

## Diverse effects of dimethyl sulfoxide (DMSO) on the differentiation potential of human embryonic stem cells

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**Abstract** In vitro disease modeling using pluripotent stem cells can be a fast track screening tool for toxicological testing of candidate drug molecules. Dimethyl sulfoxide (DMSO) is one of the most commonly used solvents in drug screening. In the present investigation, we exposed 14- to 21-day-old embryoid bodies (EBs) to three different concentrations of DMSO [0.01% (low dose), 0.1% (medium dose) and 1.0% (high dose)] to identify the safest dose that could effectively be used as solvent. We found that DMSO treatment substantially altered the morphology and attachment of cells in concurrence with a significant reduction in cell viability in a dose-dependent manner. Gene expression studies revealed a selective downregulation of key markers associated with stemness (Oct-4, Sox-2, Nanog and Rex-1); ectoderm (Nestin, TuJ1, NEFH and Keratin-15); mesoderm (HAND-1, MEF-2C, GATA-4 and cardiac-actin); and endoderm (SOX-17, HNF-3 $\beta$ , GATA-6 and albumin), indicating an aberrant and untimely differentiation trajectory. Furthermore, immunocytochemistry, flow cytometry and histological analyses demonstrated substantial decrease in the levels of albumin and CK-18 proteins coupled with a massive reduction in the number of cells positive for PAS staining, implicating reduced deposits of glycogen. Our study advocates for the first time that DMSO exposure not only affects the phenotypic characteristics but also induces significant alteration in gene expression, protein content and functionality of the

differentiated hepatic cells. Overall, our experiments warrant that hESC-based assays can provide timely alerts about the outcome of widespread applications of DMSO as drug solvent, cryoprotectant and differentiating agent.

**Keywords** Pluripotent stem cells · Embryoid bodies · Differentiation · Drug solvent · Toxicity

### Introduction

Embryonic stem cells (ESC) are derived either from inner cell mass (ICM) of blastocyst stage embryos (Thomson et al. 1998). Under suitable in vitro conditions, these cells can maintain an undifferentiated state indefinitely and differentiate into derivatives of all three germ layers: namely the ectoderm, endoderm and mesoderm. Because of their plasticity and virtually unlimited self-renewal capacity, ESC-based replacement therapies have been shown to treat genetic disorders and degenerative conditions (Carpenter et al. 2009). However, therapeutic applications of ESC are marred by several limitations including teratoma formation, immune rejection, issues regarding homogeneity and scalability of the transplantable cells. Nevertheless, it has been realized lately that ESCs may find a more immediate application for screening of potential toxicants on human cells.

Since the differentiation of ESC mimics early development, these cells could potentially permit the detection of embryotoxicants which interfere with this process. Although reliable tests based on murine embryonic stem cells exist, no such methods are available for human embryonic stem (hES) cells. Nonetheless, to avoid the false classification of substances due to inter-species differences, human-relevant toxicity tests are needed (Adler et al.

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2008). Therefore, hESC-based in vitro testing may offer physiologically and clinically, a more relevant model compared to classical rodent models.

ESCs are potentially informative in the context of toxicity testing due to their reliance on many key pathways in morphogenesis and differentiation. However, most of the hESC lines available today were cryopreserved using dimethyl sulfoxide (DMSO) in liquid nitrogen at  $-196^{\circ}\text{C}$  for future research and applications. At the same time, few reports showed that the usage of DMSO at concentrations much lower than that used during cryopreservation can induce differentiation of embryonic carcinoma (EC) and hESC (Jasmin et al. 2010; Inamdar et al. 2009).

Biochemically, DMSO is an amphipathic molecule and one of the most commonly used solvents for water-insoluble substances. DMSO plays multiple roles on cellular functions (e.g., metabolism and enzymatic activity) and cell growth by affecting cell cycle and apoptosis (Santos et al. 2003). Despite being frequently used as a vehicle for drug therapy, the side-effects of DMSO, although apparent, are rarely studied or reported. Biologically, DMSO is a hydrogen-bound disrupter, cell-differentiating agent, hydroxyl radical scavenger, intercellular electrical uncoupler, intracellular low-density lipoprotein-derived cholesterol mobilizing agent, cryoprotectant, antidote to the extravasation of vesicant anticancer agents and topical analgesic. Additionally, it is used in the treatment for brain edema, amyloidosis, interstitial cystitis and schizophrenia. However, its widespread use has been restricted due to its toxic effects, which vary depending on the cells in question and concentration. Looking at the diversity of applications using DMSO, we propose that it is imperative to understand the effect of DMSO on human cells. To address these questions, we used a hESC-based in vitro model to show the effects of different concentrations of DMSO on cell survival, attachment, proliferation rate and differentiation potential on embryoid bodies (EB) by employing techniques like phase-contrast microscopy, flow cytometry, immunocytochemistry, gene expression analysis and biochemical assays.

## Materials and methods

### hESC culture and differentiation

hESC lines HUES-7 and HUES-9 were obtained as a generous gift from Harvard University Stem Cell Institute (Prof Douglas Melton) in 2007. All experiments using hES cells were conducted after approval from Institutional Ethics Committee (IEC) as well as Institutional Committee for Stem Cell Research and Therapy (IC-SCRT) following the guidelines of Indian Council of Medical Research. Our laboratory has requisite infrastructure to undertake hESC

experimentation approved by Manipal University. Undifferentiated hESC were grown on a feeder layer of mouse embryonic fibroblast (MEF) cells inactivated with  $10\ \mu\text{g}/\text{ml}$  Mitomycin C (Sigma) in 35-mm gelatin-coated tissue culture dishes (Falcon). The culture medium consisting of 80% Knockout Dulbecco's modified Eagle's medium (KO-DMEM; Invitrogen), 15% ES-tested FBS (Hyclone), 5% serum replacement (SR, Invitrogen), 1% non-essential amino acid solution (Invitrogen), 1 mM glutamine (Invitrogen), 0.1%  $\beta$ -mercaptoethanol (Sigma) and 4 ng/ml human basic fibroblast growth factor (bFGF; Sigma) under standard conditions ( $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$ , saturated humidity). The hES cells were dissociated manually and sub-cultured on fresh MEF feeder layers every 4–6 days. We expanded the cell line and froze enough vials before we used the cells for experimental purposes.

