bone marrow are reported to possess pluripotent gene expression and also show tri-lineage differentiation [9–12]. Therefore, it appears that BMSC can serve as a model that is used for screening various cell types for their degree of stemness. Hence, cells which portray specific features that are in accordance with the parameters mentioned earlier can be designated as pluripotent cells.

In a very recent report, Wang et al. exemplify that ONS genes are the core regulators of pluripotency. This group showed that *Oct4* regulates and interacts with the BMP4 pathway to specify four developmental fates. High levels of *Oct4* enable self-renewal in the absence of BMP4; *Nanog* represses embryonic ectoderm differentiation but has little effect on other lineages, whereas *Sox-2* is redundant and represses mesendoderm differentiation. Thus, instead of being panrepressors of differentiation, each factor controls specific cell fates [13]. Numerous cell types isolated from tissues and organs such as heart, kidney, liver, lungs, brain, pancreas, spleen, muscles, adipose tissue, dental pulp, placenta, and amnion are now being tested on the parameters mentioned earlier with an aim of generating patient-specific pluripotent stem cell lines for treating various incurable degenerative diseases.

With numerous published reports, it becomes a general belief that pluripotency, stemness, and differentiation potential into trigermlinal lineages are associated with high levels of ONS genes, but this does not explain the fact that many low ONS expressing cells can also demonstrate multilineage differentiation potential, high ALP staining, and a teratoma-like structure formation and, hence, generate valid questions. (1) What should be the threshold of ONS gene expression for any cell to be pluripotent? (2) Is pluripotency really a function of high ONS gene expression or can basal level expressing cells also show lower but sustained pluripotency? Attempts are made in the present study to answer these questions scientifically using NIH3T3 as a model and are compared with high ONS expressing cells.

NIH3T3 cells, isolated from Swiss albino mice [14], are not known as stem cells due to the reduced expression of pluripotency-associated genes despite being embryonic in nature. These cells have been extensively used as a model system for various molecular, cellular, and toxicological studies [15–18] and are considered differentiated dermal fibroblast cells. Many groups used NIH3T3 as control cells that fail to differentiate [19–21]. However, their differentiation into adipocytes, myocytes, and neural cell types has been reported either after transforming genetically or if differentiated under defined media conditions [19–21]. Piestun et al. reported that NIH3T3 cells after being transfected with *Nanog* show induced pluripotency and expressed markers that are specific to various differentiating cell types [22]. In 2006, Yamanaka and colleagues also compared pluripotent ESCs and untransformed NIH3T3 by a microarray analysis and concluded that although these cells did not express pluripotency genes, especially the four crucial ones, that is, *Oct3/4*, *Nanog*, *Sox-2*, and *Klf4*; they expressed *c-Myc* [3]. However, this group neither examined nor commented on the multipotent nature of these cells. Later, in 2008 and 2009, Deng and colleagues published two reports demonstrating differentiation of untransformed NIH3T3 cells into osteocytes and

neural cell types in bone and spinal cord injury mouse models [1,23,24]. Two other groups also reported that NIH3T3 can give rise to neural cells and osteoblasts, respectively [21,25]. Recently, our group also reported pancreatic islet cell differentiation of untransformed NIH3T3 with appropriate induction [26].

To answer these questions and to understand whether pluripotency can also be associated with basal ONS gene expression, we carried out a comparative study of basal ONS expressing NIH3T3 cells with pluripotent mouse BMSC (mBMSC) and mouse ESC (mESC). We carefully examined NIH3T3 cells for pluripotent and multipotent stem cell specific markers and compared them with those of mBMSC and mESC with regard to morphological features, expression profile of stemness related genes, ability to undergo multilineage differentiation, and in vivo teratoma formation.

Materials and Methods

Animals

Male *Balb/c* mice, 3–4 weeks old, weighing around 25–30 g, were used for transplantation and bone marrow cell isolation experiments. All animal experiments were performed in accordance with our institutional “Ethical Committee for Animal Experiments” and CPCSEA guidelines and regulations.

Chemicals and cell culture media

All chemicals, media, and antibodies used in this study were purchased from Sigma Aldrich. Molecular biology reagents and cDNA and PCR kits were procured from Fermentas Inc. All plasticwares were purchased from Nunc, Inc.

Isolation of marrow-derived MSCs

Bone marrow-derived mononuclear cells were isolated from 4 week-old *Balb/c* mice according to Zhu et al. [10,27]. Mice were sacrificed after proper anaesthesia. Briefly, tibia and femur bones were dissected under sterile conditions. The metaphyseal ends of the bones were cut, and the marrow plugs were flushed out by passing low glucose Dulbecco’s modified Eagle’s medium (L-DMEM) (Sigma) through a needle inserted into one end of the bone. Bone marrow cells from mice were collected; big tissue debris were removed with the help of a cell strainer and were then seeded in low-glucose RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) for 2 days.

Purification and propagation of MSCs

MSCs were purified as described earlier [10,27]. Briefly, the cultured cells were seeded at a density of 10,000 cells/mL in complete medium (high glucose DMEM supplemented with 10% FBS (Sigma), penicillin (100 IU/mL), streptomycin (100 IU/mL; MP Biological, Inc.) in polystyrene T25 culture flasks and incubated in a humidified 5% CO₂ incubator at 37°C for 48 h. Loosely attached hematopoietic cells were discarded on trypsinizing adherent cells for 5 min. Cells that remained adhered were trypsinized for another 7 min [27] and then cultured in complete medium for 10 days. The cell media was replaced with fresh one every third day. The cells were trypsinized using Trypsin-EDTA (Sigma-Aldrich, USA) and passaged in T75 flasks until the second generation

became 70%–90% confluent. Cultured mBMSC were then characterized and used for a comparative study with NIH3T3 after ensuring their pluripotency.

Cell culture and maintenance

NIH3T3 cells were obtained from three different sources (Cell Repository, National Centre for Cell Science, Pune, India, ATCC procured cells from Zydus Pharmaceuticals, Ahmedabad, India and a generous gift from Dr. Girish M. Shah's lab, Laval University, Quebec, Canada) and were maintained in high-glucose DMEM supplemented with 10% FBS. Cells were split into a 1:1 ratio in new tissue culture flasks on being about 90% confluent. Culture media was changed after every 2 days.

