Another face Of DMSO: Effects of dimethyl sulfoxide (DMSO) on the pluripotency and differentiation capacity of mouse embryonic stem cells

JunKoo Yi (79lee38@korea.kr)
Centre National d'Elevage et de Recherches Veterinaires https://orcid.org/0000-0003-2593-6529
Jae-Jung Ha
Gyeongbuk Livestock Research Institute
Dae-Hyun Kim
Gyeongbuk Livestock research Institute
Zae-Young Ryoo
Kyungpook National University College of Natural Sciences
Sung-Hyun Kim
Korea Polytechnic University
Myoung-Ok Kim
Kyungpook National University

Research article

Keywords: mESCs, DMSO, LIF, pluripotency markers

Posted Date: October 3rd, 2019

DOI: https://doi.org/10.21203/rs.2.15531/v1

Abstract

Background Mouse embryonic stem cells (mESCs) go through self-renewal in the existence of the cytokine leukemia inhibitory factor (LIF). LIF is add to the mouse stem cells culture medium, and its removal results in fast differentiation. Dimethyl sulfoxide (DMSO) is one of the most general used solvents in drug test.

Methods We exposed 4-day mESC cultures to different concentrations of DMSO (0.1%, 0.5%, 1.0%, and 2.0%) to identify the safest dose exhibiting efficacy as a solvent. mESCs grown under general pluripotency conditions in the absence of LIF were treated with DMSO. In addition, as a control for differentiation, mESCs were grown absence of LIF.

Results DMSO downregulated the mRNA expression level of pluripotency markers. Moreover, DMSO reduced the mRNA expression levels of ectodermal marker (β-tubulin3) and a mesodermal marker (Hand1) and endodermal markers (Foxa2 and Sox17) in mESCs. These results indicate that DMSO treatment enhances the pluripotency and disrupts the differentiation of mESCs. We also show that members of the Tet oncogene family are critical to inhibiting the differentiation and methylation of mESCs.

Conclusion DMSO is appropriate to sustain the pluripotency of mESCs in the absence of LIF, and that mESCs can be sustained in an undifferentiated state using DMSO. Therefore, DMSO may, in part, function as a substitute for LIF.

Background

Dimethyl sulfoxide (DMSO) is a usual industrial solvent with amphiphilic properties, which can solvate a widespread substances. Though, several studies have shown that DMSO can induce differentiation of various cells, including embryonic teratocarcinoma cells and embryonic stem cells (ESCs) [1].

Mouse ESCs (mESCs) have pluripotency and unlimited capacity for differentiation and self-renewal. Pluripotency markers (Oct4, Sox2, Lin28, Nanog), and alkaline phosphatase (AP) are significant for sustaining the cells' pluripotency and self-renewal capacity [2–4]. mESCs obtained from preimplantation embryos can be cultured in an undifferentiated state, and are considered an suitable model for preimplantation toxicity test. ESCs have been generally used in the study of early embryonic state of pluripotent cell and development [5–8].

On removal of leukemia inhibitory factor (LIF) and feeder cells grown on non -adhesive dishes, mESCs differentiate into cells of three primary germ layers: the endoderm, mesoderm, and ectoderm. LIF is indispensable for maintaining mESC pluripotency. Removal of LIF allows stem cells to differentiate, but these cells retain their proliferative potential and pluripotency [9]. Therefore, LIF is used in the culture of mESCs. LIF is necessary to sustain stem cells in an undifferentiated state. Though, genetic manipulation

of ESCs enables LIF-independent growth, particularly by over-expression of the gene Nanog [10]. LIF is added to stem cell culture medium to decrease inherent differentiation [11].

DNA methylation is a stable epigenetic mark that is critical for diverse biological processes including gene and transposon silencing, imprinting, and the suppression of retrotransposons[12]. DNA demethylation come about in the early embryo and the germ cells [13, 14], and may be mediated by the ten eleven translocation Tet family of enzymes (Tet1,2,3) [15–17], which convert 5-methylcytosine (mC) to 5-hydroxymethylcytosine (hmC) [18]. Tet enzymes have been extensively studied in mESCs [19–22]. Here, we report that the addition of DMSO to mESCs promotes Tet activity, leading to a increase in the number of mESCs.