### EB formation

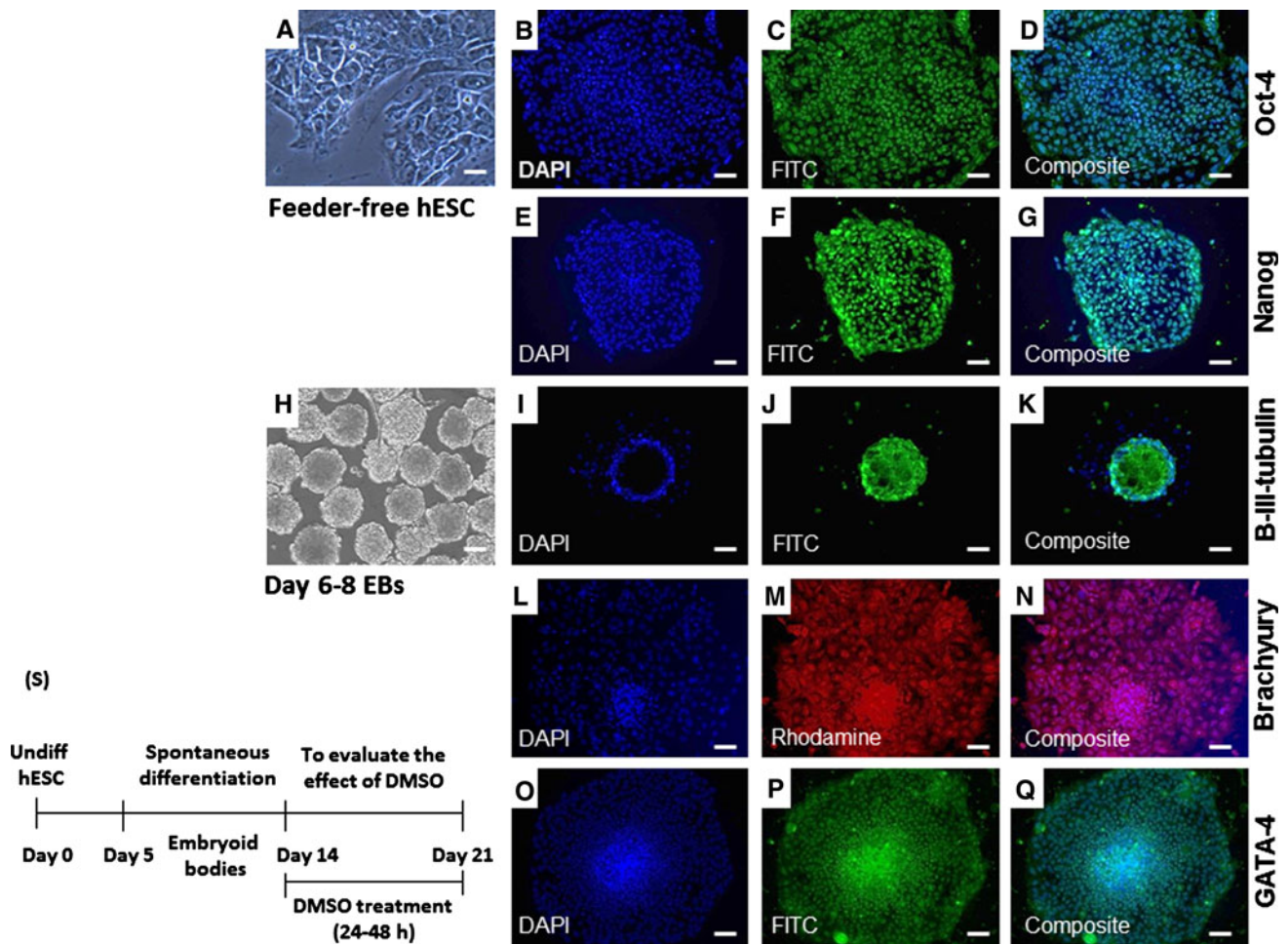
EBs formation was induced by mechanically dissecting undifferentiated hES colonies into pieces less than  $200\ \mu\text{m}$  in size using the sharp edge of a flame-pulled Pasteur pipette under the stereomicroscope as described by us earlier (Pal et al. 2009). Pieces were transferred to 60-mm sterile petri culture dishes (Corning) to allow their aggregation and prevent adherence to the plate. Equal size EBs were grown in suspension culture at approximately 30 EBs per dish. The EBs were grown in the same hESC culture medium, except that it lacked bFGF. Culture media was changed every alternate day with media equilibrated for 24 h at  $37^{\circ}\text{C}$  and 5%  $\text{CO}_2$  before use. After 4–5 days of culture, EBs were plated on coated dishes for spontaneous differentiation.

### DMSO treatment

hES cell line (HUES-7) and in-house-derived human fibroblasts were used to evaluate the dose-dependent impact of dimethyl sulfoxide (DMSO; Sigma) in order to study the solvent toxicity at room temperatures. Concentrations of DMSO tested were 0.01% (v/v), 0.1% (v/v) and 1.0% (v/v) corresponding to low, medium and high doses, respectively. DMSO was directly diluted in the culture medium to the final test concentrations. We exposed the DMSO to the EBs in suspension as well as after plating for 24–48 h and examined the effect caused by different doses of DMSO and compared the response with untreated controls and treated fibroblast cells (Fig. 1s).