Cell count and growth curve

Fully confluent mBMSC and NIH3T3 cells were trypsinized with 0.1% Trypsin EDTA solution and counted under an inverted phase-contrast microscope (Nikon TE2000). One hundred thousand cells were seeded into each well of 24-well plates for growth curve studies. Cells were eventually trypsinized and counted at different time points (0, 24, 48, 72, 96, and 120 h). Cell counts were then plotted versus time to establish the growth curve of cells. Growth curve of NIH3T3 cells was compared with that of mBMSC. Doubling time of both the cells were also determined using the algorithm $\ln(N_t - N_0) / \ln(t)$, where N_t and N_0 were number of cells at final time point and at initial seeding point, respectively, and t was time period in hours for which cell counts were recorded.

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay

Cell proliferation rate and effect of cytotoxic agent [dimethyl sulphoxide (DMSO)] were determined by MTT assay (Roche). NIH3T3 cells were seeded into 96-well plates at a density of 50,000 cells per well in complete medium. MTT reagent was added in culture in each well after 24 h of incubation with different concentrations of DMSO. The optical density values were analyzed 4 h after the MTT reaction using Multiskan PC (Thermo Lab), and cell survival curves were then plotted against time in culture.

H&E staining

Cells were trypsinized and fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) solution. A histological examination was carried out by standard histological techniques. Fixed cells were stained with H&E stain, and observations were recorded under a light microscope.

Transfection and GFP labeling

Before carrying out an in vivo teratoma assay, we transfected NIH3T3 cells with GFP harboring plasmid p-eGFP-N1 (Clontech), so that the cells could be differentiated from those of the host. One hundred thousand cells were transfected with 1 μ g p-eGFP-N1 plasmid DNA using lipofectamine 2000 (Invitrogen) as a transfecting agent in a 1:3 volume ratio in a 3.5 cm² dish, according to the manufacturer's protocol. Following the transfection, stably GFP expressing clones were selected by growing them in media

containing G418 at 300 μ g/mL concentration for the first 2 days and thereafter in 900 μ g/mL G418 for the next 7 days. Clones were purified from isolated colonies using clonal discs and scaled up for subcutaneous transplants with agarose plugs in *Balb/c* mice.

Teratoma formation

For each graft, ~0.2 million GFP expressing NIH3T3 cells, washed and resuspended in 300 μ L DMEM complete medium, were injected subcutaneously into *Balb/c* mice (maintained in MSU in-house animal house facility) in 1.5% melted agarose [28–30]. Visible tumors, after 2 weeks of transplantation, were dissected and fixed overnight with 4% PFA solution. The tissues were then paraffin embedded, sectioned, stained with H&E, and, the sections were examined for the presence of cells that were representatives of all three germ layers produced by NIH3T3-eGFP cells [31].

ALP staining

ALP staining of spheroids of both NIH3T3 and mBMSC origin was carried out with BCIP/NBT stain. Cell clusters were mechanically isolated, and cells were dissociated with trypsin EDTA digestion for 5 min at 37°C. After washing with 10% FBS and then with PBS, the cells were fixed with 2% PFA solution for 5 min and stained with NBT/BCIP [1]. 3T3L-1 cells served as controls.

Immunocytochemistry

An immunocytochemical analysis was performed for various stem/progenitor markers in both cell types as previously described [32,33]. Cells were seeded on glass coverslips in high-glucose DMEM complete medium and allowed to adhere for 3–4 h. After attachment, cells were fixed with 4% PFA solution for 10 min at room temperature. After permeabilizing them with 0.1% Triton X-100 in PBS for 5 min, the cells were incubated in blocking buffer (1% bovine serum albumin and 4% FBS in PBS) for 1 h at room temperature. Primary antibodies were added in blocking solution, and coverslips were incubated overnight at 4°C. A list of the antibodies used and their dilutions are given in Table 1. Cells were then washed thrice with washing buffer (1:10 dilution of blocking buffer in PBS) and incubated with secondary antibodies conjugated to FITC and TRITC fluorophores (Sigma Aldrich) in the dark for 30 min at room temperature. For negative controls, cells were incubated with normal IgG. Cells were mounted with Vectashield mounting medium containing 4'-6'-diamidino-2-phenylindole dihydrochloride (DAPI) (Vector Laboratories, Inc.). Fluorescence imaging was carried out by using a Nikon TES2000 microscope. All images were analyzed with the help of NIH element software supplied by Nikon Japan.

RNA extraction and semi-quantitative reverse transcriptase–polymerase chain reaction

Total RNA was isolated from cells using TRIzol Reagent (Sigma Aldrich), and 5 μ g of total RNA was reverse transcribed into first-strand cDNA and subjected to polymerase chain reaction (PCR) amplification for various stem cell genes according to conditions mentioned in Tables 2 and 3.

TABLE 1. ANTIBODY INFORMATION CHART

Name of antibody	Company	Mono/polyclonal	Molecular weight (kDa)	Source	Dilution for WB	Dilution for ICC
Beta-actin	Thermo Scientific	Mono	42	Mouse	1:5,000	NA
E-Cadherin	Cell Signaling	Mono	135	Rabbit	1:1,000	1:200
Fibronectin	Sigma	Mono	220	Rabbit	1:1,000	1:800
Ki-67	Sigma	Mono	345/395	Mouse	1:1,000	1:800
Ngn-3	Santa Cruz	Mono	27	Mouse	1:1,000	NA
Nestin	Sigma Aldrich	Mono	177	Rabbit	1:1,000	1:250
Pdx1 antibody	Cell Signaling	Mono	42	Rabbit	1:1,000	1:250
α -SMA	Sigma	Mono	42	Mouse	1:1,000	1:250
Vimentin	Sigma	Mono	53	Mouse	1:1,000	1/250
Oct3/4-PE	BD	Mono	32	mouse	1:1,000	1:200
Nanog	BD	Mono	42	mouse	1:1,000	1:200
Sox-2-APC	BD	Mono	38	Mouse	1:1,000	1:200
CD44-PE	BD	Mono	—	Mouse	—	1:200

SMA, smooth muscle actin; PDX-1, pancreatic duodenal homeobox gene-1; Ngn-3, neurogenin-3; WB, western blot; ICC, immunocytochemistry.

Optimal conditions for PCR were determined by running gradient PCR performed with a range of annealing temperature from 51°C–60°C. One microliters of cDNA products was used to amplify genes using Fermentas 2×master mix containing 1.5 μ L Taq Polymerase, 2 mM dNTP, 10×Tris, glycerol reaction Buffer, 25 mM MgCl₂, and 20 pM appropriate forward and reverse primers for each gene. PCR products were then resolved on a 1.5% Agarose gel (Sigma Aldrich) and visualized with ethidium bromide staining. Images were analyzed using Alpha Imager software (UVP Image Analysis Software Systems).

Real-time quantitative PCR

cDNA, synthesized by using Maxima First-Strand Reverse Transcription System Kit (Fermentas, Inc.), was used for real-time analysis (Applied Biosystem Step One™ Real-Time PCR Sequence detection System) of pluripotent stem cell gene expression. The primer sequences used for quantitative real-time PCR (qRT-PCR) are listed in Table 4. qRT-PCRs were performed in two independent experiments in triplicates. Five microliters of total reaction volume containing 2.5 μ L Maxima SYBR-Green Master-mix (Fermentas, Inc.), 20 pM of each forward and reverse primers using 100 ng cDNA (1/20th of total cDNA preparation) was used. PCR amplification program was 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C, and 1 min at 54°C–57°C). All qRT-PCR results were normalized to the level of beta-actin determined in parallel reaction mixtures to correct for differences in RNA

input. Fold changes by qRT-PCR gene expression were analyzed using Step One™ software V.2.2.2 (Applied Biosystems, Inc.), which led to a possible estimation of the actual fold change (down-regulation after setting mBMSC expression to base in log10). The qRT-PCR results are mean $\pm$ SEM of RQ values versus target gene.

FACS analysis for ONS markers

Cultures of fixed mBMSC and NIH3T3 cells were examined for the presence of ONS markers in their normal cell culture condition. Cells were stained in solution using a mixture of OCT-3/4-PE, Nanog (unlabeled), and Sox-2-APC-labeled antibodies. Unstained cell samples included unlabeled cells, cells labeled with secondary FITC-conjugated antibody alone (for Nanog only), and single fluorochrome-labeled cells. Cells were analyzed using an FACS Aria III (BD Biosciences) with a 70 μ m nozzle and 70 PSI pressure conditions. Cells were first gated based on forward and side scatter properties and then compared with unstained negative cells for levels of OCT-3/4 and Sox-2 labeling. Cells stained with Nanog were compared with secondary antibody control cells at FITC channel. All the calculations and statistics were performed using FlowJo software (Treestar, Inc.).

Protein extraction and western blotting

Western blotting analysis of both NIH3T3 cells and mBMSC at two different passages was performed as

TABLE 2. PRIMER SEQUENCES FOR REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Name of gene	Gene accession no.	Primer sequence forward	Primer sequence reverse	PCR conditions (Tm)	Amplicon size (bp)
Oct-3/4	NM_013633.2	GGCGTTCGCTTTGGAAAGGTGTTC	CTCGAACACATCCTTCTCT	60	431
Nanog	NM_028016.2	TACCTCAGCCTCCAGCAGAT	GAAGTTATGGAGCGGAGCAG	57	411
α -SMA	NT_039687.7	AGTCGCCATCAGGAACCTCGAG	ATCTTTTCCATGTCGTCCCAGTTG	60	296
Vimentin	NT_039202.7	AGCGGGACAACCTGGCCG	GGGAAGAAAAGGTTGGCAGAGGC	58	744
Nestin	NT_039240.7	GCGGGGCGGTGCGTGACTAC	AGGCAAGGGGGAAGAGAAGGATGT	58	326
PDX-1	NT_039324.7	CTC GCT GGG AAC GCT GGA ACA	GCT TTG GTG GAT TTC ATC CAC GG	55	229
Ngn-3	NT_039500.7	ACTAGGATGGCGCCTCATCCCTTG	GGTCTCTTCACAAGAAGTCTGAGA	57	658
GAPDH	NT_166349.1	CAAGGTCATCCATGACAACCTTG	GTCCACCACCCTGTTGCTGTAG	58	496

TABLE 3. PRIMER SEQUENCES FOR mESC PLURIPOTENCY GENE PROFILE BY REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Serial No.	Gene accession no.	Gene symbol	Primer sequences (forward & reverse)
1	NT_166349.1	<i>GAPDH</i>	5'-TCAACAGCAACTCCCCTCTTCCA-3' 5'-ACCCTGTTGCTGTAGCCGTATTCA-3'
2	NM_013633.2	<i>Oct-4</i>	5'-GCTCTCCCATGCATTCAAAC-3' 5'-TGTCTACCTCCCTTGCCTTG-3'
3	NM_011443	<i>Sox-2</i>	5'-ATGGCCCAGCACTACCAGAG-3' 5'-CTTCTCCAGTTCCGAGTCCA-3'
4	NM_028016.2	<i>Nanog</i>	5'-TTTGGGAAGCCACTAGGGAAAG-3' 5'-AAGCCCAGATGTTGCGTAAAGT-3'
5	NM_011920	<i>ABCG2</i>	5'-CCATAGCCACAGGCCAAAGT-3' 5'-GGGCCACATGATTCTTCCAC-3'
6	NM_006086	<i>β-III Tubulin</i>	5'-GGAACATAGCCGTAAACTGC-3' 5'-TCACTGTGCCTGAACCTAACC-3'
7	NM_144841	<i>Otx2</i>	5'-GCTGGCTCAACTTCTACT-3' 5'-TCCAAGCAGTCAGCATTGAAG-3'
8	NM_008090.5	<i>GATA-2</i>	5'-GCAGAGAAGCAAGGCTCCG-3' 5'-CAGTTGACACACTCCCGGC-3'
9	NM_003181	<i>Brachyury (T)</i>	5'-ATAACGCCAGCCACCTACT-3' 5'-TGGTACCATTGCTCAGAGACC-3'
10	NM_008092	<i>GATA-4</i>	5'-TCAAATTCTGCTCGGACTTGGGA-3' 5'-GTTTGAACAACCCGGAACACCCAT-3'
11	NM_008814	<i>PDX-1</i>	5'-ACTTAACCTAGGCGTCCGCACAAGA-3' 5'-GGCATCAGAAGCAGCCTCAAAGTT-3'