### Cell viability assay using 7-AAD

Cell viability was determined by the 7-amino actinomycin (7-AAD) a fluorescent DNA intercalator, which binds to



**Fig. 1** Quality assurance of hESC and hEB. **a** Typical morphology of feeder-free hESC colony; colonies demonstrated uniformly distributed fluorescence when immunostained against **b–d** Oct-4 and **e–g** Nanog. **h** Phase-contrast micrograph of 14-day-old EBs. Immunological characterization of EBs **i–k** showing positive immunofluorescence for B-III tubulin; **l–n** Brachyury; and **o–q** GATA-4 representing ecto-, meso- and endoderm lineage, respectively. The *blue* color

represents nuclei counterstained with DAPI, while *green* and *red* color represent FITC and Rhodamine conjugation, respectively. Negative controls were performed with corresponding immunoglobulin G (IgG). Representative data from one of three experiments are shown ( $n = 3$ ). Scale bar: 100  $\mu$ m. **s** A schematic presentation of the experimental setup to examine the effects of DMSO on spontaneous differentiation of hESC

double-stranded DNA of the dead and damaged cells. Following ethanol treatment, cells were washed twice with PBS, 100  $\mu$ l of the cell suspension consisting of  $1 \times 10^5$  cells were transferred to a 15-ml centrifuge tube and 5  $\mu$ l of 7-AAD fluorescent dye was added to each tube. Cells were gently vortexed and incubated for 15 min at room temperature (25°C) in the dark. Cell suspension was subsequently analyzed by flow cytometry (excitation at 488 nm, emission at 520 nm), and appropriate compensation controls were performed in conjunction to establish the gates with the reference values. Ten thousand cells were analyzed for each sample, and data were acquired using Cytosoft, Version 5.2, Guava Technologies (Millipore).

#### Isolation of total RNA, preparation of cDNA and RT-PCR

Cell pellets were collected and total RNA was isolated by the Trizol method (Invitrogen) as per the manufacturer's protocol and was quantified using spectrophotometer (Agilent, NanoDrop, Technologies Inc.), and the purity was assessed by the 260/280 nm ratio. The RNA was stored at  $-80^\circ\text{C}$ . The first strand of cDNA was synthesized using 1  $\mu$ g of RNA treated with RNase-OUT ribonuclease inhibitor and Superscript II First Strand Synthesis system (Invitrogen) as per the manufacturer's instructions. PCR was performed in 0.2-ml eppendorf tubes (Axygen) with a final volume to 12.5  $\mu$ l. The amplification program for

PCR consisted of an initial denaturation step at 94°C for 5 min, followed by 35 cycles of 30 s denaturing (94°C), 45 s annealing (temperature of the respective gene primer), 45 s elongation (72°C); a final extension at 72°C for 10 min and finally soaking at 4°C. Amplified PCR products were analyzed on 1.5% agarose gel stained with ethidium bromide (Sigma), viewed under a UV trans-illuminator, and photographed on a Gel documentation system (Pharmacia Biotech, Piscataway, NJ). GAPDH was used as the housekeeping gene, and 100-bp ladder (Invitrogen) was used as molecular weight markers.

### Immunocytochemistry

hESCs and hEBs were plated on two-well chamber slides (BD Biosciences) and allowed to grow for 2 days. Attached cells were rinsed twice with PBS, microphotographed in bright field (BF) on an inverted microscope and fixed with 4% paraformaldehyde (PFA) diluted in PBS for 20 min at room temperature (RT). Cells were permeabilization with 0.2% Triton X-100 (Sigma) and blocked with 0.1% (w/v) bovine serum albumin (BSA; Sigma) to minimize non-specific binding of antibodies. Then, the samples were incubated with primary antibodies such as Oct-4, Brachyury, GATA-4 (all Abcam); Nanog, CK-18 (Millipore) and albumin (Sigma) overnight at 4°C and washed twice in PBS to remove non-specific binding of the primary antibody. Please refer to Table 1 for other details of the antibodies used in this study. Fluorescein isothiocyanate (FITC) or rhodamine (Millipore)-conjugated secondary antibody incubation was performed at room temperature for 1 h in the dark. Cell nuclei were counterstained with 4',6-Diamidino-2-phenylindole (DAPI, 10 µg/ml; Sigma, D9542) after through washing with PBS. Cells were mounted in mounting medium (Sigma), and a glass

coverslip was mounted over the chamber slides. The photographs were captured using BX-51 microscope (Olympus, UK) equipped with an Olympus DP50 digital camera (Olympus Optical Co) and analyzed using analySIS® imaging software (Soft Image System GmbH).

### Flow cytometry analysis

For flow cytometry analysis, DMSO-treated cells along with untreated control cells were subjected to Tryple-LE (Invitrogen) at 37°C to prepare single cell suspension. Briefly, single cell suspensions were washed with DPBS (Invitrogen) and fixed with 4% paraformaldehyde at 4°C for 30 min. Cell suspension blocked with 0.1% BSA (Sigma) in 1XPBS (Invitrogen) for 30 min at room temperature. Cells were incubated on ice with primary antibodies albumin (Sigma) and Cytokeratin-18 (Millipore) for 1 h, washed with 1XPBS and subsequently stained with FITC-conjugated secondary antibodies along with a separate isotype control in the dark. FACS was performed on Guava Easy-Cyte Plus (Millipore), and data were analyzed by Cytosoft v5.2 software. A minimum of 10,000 viable cells were analyzed, and the percent positivity of specific markers was calculated based on the respective isotype control.

### Periodic acid Schiff (PAS) staining

PAS staining method was used to detect glycogen in cells and tissues. The reaction of periodic acid selectively oxidizes the glucose residues, creates aldehydes that react with the Schiff reagent and creates a purple-magenta color. Samples were fixed in 10% formalin in methanol for 1 h at room temperature and subjected to PAS staining according to the manufacturer's instructions (Sigma; 395B). Cells were counter stained with acidified Harris Hematoxylin

**Table 1** Represents the list of antibodies used in the study

Serial no.	Antigen	Antibody details	Dilution used	Brand	Application of the antibody in the study
1	Oct-4	Polyclonal rabbit immunoglobulin (ab19857)	1:50	Abcam Inc. Cambridge, USA	Cultured cells (in situ staining)
2	Nanog	Monoclonal mouse anti-human immunoglobulin (ab62734)	1:50	Abcam Inc. Cambridge, USA	Cultured cells (in situ staining)
3	B-III-tubulin	Monoclonal mouse immunoglobulin (MAB1637)	1:100	Millipore MA 01821, USA	Cultured cells (in situ staining)
4	Brachyury	Polyclonal rabbit immunoglobulin (ab20680)	1:50	Abcam Inc. Cambridge, USA	Cultured cells (in situ staining)
5	GATA-4	Polyclonal rabbit immunoglobulin (ab61767)	1:50	Abcam Inc. Cambridge, USA	Immunocytochemistry; flow cytometry
6	Albumin (ALB)	Monoclonal anti-human immunoglobulin(A6684)	1:100	Sigma-Aldrich St. Louis, MO 63103, USA	Immunocytochemistry; flow cytometry
7	Cytokeratin-18 (CK-18)	Monoclonal anti-human immunoglobulin(MAB3234)	1:100	Millipore MA 01821, USA	Immunocytochemistry; flow cytometry

basic stain. Specimens were observed with an inverted light microscope (Olympus).

### Statistical analysis

Data are presented as mean  $\pm$  SEM, and results were analyzed by one-way ANOVA with Tukey's multiple comparison post-test for more than two groups. Differences were considered statistically significant when  $P < 0.05$ .

## Results

### Quality testing of hESC

We analyzed the hESCs at every 5 passages to confirm their characteristics in terms of self-renewal and pluripotency. This provides a stringent quality testing of these cells before they are used for studying the cytotoxic effects of DMSO exposure. Figure 1a represents 5-day-old hESC colonies at passage 40 grown without feeders, showing high nucleus-to-cytoplasm ratio and shiny borders. Upon immunocytochemistry, these hESC demonstrated strong expression of stemness-related transcription factors such as Oct-4 (Fig. 1b–d) and Nanog (Fig. 1e–g), which ensured the maintenance of undifferentiated state. It was further observed that undifferentiated hESC formed typically compact, round and uniform-sized EBs in suspension cultures (Fig. 1h). To confirm the pluripotent capacity of HUES-7 cell line, we demonstrated conspicuous expression of markers associated with the formation of three germ layers such as ectoderm, B-III tubulin (Fig. 1i–k); mesoderm, Brachyury (Fig. 1l–n); and endoderm, GATA-4 (Fig. 1o–q). We also performed gene expression analysis by RT-PCR using the same set of candidate markers (data not shown) and karyotype analysis (data not shown) to lend support to our initial finding. Moreover, based on our earlier experience (Pal et al. 2009; Mamidi et al. 2010), we preferentially selected this well-characterized cell line, HUES-7, to study the effect of DMSO.

### Morphological changes in EBs caused by DMSO treatment leads to cell mortality

In untreated controls, about 90–95% of EBs retained their normal morphology and shape; and they attached within 6–8 h of plating. Upon attachment, they showed spontaneous differentiation as expected (Fig. 2a, e); we consistently observed healthy morphology and normal attachment of the EBs in all of our experiments conducted as part of the work reported here. In contrast, DMSO exposure for 24–48 h produced a dose-dependent inhibition in EB formation. Six- to 8-day-old EBs after exposure

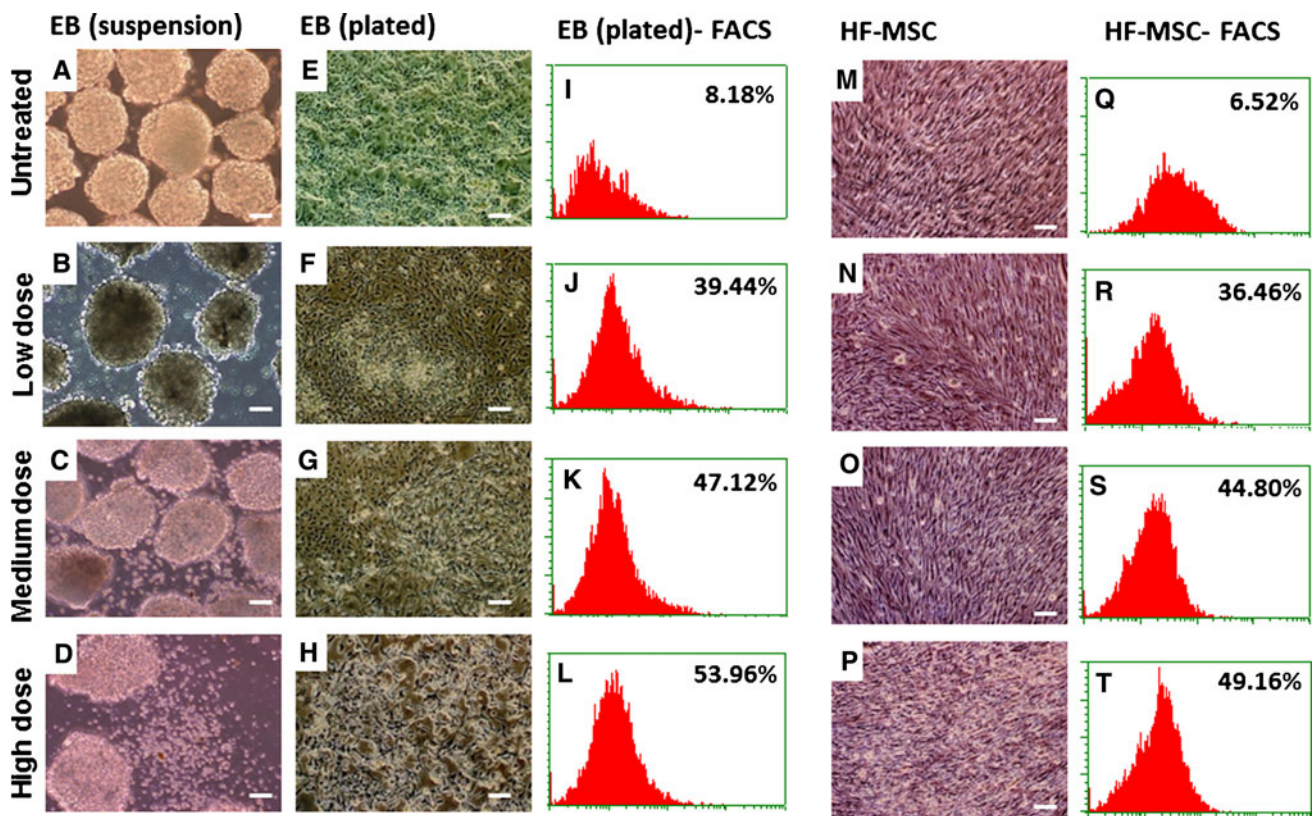
to medium and high doses of DMSO exhibited shrunken, disintegrated and dark-colored aggregates when compared to low dose of DMSO and untreated control (Fig. 2b–d). Consistent with this observation, the plated EBs after DMSO treatment continue to show adverse effects including loss of attachment and cell death particularly in response to high and medium doses of DMSO (Fig. 2f–h). Furthermore, we examined the dose-dependent effect of DMSO on human foreskin-derived mesenchymal stem cells (HF-MSC) (Fig. 2m–p) as an independent control.