previously described [3]. Both NIH3T3 cells and mBMSC were lysed with urea containing lysis buffer (1 mM EDTA, 50 mM Tris-HCl pH 7.5, 70 mM NaCl, 1% Triton, and 50 mM NaF) supplemented with protease inhibitor cocktail (Fermentas, Inc.). Protein estimation in all samples was carried out using Bradford reagent according to the manufacturer's suggestions (Bio-Rad). Cell lysates (50 µg) were separated on Polyacrylamide gel using Mini-tetrapod electrophoresis system (Bio-Rad) and transferred onto nitrocellulose blotting membrane (Thermo, Inc.). Blots were then incubated with blocking milk buffer (5% fat-free skimmed milk with 0.1% Tween-20 in PBS). Dilutions of primary antibodies used, against various stem cell genes, is listed in Table 1. Primary antibodies were added to blots and incubated overnight at 4°C. Anti-rabbit and Anti-mouse IgG conjugated with HRP were used to develop the blots using ultra-sensitive-enhanced chemiluminescence reagent (Millipore) and imprinted on X-ray films.

Results

To determine whether pluripotency is a function of ONS gene expression and whether basal ONS gene expression can contribute to multilineage differentiation potential, a comparative study of NIH3T3 cells was done with pluripotent

mBMSC and mESC. Stemness in NIH3T3 was monitored in cells procured from three independent sources (as mentioned earlier, cells showed normal karyotyping, see Supplementary Fig. S3; Supplementary Data are available online at www.liebertpub.com/scd), after which the presence of stemness-related genes was screened separately with cells obtained from each individual source at both mRNA and protein levels. In this study, we have monitored and compared growth curve, doubling time, and growth kinetics of NIH3T3 cells with that of mBMSC. Presence of various proteins and gene expression profile of pluripotent, multipotent, self-renewal, and differentiation-related genes were also carried out. Here, we also examined in vivo differentiation potential and phenotypic features for pluripotent stem cells with specific reference to teratoma formation and trigeminal layer representatives.

BMSC isolation provided purified MSC

We successfully isolated mBMSC from bone marrow of mice by employing the surgical procedure used by Hsiao and Cheng et al. [27]. A homogeneous population of mBMSC, without hematopoietic or macrophage contaminations, was achieved by differential trypsinization technique, one of the major steps in the isolation and purification

TABLE 4. PRIMER SEQUENCES FOR QUANTITATIVE POLYMERASE CHAIN REACTION

Name of gene	Gene accession no.	Primer sequence forward	Primer sequence reverse	PCR conditions (Tm)
<i>Oct-3/4</i>	NM_013633.2	AACCTTCAGGAGATATGCAAATCG	AACCTTCAGGAGATATGCAAATCG	54
<i>Nanog</i>	NM_028016.2	TACCTCAGCCTCCAGCAGAT	GAAGTTATGGAGCGGAGCAG	57
<i>Sox-2</i>	NM_011443.3	CTGCAGTACAACCTCCATGACCAG	GGACTTGACCACAGAGCCCAT	54
<i>Beta-actin</i>	NM_007393.3	ACGAGGCCCCAGAGCAAGAG	GGTGTGGTGCCAGATCTCCTC	57

FIG. 1. Phase contrast and H&E stain photomicrographs of NIH3T3 cells and mBMSC. (a) Represents cells in high glucose Dulbecco's modified Eagle's medium complete medium at 37°C and 5% CO₂. (b, c) Represent H&E stained NIH3T3 cells at 200× magnifications. Images represent fibroblastic cell morphology. Encircled arrow marked cells show mitotically dividing nuclei with high chromatin content. (d) Represents phase-contrast photomicrograph of mBMSC at P10. mBMSC, mouse bone marrow mesenchymal stem cells.

procedure, and as a result of it, long-term cultures of mBMSC could be established. The number of nucleated marrow cells obtained after isolation was around $3.26 \pm 0.31 \times 10^7$ per mouse, which was comparable to that reported by Hsiao and Cheng et al. 2010 [29]. One of the most important factors in determining the establishment of a long-term culture of mBMSC was the cell density in the starting culture. mBMSC were seeded at a density of 0.2 million nucleated cells/cm² and were then allowed to attach to the surface of the plates for 2 days in low-glucose RPMI 1640 medium supplemented with 10% FBS, which prevented the adherence of contaminating undesired cells. Nonadherent cells were easily removed when the medium was changed on day 3, and confluent colonies were observed by day 8 (P1). Once passaged, in addition to mBMSC, a large number of contaminating small spherical cells, called lymphohaemopoietic cells, engaged in mBMSC colonies as a second layer were also observed (Fig. 2. P#3; P#5). Purification of mBMSC was achieved with the property that they can be removed from the plastic surface within 5 min in response to trypsin, while for hematopoietic ones, it takes 8 min. However, trypsin-lifted cells still contained a few hematopoietic cells. When seeded at a density of 50,000 cells/cm² in the next passage, these hematopoietic cells did not adhere to the plastic surface and were eradicated from the culture on

subsequent passaging (Fig. 2: P3 to P10 for gradual purification of mBMSC). The differential trypsinization steps were repeatedly done till P8. To ensure pure population of mBMSC, different characterization and comparative studies were carried out on cells obtained after P10.

Morphological studies and H&E staining showed stem-cell-like features in NIH3T3 cells

Both NIH3T3 and mBMSC were cultured in high-glucose DMEM with 10% FBS in the absence of any growth factor or growth supplements and sub-cultured when they became about 70% confluent to ensure continued availability of log phase culture conditions for both the cell types. All the characterizations for comparative studies were performed in their native and untransformed state. Morphologically, NIH3T3 cells attain basic fibroblastic morphology, as explained by Todaro and Green [14], similar to that of fibroblastic mBMSC (Fig. 1a, d). In confluent cultures, NIH3T3 cells progressively formed a monolayer and got interlaced with each other, which was similar to the case of mBMSC. Since these cells showed neoplastic behavior, we observed an absence of contact inhibition and cells continued to grow to form multiple layers when cultured for a longer period of time. H&E staining of NIH3T3 cells showed rapidly dividing nuclei

FIG. 2. Phase-contrast images demonstrate step-wise purification of mBMSC from P0 to P10: figure P0 represents mBMSC after isolation at day 1 with heterogeneous population of cells. The other images represent step-wise purification, subsequent removal of stem cells, and gradual depletion of hematopoietic in cultures of mesenchymal cells from P1 to P9. Phase-contrast photomicrograph at P10 shows purified population of bone marrow mesenchymal cells.