Cytotoxic effect of DMSO on EBs was analyzed using flow cytometry-based 7-AAD staining method as shown earlier (Pal et al. 2011). Our data revealed that DMSO even at the lowest dose is able to induce cell death as witnessed by an increase in 7-AAD positivity when compared to untreated control cells (Fig. 2i, j). The percentage of cell mortality in medium and higher concentration of DMSO was drastically high (Fig. 2k, l). In addition, we employed HF-MSCs as an independent control to evaluate our cytotoxicity results. Although a similar pattern of cell mortality was observed in HF-MSC, the percentage of cell death induced by DMSO was a slightly lower for all three concentrations when compared to EBs (Fig. 2q–t). Further, we drew a direct comparison between hESC versus MSC, which clearly demonstrates the dose-dependent cytotoxic effect of DMSO (Fig. 3a). Therefore, we concluded that DMSO produces serious adverse effects on the EBs both before and after plating, which results in high level of cell mortality in a concentration-dependent manner.

### Differential expression of gene markers associated with tri-lineage differentiation

As a downstream event of DMSO-induced cytotoxicity, we observed a differential regulation of a subset of undifferentiated stem cell-, lineage- and tissue-specific markers in the day 14 EBs. We analyzed the lineage-specific effects of DMSO on hESC differentiation in terms of gene expression and found a uniform expression of all the gene markers in HUES-7-derived day 14 EBs (Fig. 3b, c) (Table 2). We also found that the expression of Oct-4, Sox-2 and Rex1 was maintained unambiguously among the control and DMSO-treated samples. However, the mRNA levels of Nanog were downregulated in the medium and high dose of DMSO in comparison with the control group (Fig. 3b).

In the absence of DMSO, Nestin (a primitive neural stem cell marker), TuJ1 (neuron-specific class III beta-tubulin), NEFH (neurofilament heavy polypeptide) and Krt-15 (an epithelial cell marker) were found to show uniform expression (Fig. 3c). However, in the cells treated with DMSO, the expression levels of Nestin, TuJ1, NEFH and Krt-15 exhibited a trend of downregulation in response to the highest dose; nevertheless, the expression of all these



**Fig. 2** Effect of DMSO on morphology and viability of cells. **a–d** Photomicrographs showing morphological changes in HUES-7-derived EBs treated with low, medium and high doses of DMSO in comparison with untreated healthy EBs; **e–h** abnormal cell attachment after plating of 6-day-old EBs treated in response to DMSO treatment. **m–p** As an independent control, HF-MSc was treated with same doses of DMSO and similar adverse effects on their

morphological features were observed. Effect on cell survival was evaluated by calculating the percentage of cells positive for 7-AAD by flow cytometry. **i–l** Massive reduction in cell viability is shown after DMSO treatment in a dose-dependent fashion for **i–l** plated EBs; and **q–t** HF-MScs in comparison with untreated cells. Representative data from one of three experiments are shown ( $n = 3$ ). Scale bar: 100  $\mu$ m

markers was unaltered in low and medium doses of DMSO (Fig. 3c). Interestingly, the maximum impact of DMSO was seen on the mRNA transcript levels of NFH. Overall, we noticed significant downregulation of neuroectoderm markers in case of higher doses of DMSO.

Interestingly, we observed a considerable upregulation of mesodermal markers HAND1, MEF2C, GATA-4 and C-actin in low dose of DMSO, which gradually became transient upon increase in the concentration of DMSO (Fig. 3c). In response to the highest dose of DMSO, substantial downregulation of all four mesoderm markers was witnessed (Fig. 3c). Our results reassure the long-standing concept that DMSO at low and medium concentrations can act as an inducing agent for the formation of mesodermal phenotypes (Wang et al. 2010).

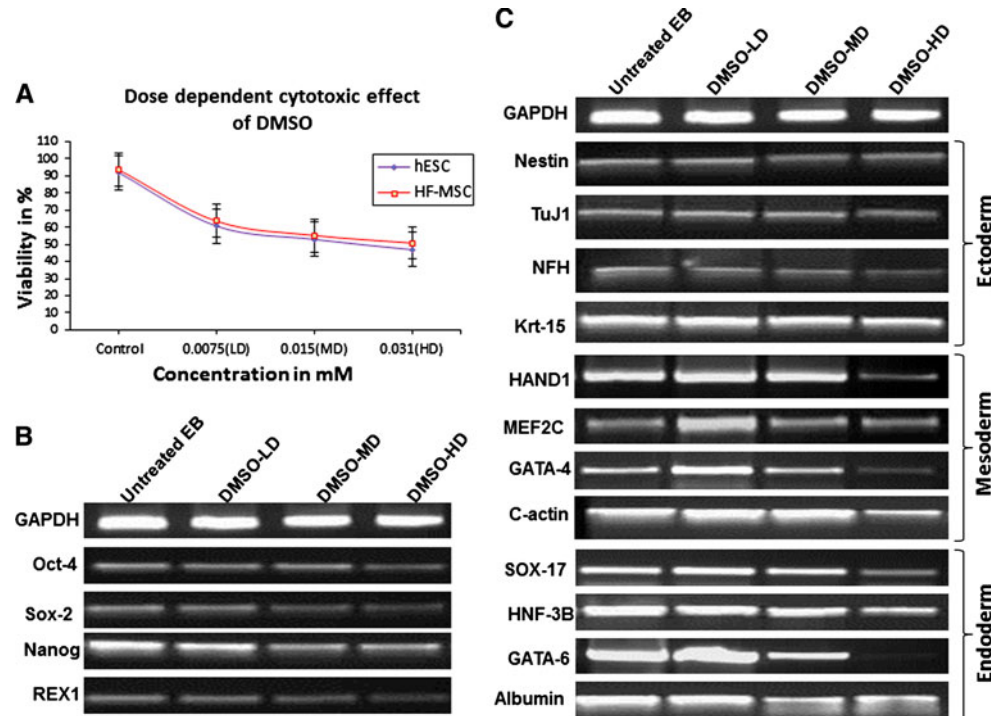
Finally, we examined the effect of DMSO on day 14 EBs in terms of their developmental propensity toward endoderm lineage using four crucial gene markers including Sox-17, HNF-3B, GATA-6 and albumin all associated with the formation of definitive endoderm through hepatocytes.