FIG. 3. ALP staining in NIH3T3 and mBMSC: (a, b) represents typical spheroid formation after 8 day culture in absence of any growth factor supplement (arrowhead). (c, d) Represent positive ALP staining in cell clusters generated in both cell types (marked with arrowhead). (e, f) Represent magnified view of clusters (marked with arrowhead). (g) Represents stained embryoid bodies from mESC (shown with arrowhead) and (h) represents 3T3-L1 cells as negative control. mESCs, mouse embryonic stem cells; ALP, alkaline phosphatase.

and cells with high chromatin content (Fig. 1c). All these observations are in congruence with the previous report by Todaro and Green [14]. A comparison of the morphological behavior of NIH3T3 cells with mBMSC demonstrated many similarities between the two cell types in cell shape, pattern of growth, cell contacts, and proliferative nature.

Spheroid body formation with ALP staining indicated in vitro differentiation potential in NIH3T3 cells

Next, in order to determine the in vitro differentiation potential of NIH3T3 cells, we carried out ALP staining of the spheroid body from two cell types, as it is more important to characterize these cells based on recent ISSCR guidelines and other reports available from current literature. Spheroid bodies were generated by seeding high cell density in a minimum surface area in plates without any coating with growth factors. As expected, most of the cells adhered, and some of them died; these were excluded from the culture on medium replenishment. Varying sizes of small to large spheroid bodies were formed in each cell type by day 3–5, and these were very similar to embryoid bodies (EBs) formed from ESCs. On comparing, NIH3T3 cells showed similar features to mBMSC, which are in accordance to Yamanaka and Riektina's group [2]. The floating spheroids from both cell types were collected and stained for ALP. We noticed that NIH3T3 cells formed spheroids and remained intensely positive for ALP, similar to mBMSC and mESC, confirming the pluripotent nature of these cells [2]. Normal 3T3-L1 cells served as a control that was negative for ALP (Fig. 3).

Growth curve and kinetics (cumulative duplication number and doubling measurement) of NIH3T3 demonstrated growth pattern identical to mBMSCs

Healthy cells from sub-confluent cultures of both cell types were plated in 24-well plates and used for determining the population doubling potential, progression, and proliferative activity. Cumulative population doublings were calculated by considering the initial number of NIH3T3 and

mBMSC cells seeded at 0 h and the number of cells harvested at each destined time point, respectively, without passaging (Graph 2). These observations provide a theoretical growth curve that is directly proportional to the cell number. With the help of the curve generated, doubling time was found to be 27.53 ± 3.4 h and 21.87 ± 2.39 h for NIH3T3 and mBMSC, respectively, suggesting that both the cells have more or less a similar population doubling time. A small but insignificant 1.2-fold difference was found in their doubling time (Graph 3), which is in accordance with Todaro and Green [14]. A correlation analysis curve of both cell systems, from a log-scale graph, gave a trendline that agreed with the linear curve equation, that is, $y = mx + c$. The regression value (R^2 value) of both curves was found to be 8.9 and 8.2, respectively, suggesting no significant difference in growth patterns of the two cell systems.

Cell proliferation and cytotoxicity assays revealed stemness behavior of NIH3T3 cells

For determining the proliferative nature of NIH3T3 cells and their tolerance to a cytotoxic material such as DMSO, MTT assay was employed. NIH3T3 cells were cultured in 96-well plates and were allowed to grow for 72 h. At regular time intervals of 24 h each, MTT reagent was added and optical density was recorded. We found that starting from 24 to 72 h, NIH3T3 cells proliferated aggressively with a fold change of 2.8 from 0 to 24 h and of 2.78 from 24 to 72 h, respectively (Graph 4). It has been notably shown that cytotoxicity tolerance of DMSO is evidently much higher in stem cells rather than in normal differentiated ones. It is worth mentioning that differentiated cells can tolerate optimal DMSO concentration up to 0.5%; however, peripheral blood mononuclear cells and MSCs are shown to tolerate DMSO even till 5% [34,35]. When carefully observed, these cells were found to have enormous tolerance for potent cytotoxic agents DMSO. Unexpectedly, NIH3T3 cells showed tolerance to DMSO even at 5% with ~50% viability. The cells were growing normally in media containing 5% of DMSO. Almost 20% cells even survived in very high doses (upto 10%) of DMSO (Graph 5).

GRAPH 1–5. Growth curves and growth kinetics of NIH3T3 and mBMSC: Graphs represent comparative growth pattern and proliferation of NIH3T3 cells and mBMSC. Kinetics graph represents the correlation of growth pattern of both cell systems fitting in a linear curve equation. $n = 4$.

Immunocytochemistry confirms pluripotency in NIH3T3 cells

To determine whether NIH3T3 cells express pluripotent or multipotent markers, cells were cultured on glass coverslips

and immunostained after 24 h for ESC (ONS) and various other markers associated with stemness and progenitor state. We observed intense positive staining in both mBMSC and NIH3T3 for *Oct3/4*, *Nanog*, and *Sox-2*, which was similar to mESC (Fig. 4a). Moreover, we also found that NIH3T3

FIG. 4. Immunocytochemistry of mESC, mBMSC, and NIH3T3: (a) represents pluripotency marker staining *OCT3/4* (red), *Nanog* (green), and *Sox-2* (yellow) in mESC, mBMSC, and NIH3T3 cells, respectively. (b, c) represents comparative immunophenotyping of other stem cell markers in mBMSCs and NIH3T3 cells. Color images available online at www.liebertpub.com/scd

cells were positive for *Nestin*, *Vimentin*, *Smooth Muscle Actin (SMA)*, *Ki-67*, *Fibronectin*, *E-cadherin*, and *pancreatic duodenal homeobox gene-1 (PDX-1)* (Fig. 4b, c). *Nestin* and *Vimentin* are markers of neural and MSC, respectively, whereas *Fibronectin* and *SMA* confirm MSC-like features. In addition, we noticed that NIH3T3 cells were strongly positive for *PDX-1*, which is not only a pancreatic progenitor marker but is also expressed by pluripotent stem cells [2].