Owing to the inherent propensity of HUES-7 toward hepatic lineage (Pal et al. 2009), the expression of these markers in the control EBs was ubiquitous (Fig. 3c). However, we detected a clear downregulation in the expression of Sox-17, HNF-3B and albumin in EBs treated with medium and higher doses of DMSO (Fig. 3c). Furthermore, the presence of GATA-6 transcript was completely obliterated in response to the highest dose of DMSO (Fig. 3c). Therefore, it is evident from these experiments that DMSO at higher doses imparts severe adverse effects on the expression of lineages- and tissue-specific markers which may lead to abnormal differentiation.

DMSO affects the spontaneous differentiation of HUES-7 and hinders the formation of hepatic progenitors

Our results clearly demonstrated that the exposure of higher concentrations of DMSO had an adverse effect on the morphology, cell adhesion, cell viability and gene

**Fig. 3** DMSO-induced cytotoxicity leads to differential gene regulation. **a** Graphical representation of DMSO-induced dose-dependent cytotoxic effect was found to be similar in hESC and MSC and hence is independent of the cell type. A candidate set of markers showing differential gene expression pattern in the differentiated hESCs upon DMSO exposure was found by RT-PCR studies. **b** Expression of genes associated with undifferentiated state and; **c** lineage- as well as tissue-specific markers expression in day 14 EBs DMSO-treated and untreated cultures



expression pattern of hESC. Further, we wanted to examine whether DMSO treatment affected the inherent propensity of HUES-7 toward hepatic endoderm as reported earlier (Pal et al. 2009). We employed two separate assays to determine the impact of DMSO on spontaneous differentiation of HUES-7; namely immunocytochemistry and flow cytometry by analyzing the content of albumin and CK-18 proteins coupled with PAS staining to measure the glycogen storage of hESC-derived hepatocyte-like cells. The number of albumin-positive cells decreased dramatically, and extensive nuclear fragmentation was observed in the medium and higher doses of DMSO as witnessed by immunocytochemistry (Fig. 4a–d). Likewise FACS analysis revealed a drastic reduction in the albumin-positive population in the medium (40.4%) and high (12.7%) doses compared to untreated control (71%); however, the low dose of DMSO was unable to induce any changes in albumin immunoreactivity (Fig. 4e–h). The results showed a similar trend in case of CK-18 except that the adverse effect of the highest dose was slightly milder (21.8%) compared to albumin (Fig. 4i–l). As a second criterion, the number of cells positive for PAS staining was found to decline significantly in a dose-dependent manner (Fig. 4m–p). The cultures treated with the highest dose of DMSO failed to display barely any cells positive for PAS staining except a disintegrated mass (Fig. 4p). These results suggest that DMSO imparts a selective deleterious effect on differentiating hepatocytes by inducing cell death.

## Discussion

DMSO has been known as an organic solvent for lipophilic drugs, a differentiating agent and an excellent cryoprotectant. More specifically, DMSO is known to induce differentiation in ES and EC cells (Jasmin et al. 2010). We undertook the present study in order to clarify whether solvents like DMSO have an influence on in vitro developmental toxicity test systems. This study was designed to evaluate the effect of DMSO on development and differential potential of hESCs. We found that DMSO treatment substantially altered the morphology and attachment of cells, resulting in substantial decrease in cell viability in a dose-dependent manner. Earlier reports also describe morphologic and biochemical changes in the colonic epithelial cell line SW620 following DMSO incubation. Cells cultured in the presence of DMSO showed striking changes in morphology characterized by enlargement, elongation and formation of process-like structures by light microscopy and a propensity to form microvillus-like structures by electron microscopy (Omary et al. 1992). Similar concentrations of DMSO were found to alter locomotor activity of larval Zebra fish in a more recent study. Although no developmental defects were detected at the 0.01 and 0.1% concentrations, significantly higher deformity rates occurred with 1% DMSO groups (Chen et al. 2011). These results are in agreement with our data, indicating concentration-dependent degree of damage induced by DMSO treatment. It is well known that DMSO can

**Table 2** Details of the reaction conditions and semi-quantitative RT-PCR primers analyzed during hepatic differentiation of hESC