Gene expression analysis indicates pluripotent nature of NIH3T3 cells

Subsequently, when we found NIH3T3 cells to be positive for stemness markers by immunocytochemistry, we tested them for the presence of these pluripotent, multipotent, and differentiation markers at transcript (mRNA) levels by semi-quantitative reverse transcriptase and qRT-PCR in NIH3T3 cells procured from second-source Zydus Pharmaceuticals, Ahmedabad, India. Expression profiles of all stem cell markers such as *Oct3/4*, *Nanog*, *Sox-2*, *Nestin*, *Vimentin*, *SMA*, *PDX-1*, and *Neurogenin-3 (Ngn-3)* were carried out. mBMSC at P10 were found to be positive for all *Oct3/4*, *Nanog*, *Sox-2* (by q-PCR), *Nestin*, *Vimentin*, *SMA*, and *PDX-1*, and *Ngn-3* (by RT-PCR) (Fig. 5). These marker profiles, determined by q-PCR, clearly demonstrate the pluripotency of mBMSC as *Oct3/4*, *Nanog*, and *Sox-2* expression were up-regulated in them. Expression pro-

FIG. 5. Gene expression analysis of mESC, mBMSC, and NIH3T3: (a) represents comparative gene expression profile of pluripotency markers and trigeminal layer markers in mESC and NIH3T3 cells. (b) Represents mRNA expression of other stem cell genes in mBMSC and NIH3T3 cells.

FIG. 6. Comparative q-PCR Plot for gene expression of NIH3T3 and mBMSC: Figure represents a comparative study of gene expression profile of 3 crucial stem cell markers—*Oct3/4*, *Nanog*, and *Sox-2*, in NIH3T3 and mBMSC by real-time PCR. Results are the mean of triplicates performed independently in two sets of experiments using cells at a different passage number. q-PCR, quantitative polymerase chain reaction.

files of the same markers were screened in NIH3T3 cells of P15. The cells were found to be positive for *Oct3/4*, *Nanog*, *Sox-2*, *Vimentin*, *SMA*, *Nestin*, *PDX-1*, and *Ngn-3* by RT-PCR; whereas contradictorily, down-regulation of *Oct3/4*, *Nanog* (100 times), and *Sox-2* (1,000 times) was also observed in these cells by q-PCR when compared with mBMSC expression (Fig. 6). To further validate and confirm that NIH3T3 cells retain pluripotent nature, we compared NIH3T3 with mESC by RT-PCR; interestingly, we found these cells to express all three ONS genes, which was similar to mESC. Further, NIH3T3 cells were also found to express *ABCG2*, which is an important transporter molecule in ESCs. We also examined the expression of tri-lineage germ layer markers such as β -III tubulin, *Otx-2* for Neuroectodermal layer, *GATA-2*, *Brachyury (T)* for Mesodermal layer, and *GATA-4* and *Pdx-1* for Endodermal layer in NIH3T3 aggregated clusters, which was similar to EBs generated from mESC (Fig. 5a).

Flow cytometry analysis supports pluripotent nature of NIH3T3 cells

FACS analysis of ONS markers in both mBMSC and NIH3T3 cells was carried out. We clearly observed that both mBMSC and NIH3T3 cells showed ONS positivity. On comparing the percentage population of mBMSC, we found that 7.91% cells were positive for *Oct-3/4*, 70.87% cells for *Nanog*, and 75.37% cells for *Sox-2* (Fig. 7). Likewise, in the case of NIH3T3, 6.13% cells were positive for *Oct-3/4*, 70% cells for *Nanog*, and 7.96% cells for *Sox-2* (Fig. 7).

Western blot profile validates pluripotent stem cell-like nature of NIH3T3 cells

For confirming the presence of ONS genes at the protein level, western blot was carried out with the cell lysate of mBMSC and NIH3T3. As expected, both the cell types from two different passage numbers (P10 and P12 in mBMSC and P31 and P33 in NIH3T3; see Fig. 8) were positive for

FIG. 7. FACS histograms of ONS markers in mBMSC and NIH3T3 cells: figure represents comparative histograms of pluripotency marker profile from mBMSC and NIH3T3 cells. ONS, *Oct-3/4*, *Nanog*, and *Sox-2*.

Vimentin, *Fibronectin*, *SMA*, *Ki-67*, *PDX-1*, and *Ngn-3*. Both mBMSC and NIH3T3 cells showed high expression levels of both precursor and mature isoforms of *Fibronectin* protein, which were 170 and 90 kDa, respectively. The level of *SMA* expression in NIH3T3, however, was a little lower than that in mBMSC; whereas that of *Ki67* isoforms of 395 and 365 kDa was higher in NIH3T3 cells. Further, the expression of *Nestin* was seen in NIH3T3 cells only but not in mBMSC. In fact, the expression levels of *Vimentin*, *PDX-1*, and *Ngn-3* were also very high in NIH3T3 compared with those in mBMSC (Fig. 8). More interestingly, we further found that mBMSC cells demonstrated increased protein expression of ONS markers from P10 to P12; nonetheless, NIH3T3 cells showed bands for *Oct-3/4* of 34 kDa, *Nanog* of 42 kDa, and *Sox-2* of 38 kDa at both P31 and P33. We also checked expression of ONS in GFP-labeled NIH3T3 cells before teratoma formation during

in vivo studies to ensure pluripotency. NIH3T3-eGFP cells as per our expectation showed ONS protein bands even after GFP labeling (Fig. 8).

In vivo teratoma formation reconfirms pluripotency in NIH3T3 cells

To further validate the phenotypic properties of NIH3T3 cells in terms of pluripotency, an in vivo experiment for teratoma formation in *Balb/c* mice was carried out. NIH3T3-eGFP cells in a small aliquot of medium with 1.5% warm agarose plugs were transplanted subcutaneously on the left side of ten *Balb/c* mice [28–30,36,37]. The right side of the same animals was used for control or placebo that is, only agarose plugs were injected at that site. After 15 days of transplantation, mice were sacrificed after teratoma

FIG. 8. Western blotting profile of NIH3T3 and mBMSC: figure represents comparative protein expression profile of pluripotency and other stem cell markers from mBMSC (P10 and P12) and NIH3T3 (P31 and P33). Figure also represents ONS expression in NIH3T3-eGFP cells post-transfection at P5.

FIG. 9. In vivo study for teratoma formation in *Balb/c* mice: (a) shows NIH3T3-eGFP produced teratomas in *Balb/c* mice on the left side of mice, while saline showed no outgrowth on the right side of the body. (b) Represents no other organ affected by teratoma as transplanted cells did not metastasize. (c) and (d) Shows subcutaneous teratoma bulging on left and right peritoneum of mice respectively. (e) Indicates difference in the size of the teratomas between transplanted cells in saline and those in agarose plugs. (f) Represents ALP staining of cells transplanted in agarose plug after 15 day teratoma formation.

formation from NIH3T3-eGFP cells had been observed in these animals. All of the 10 mice developed evident teratomas (left side) wherein skin bulges were bigger in size than those of agarose plugs alone (right side) (Fig. 9). In addition, we noted that teratomas formed by NIH3T3-eGFP cells injected in agarose plugs were significantly larger (average weight of 0.999 ± 0.1 g and volume 2.70 ± 0.48 mm³) than those formed by agarose plugs alone (average weight

FIG. 10. Comparative graph for weight and volume of NIH3T3-eGFP induced teratoma and agarose plug alone after 15 days. Results are expressed as mean $\pm$ SEM with $n=6-10$ animals. * and ** represents $P < 0.01$ and $P < 0.001$ versus control plugs (Saline), respectively.

0.70 ± 0.03 g and volume $1.5-0.16$ mm³) (Fig. 10). GFP imaging of teratoma sections demonstrated that a major mass of tumor plugs was mainly derived from NIH3T3-eGFP cells only and not from host dermal cells (Fig. 11). Immunohistochemistry of tri-lineage markers clearly depicted the presence of pluripotency markers ONS in developed tumor tissues. Representative regions of slides showed colabeling of *Oct-3/4*, *Nanog*, and *Sox-2* with GFP staining (Fig. 13). Histological studies confirmed the in vivo functionality and representation of tri-germinal layers in developed teratomas (Fig. 12; see Supplementary Fig. S2 showing Alizarin red and Oil O Red staining in excised teratoma sections after 15 days. Fig. S1 showed staining for oestoe and adipocytic differentiation in vitro). The infused cells were found to be confined to the transplanted areas only, as all other organs in the alimentary canal were normal and no significant metastasis was observed. Since the cells were encapsulated in agarose and transplanted subcutaneously beneath the forearms and not directly in vasculature, hence, the chances of spreading were minimal to begin with. Apart from this, in order to effectively validate the signatures of all three germ layers in the excised teratomas, we confirmed the markers of ectoderm, mesoderm, and endoderm using immunohistochemistry with GFP colabeling. NIH3T3-eGFP teratoma slices stained positive for *Nestin*, neurofilament markers for ectodermal germ layer, *CD44* specific marker for mesodermal cell types, and *PDX-1* for endodermal germ cells (Fig. 14).

Discussion

NIH3T3 cells, till 2005, were not known to possess stemness properties. Recent studies have shown stemness in NIH3T3,

FIG. 11. Histological images of teratoma: figure represents histological and GFP fluorescence in panel (a) agarose plug alone and (b) NIH3T3-GFP-developed teratoma. Teratoma sections of agarose plugs with NIH3T3-eGFP cells showing GFP fluorescence with representatives of three germinal layers in teratoma sections. Color images available online at www.liebertpub.com/scd

as the cells are capable of differentiating into various cell types such as neural cells, osteocytes, and adipocytes, including insulin-producing beta cells [1,19–21,24–26].

The present study is an attempt to demonstrate whether pluripotency and stemness is associated with basal levels of ONS genes. Efforts were made to unravel these facts by demonstrating characteristic features of NIH3T3 in terms of stem-

ness by undertaking a comparative analysis of NIH3T3 with mBMSC employing cellular and molecular techniques. Morphologically, NIH3T3 showed more or less similar shape-like fibroblastic mBMSC with the ability to survive in culture conditions for numerous passages. We also found that NIH3T3 became progressively hyperplastic and that they get interlaced with one another with an increase in the number of days in culture. Multiple layers of cells are produced, as the cells'

FIG. 12. Histological images of teratoma: figure represents histological representatives for trigeminal lineages, panel (a) ectoderm (b) endoderm (c) mesoderm (skeleton muscles), and (d) mesoderm (adipose tissue). Color images available online at www.liebertpub.com/scd

FIG. 13. Immunohistochemistry of NIH3T3-eGFP teratoma sections: figure represents pluripotency markers OCT-3/4, Nanog, and Sox-2 staining in NIH3T3-eGFP teratoma sections with GFP colocalization. Panel represents ONS staining in red, GFP staining in green, and DAPI nuclear staining in blue with merged image. DAPI, 4',6'-diamidino-2-phenylindole dihydrochloride. Color images available online at www.liebertpub.com/scd

FIG. 14. Immunohistochemistry of NIH3T3-eGFP teratoma sections: figure shows ectodermal; Nestin (red) expression in a few GFP (green) positive cells representing neuroectodermal cell lineage, mesoderm; CD34 (red) positive nuclei marker of chondrocyte cells, and endoderm; PDX-1 positive (red) nuclei depicting presence of pancreatic endodermal layer. DAPI (blue) was used for nuclear staining. Color images available online at www.liebertpub.com/scd

growth is not inhibited by contact inhibition as described earlier [14]. A histological observation also supported the proliferative rate and plasticity of NIH3T3 cells. High chromatin content and frequent appearance of dividing nuclei indicated progenitor-like features, which are in accordance with earlier studies done with pluripotent stem cells [1,5,38–40].

ISSCR guidelines suggest ALP activity as one of the critical criteria of pluripotency, which has also been emphasized by Riekstina et al. [2]. On these grounds, we demonstrated that NIH3T3 cells formed spheroid bodies that stained positive for ALP staining. The purple color in NIH3T3 spheroids indicated a characteristic feature of EBs derived from pluripotent ESCs, reinforcing their enhanced stemness. These findings are in concurrence with those of Li and Riekstina et al. [2,5].