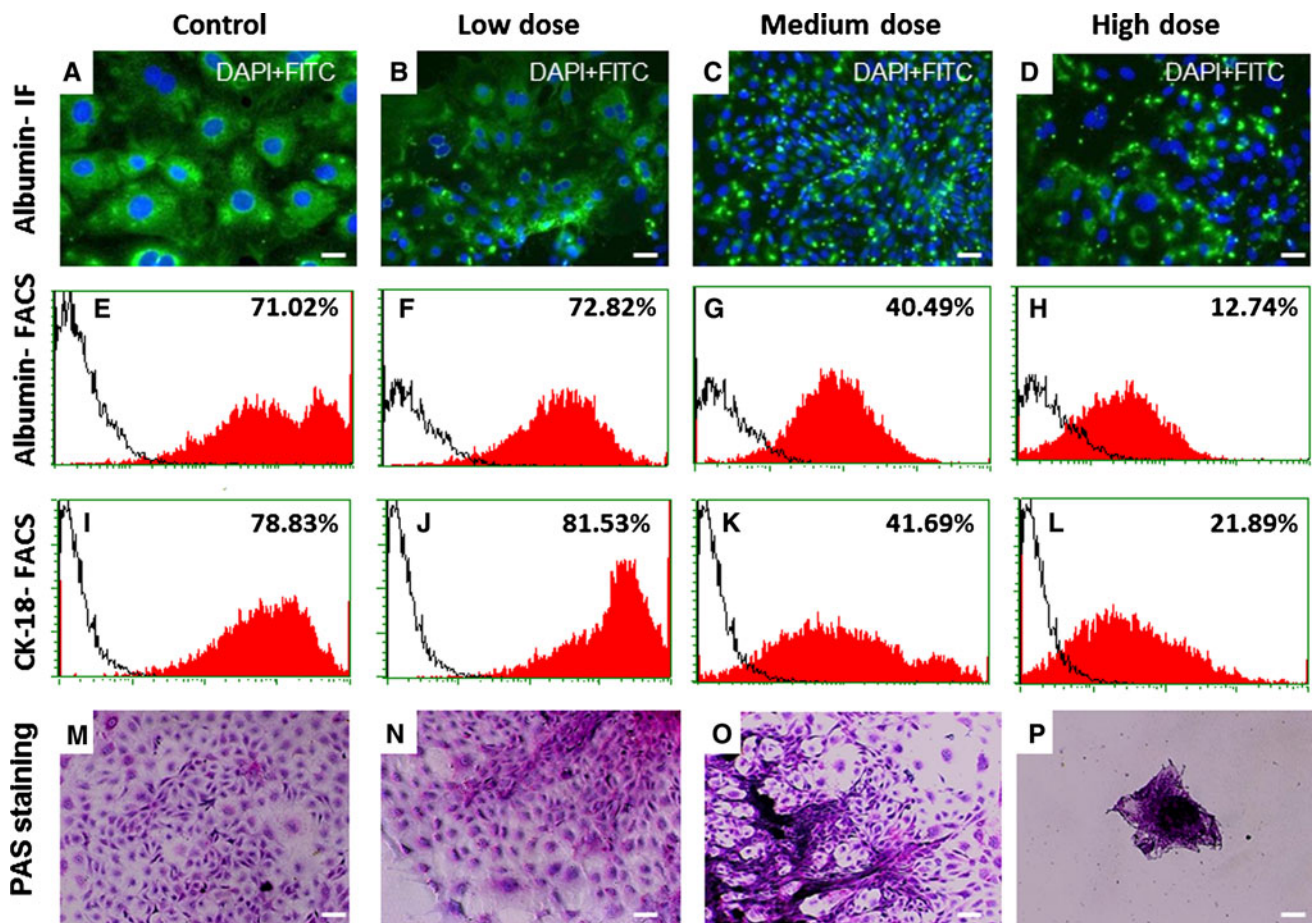
Serial no.	Gene symbol	Primer sequences (5'–3')	Annealing temperature (°C)	Product size (bp)	Gene bank accession number
1	GAPDH	5'-TGAAGGTCGGAGTCAACGGATT-3' 5'-CATGTGGGCCATGAGGTCCACCAC-3'	60°C	983	NM_002046.3
2	Oct-4	5'-CGACCATCTGCCGCTTTGAG-3' 5'-CGACCATCTGCCGCTTTGAG-3'	57°C	572	NM_203289.3
3	Sox-2	5'-CCCCGGCGGCAATAGCA-3' 5'-TCGGCGCCGGGAGATACAT-3'	55°C	447	NM_003106.2
4	NANOG	5'-TCCTCCATGGATCTGCTTATTCA-3' 5'-CAGGTCTTCACCTGTTTGTAGCTGAG-3'	58°C	259	NM_024865.2
5	Rex1	5'-GCGTACGAAATTAAGTCCAGA-3' 5'-ATCCTAAACAGCTCGCAGAAT-3'	55°C	302	NM_174900.3
6	Nestin	5'-CAGCGTTGGAACAGAGGTTGG-3' 5'-TGGCACAGGTGTCTCAAGGGTAG-3'	58°C	388	NM_006617.1
7	TuJ1	5'-AACAGCACGGCCATCCAGG-3' 5'-CTTGGGGCCCTGGGCCTCCGA-3'	58°C	242	NM_006086.2
8	NEFH	5'-ACGCTGAGGAATGGTTCAAG-3' 5'-GCCTCAATGGTTTC-3'	58°C	555	NM_006158.2
9	Krt-15	5'-CACAGTCTGCTGAGGTTGGA-3' 5'-GAGCTGCTCCATCTGTAGGG-3'	60°C	196	NM_002275.3
10	HAND1	5'- TGCCTGAGAAAGAGAACCAG-3' 5'- AGGATGAACAAACAC-3'	58°C	245	NM_004821.1
11	MEF-2C	5'- GATGCGGACGATTCCGTAGG-3' 5'- TGGTGCCTGCACCAGACGTG-3'	59°C	327	NM_002397.3
12	GATA-4	5'- CTCCTTCAGGCAGTGAGAGC-3' 5'- GAGATGCAGTGTGCTCGTGC-3'	58°C	574	NM_002052.2
13	C-actin	5'- TCTATGAGGGCTACGCTTTG -3' 5'- CCTGACTGGAAGGTAGATGG -3'	59°C	668	NM_005159.4
14	Albumin	5'-CCTTTGGCACAATGAAAGTGGGTAACC-3' 5'-CAGCAGTCAGCCATTCACCATAGG-3'	55°C	354	NM_000477.3
15	SOX-17	5'-CGCACGGAATTTGAACAGTA-3' 5'-GGATCAGGGACCTGTCACAC-3'	58°C	181	NM_022454.3
16	HNF-3 $\beta$ (FOXA2)	5'- GACAAGTGAGAGAGCAAGTG-3' 5'- ACAGTAGTGAAACCGGAG-3'	56°C	234	NM_153675.1
17	GATA-6	5'-GCCTCACTCCACTCGTGTCT-3' 5'-TCAGATCAGCCACACAATATGA-3'	58°C	541	NM_005257.3

cause growth arrest and terminal differentiation of transformed cells (Marks and Breslow 2007). In this context, our data are of significance wherein we show that DMSO also perturbs spontaneous differentiation of hESCs unraveling for the first time the broad spectrum of its action covering important phases of growth, development and differentiation. It appears from all the aforementioned studies that DMSO action not only varies from species to species and cell to cell but also depends on the stage of development and differentiation as well as on appropriate concentration of DMSO. This feature of DMSO is further evidenced by the fact that DMSO, at concentrations of

1–2%, induces terminal differentiation in several different cell types in vitro and enhances the growth of newborn mouse epidermal cells in primary culture under conditions that also permit terminal differentiation, whereas DMSO concentrations approaching 4% reversibly inhibit (with little overt toxicity) terminal differentiation of normal epidermal cells from newborn SENCAR mice (Miller et al. 1991).