Molecular and immunological characterization is important to investigate the presence of stem cell-like markers at both mRNA and protein levels. To achieve this, we characterized NIH3T3 cells from all the three different sources as mentioned earlier at gene and protein levels by RT-PCR, q-PCR, immunocytochemistry, flow cytometry, and western blotting. Based on the immunocytochemistry results, we conclude that NIH3T3 cells obtained from the first source, that is, NCCS Pune, India at P4, showed the presence of all characteristic markers which are relevant to pluripotency. They express ESC markers such as *Oct3/4*, *Nanog*, and *Sox-2*. NIH3T3 cells were highly positive for *Nestin*, a neuroendo-

crine filament protein, expressed in pluripotent neural stem cells [41,42]. It is also reported that *Nestin* is widely expressed in pluripotent stem cells from various other tissues such as the ear [5], dermal fibroblast [31], and pancreatic lineage cells [43]. NIH3T3 cells were also found to be positive for mesenchymal lineage markers such as *Vimentin*, *SMA*, and *Fibronectin*, confirming their mesenchymal or multipotent status. Various pluripotent or multipotent MSCs are known to express *Vimentin*, *Fibronectin* [1,44], and *SMA* [7]. *PDX-1*, a pancreatic progenitor marker present in pluripotent MSC [2], was also found to be expressed by NIH3T3, indicating their predisposition to give rise to definitive endoderm. Pluripotent stem cells are also known to have high *E-cadherin* content [1], which represents higher self-renewal capacity and undifferentiated status. When we examined mBMSC and NIH3T3 cells, both strongly expressed *E-cadherin*, a finding in good agreement with that of Conrad and group [1]. These findings clearly suggested that NIH3T3 cells harbor pluripotent characteristics.

We further tested NIH3T3 stemness at the molecular level at P15 obtained from the second source, that is, Zydus Pharmaceuticals. The idea was to demonstrate that the results and observations we had recorded so far in an early passage of NIH3T3 by immunocytochemistry held true in cells of higher passage numbers obtained from another source. The presence of *OCT-3/4*, *Nanog*, and *Sox-2* represented an ESC-like state as reported by Yamanaka and group [3]. *Nanog* is known to be responsible for self-renewal, pluripotency, and for maintaining an undifferentiated state of cells [45]. As per our observations evident by RT-PCR, NIH3T3 cells express *Oct3/4*, *Nanog*, and *Sox-2* albeit at slightly lower levels, thus indicating their pluripotent nature. Further, NIH3T3 cells also expressed *ABCG2*, an important transporter molecule that is abundant in ESCs. In order to reconfirm our semi-quantitative PCR data, we decided to quantify the levels of the genes by a more sensitive technique such as qRT-PCR. Results showed that NIH3T3 exhibited a 100-fold lesser level of *Oct3/4* and *Nanog* expression, while that of *Sox-2* was 1,000 fold less than that in mBMSC. We hypothesize that NIH3T3 cells sustain their pluripotency, through sub-basal levels of *Oct3/4*, *Nanog*, and *Sox-2* unlike ESCs. However, the presence of ONS can perhaps explain the differentiation potential of NIH3T3 cells into neural cells, osteocytes, and adipocytes as reported by Deng group [21,23] and pancreatic islets by Gupta et al. [26]. In one of the recent reports, we also demonstrated successful differentiation of NIH3T3 cells into islet-like cell clusters [26], further validating the pluripotent nature of these cells. Even further, flow cytometry and western blotting confirming the presence of ONS proteins in NIH3T3 cells similar to mBMSC provide additional support to the hypothesis and authenticate the fact that NIH3T3 may represent a connecting link to ESCs with regard to pluripotency.

Pluripotent cells have the ability to differentiate into cell types of all three germinal layers, namely ectoderm, mesoderm, and endoderm, under suitable culture conditions. In vitro pluripotency of cells can be assessed by their ability to form three dimensional structures called EBs or aggregates or spheroids; on the other hand, in vivo pluripotency can be characterized by the ability to form putative tumours when implanted into immunocompromised animals [37]. Non-cancerous benign tumors formed by ESCs are called

teratomas [36,37]. Our studies demonstrated interesting results in terms of spheroid formation in vitro and teratoma formation in vivo with NIH3T3 cells. GFP-expressing NIH3T3 cells, when engrafted with agarose plugs without any growth factors, showed high incidence of teratoma formation in immune compromised mice such as *Balb/c*. NIH3T3eGFP cells not only demonstrated the ability to proliferate under in vivo conditions to form noncancerous cell mass, but also stimulated angiogenesis, which was evident by new blood vessel formation within 15 days. The behavior of NIH3T3 cells in vivo was fairly identical to ESC-induced teratoma formation. An immunological confirmation of three lineage markers such as Nestin, CD44, and PDX-1 for ecto-, meso-, and endoderm layers further verified in vivo pluripotency of NIH3T3 cells. All these results demonstrate the pluripotent nature of NIH3T3 cells representing a connecting link between mBMSC and mESC.

Conclusions

Several lines of evidence in our study lend credence for the pluripotent nature of NIH3T3 cells. The most striking evidence that provided functional confirmation for their pluripotency were strong ALP staining, basal *Nanog* expression, and high teratoma-forming capacity of these cells in mice without any growth supplements. Interestingly, the acquisition of pluripotency in NIH3T3 cells was concurrent with the lower levels of *Nanog* expression in comparison with mESC rather than complete silencing of the gene as witnessed in mBMSC cells. Their potential to differentiate into neurons and insulin-producing cells furnished functional evidence of their pluripotentiality. Thus, our findings demonstrate that NIH3T3 cells have pluripotent characteristics and can be differentiated into cells of all three germ lines, which suggest that basal level of ONS gene expression can also lead to pluripotent stem cell-like feature, thereby conferring pluripotency and tri-lineage differentiation potential. These promising findings offer a new hope to look for pluripotent/multipotent stem cells from different human tissue-specific cell types already in culture that could potentially be used in clinics for autologous cell therapy in degenerative diseases.

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Author Disclosure Statement

No competing financial interests exist.

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