DMSO is generally considered to be genetically inactive and is thus very frequently used as a solvent in drug-screening assays. However, its ability to induce cell differentiation indicates that DMSO might exert an influence





**Fig. 4** Impairment in functional activity of differentiated cells after DMSO exposure. **a–d** Immunocytochemical analysis demonstrated that reactivity of cells against albumin antibody suffered a dose-dependent decrease coupled with nuclear fragmentation in the highest dose of DMSO. FITC was used as a conjugate, and the nuclei were counter stained with DAPI. Next quantitative analysis of the proteomic content by flow cytometry was performed to estimate the

percentage positivity toward hepatocyte-specific antibodies **e–h** albumin and **i–l** CK-18, which clearly displayed a remarkable reduction in the number of immunopositive cells again in a dose-dependent manner. These observations were further confirmed by functional assessment of **m–p** glycogen storage ability in the differentiated cells by PAS staining. All data are representative of three separate experiments ( $n = 3$ ). Scale bar: 100  $\mu$ m

at the genetic regulation level. Likewise, we show that there is an incremental and differential upregulation/downregulation of genes specific to three germ layers depending on the concentration of DMSO. Overall, we noticed significant downregulation of ectoderm markers (Nestin, TuJ1, NEFH and Krt-15) at higher doses of DMSO, whereas the expression of all these markers was almost unchanged in cells treated with low and medium doses of DMSO. Evaluation of mesodermal markers like HAND1, MEF2C, GATA-4 and C-actin revealed a considerable upregulation of all these genes in low and medium dose, whereas highest dose of DMSO triggered significant downregulation of HAND1, GATA-4 and C-actin. These results indicate that DMSO at low and medium doses can act as an inducing agent for the formation of mesodermal phenotypes as also evidenced by the earlier reports (Wang et al. 2010). Although DMSO is known to differentiate P19CL6 embryonic carcinoma cells

into cardiomyocyte-like cells, the low differentiation capacity of DMSO reduces its usefulness (Seya et al. 2011). Interestingly, we further observed a distinct downregulation in the levels of Sox-17, HNF-3B and albumin in cells treated with medium and higher doses of DMSO. Moreover, the expression of GATA-6 was completely abolished in response to the highest dose of DMSO, indicating its selective adverse effect on endodermal derivatives more precisely on the hepatocyte-like cells. The hepatic lineage-specific action of DMSO was confirmed by a radical reduction in the number of albumin- and CK-18-positive cells and increased nuclear fragmentation seen in the DMSO-treated groups especially with medium and high doses. Further, the number of cells positive for PAS staining diminished remarkably after DMSO treatment, indicating loss of glycogen deposits in the hESC-derived hepatocyte-like cells. Our results are in agreement with the latest report demonstrating the inhibition of CYP1A2-

mediated phenacetin O-deethylation even at low concentrations (0.1%) of DMSO (Nirogi et al. 2011), indicating hepatotoxic potential of DMSO.

Taken together, our data demonstrate for the first time that DMSO-induced toxicity severely affects the endodermal and hepatic lineage while sparing mesodermal and ectodermal lineages in a concentration-dependent manner. Although at higher concentrations DMSO adversely affects the development of all the three germ layers possibly leading to abnormal growth of embryo, the lower concentrations of DMSO enhance mesodermal differentiation, although there is no significant action on ectodermal development. This important finding of preferential behavior of DMSO has been supported by the recent study reporting no toxic effect on the neural and arterial tissues of rats when it is slowly infused into the carotid artery (Baker et al. 2011).

The action of DMSO in different cellular processes may be mediated through common molecular mechanisms. For instance, DMSO has been shown to inhibit c-myc expression (Darling et al. 1989), arrest cell cycle progression thus affecting cell proliferation in different cell types (Srinivas et al. 1991). Further DMSO may lead to the collapse of mitochondrial membrane potential, release of cytochrome c from the mitochondria and activation of caspase-9 and caspase-3 (Liu et al. 2001). We hypothesize that the possible mechanism of DMSO action involves a modulation of cytoplasmic  $Ca^{2+}$  concentration; a large spike of intracellular  $Ca^{2+}$  concentration may play a role in the induction of cell differentiation by DMSO. The enhancement in the expression of cardiac markers observed in EBs may be attributed to this mechanism. Further DMSO might increase membrane fluidity thereby altering the transport functions and permeability properties of the membrane causing the “release” of an intracellular messenger, which would itself be responsible for initiating the transcription of mRNA necessary for differentiation. It can even act at a transcriptional level, and more precisely, by interfering with the binding of the repressor to the operator portion of an operon. Likewise, the transcriptional silencing of hepatocyte markers resulting in the mitigation of hepatic differentiation from HUES-7 in response to DMSO exposure can be ascribed to a combination of these proposed mechanisms.

Apart from our findings, it is evident that there are several other reports depicting the cellular and molecular effects of DMSO such as different effects related to inflammation, lipid metabolism, apoptosis, cell cycle, protein expression, differentiation, molecular binding, enzyme activity, reactive oxygen species scavenging, cell polarization, cryopreservation and other experimental procedures. Considering the multitude of effects of DMSO, it is easy to predict how many researchers working with DMSO or studying one of its specific effects can be oblivious of the results of other groups working with it in a different context.

The lack of an immaculate understanding underpinning the diverse effects of DMSO can cause impediment toward reaching of accurate conclusions, since experimental artifacts produced by DMSO can lead to aberrant interpretation of results. We strongly believe that an increased awareness of the multidisciplinary utilization of this molecule in several research fields can offer a valuable contribution in order to avoid or minimize these issues.

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