In the present study, we developed a novel and robust method for the differentiation of mESCs into the three germ layers and evaluation of their methylation potential by the addition of DMSO. DMSO has diverse applications in cell culture [23–28], as it is used to maintain the function of cultured hepatocytes *in vitro* [29] and to generate mESC derived hepatic progenitor/hepatoblasts [30]. We added DMSO to an LIF-based medium during differentiation to efficiently and concertedly downregulate mESC pluripotency genes and upregulate germ layer markers, and to study the effects of DMSO on DNA methylation potential in mESCs.

Results

DMSO affects the morphology and pluripotency of mESCs

DMSO has been shown to regulate epigenetic modification by altering CpG methylation patterns in diverse E14 mESCs and tissues [31, 32], thus affecting their development and differentiation [33]. To study the effects of DMSO on cell morphology and differentiation, E14 mESCs were exposed to diverse concentrations of DMSO for 96 h. mESCs were collected after 4 days of culture in the presence of diverse concentrations of DMSO (0.1%, 0.5%, 1.0%, or 2.0%) or with the fundamental pluripotency factor LIF as a control for self-renewal and pluripotency. Colony growing as a round and dome shape could be seen under control conditions, whereas cells growing without LIF no longer formed these typical pluripotent colony. On the other hand, higher levels of differentiation were apparent, resulting in fewer colonies without defined borders. Rather, cultures grown without LIF in the presence of 1.0% and 2.0% DMSO produced well-defined, round colonies and exhibited a reduced number of differentiated cells in comparison with the differentiation control (without LIF) (Fig. 1A).

To determine whether DMSO influences pluripotency, we stained mESCs with AP. Differentiation of mESCs is characterized by the loss of AP staining and the emergence from a flattened cellular morphology [34]. In the present study, mESCs treated with DMSO for 96 h were resembling the control (with LIF), showing deep AP staining and a similar colony morphology (Fig. 2). Given that pluripotent cells express high levels of AP, we also performed an AP assay to monitor the number of AP-positive red colonies [35, 36] (Fig. 2). The differentiation control (without LIF) showed a low rate of AP-positive

colonies, while cells growing without LIF in the presence of DMSO had a higher rate of AP-positive colonies. Treatment with 1.0% DMSO showed the highest rate of AP-positive colonies.

DMSO regulates the expression of mESC pluripotency genes in a concentration-dependent manner

To determine whether DMSO influences mESC differentiation, cells were treated with DMSO and the effect on differentiation in the absence of LIF was evaluated. mESCs were exposed to various concentrations of DMSO (0.1%, 0.5%, 1.0%, or 2.0%) or LIF as a control for 96 h, and then collected after 4 days of culture. The expression levels of pluripotency markers such as Oct4, Sox2, and Lin28 were analyzed by qRT-PCR (Fig. 3A–C). Treatment with 0.1%, 0.5%, and 1.0% DMSO increased Oct4, Sox2, and Lin28 mRNA levels after 4 days in a dose-dependent manner. Treatment with 2.0% DMSO, however, resulted in a significant decrease in Oct4, Sox2, and Lin28 mRNA expression levels after 4 days of culture.

mRNA expression levels of markers of endoderm, mesoderm and ectoderm were also examined by qRT-PCR (Fig. 4A–D). mRNA levels of the endoderm marker Foxa2 decreased significantly when mESCs were treated with 0.5% or 2.0% DMSO (Fig. 4A). mRNA levels of another endoderm marker, Sox17, also decreased in a dose-dependent manner following DMSO treatment (0.1%, 0.5%, and 1.0%) (Fig. 4B). Treatment with 2.0% DMSO, however, significantly increased Sox17 mRNA levels in comparison with those of the control. The mRNA levels for the mesoderm marker Hand1 and the ectoderm marker β-tubulin 3 also decreased in a dose-dependent manner with DMSO treatment (Fig. 4C, D). On the whole, we find out pluripotency markers were differentially expressed, suggesting that DMSO biases differentiation towards the three germ layers.

DMSO also affects the mRNA levels of genes involved in active DNA methylation

To evaluate the effect of DMSO on the active DNA methylation of mESCs, qRT-PCR was used to assess mRNA expression levels of two members of the TET oncogene family: *Tet1 (*Tet oncogene 1) and *Tet2 (*Tet oncogene 2), following DMSO treatment. Expression of these genes after 4 days of culture increased in a dose-dependent manner; however, expression was downregulated by 2.0% DMSO treatment (Fig. 1A, B). mRNA expression of the third member of the TET family, *Tet3,* was increased significantly in a dose-dependent manner by DMSO treatment. These findings suggest that regulating the expression of the TET oncogene family members is critical for proper differentiation and methylation of mESCs.

Discussion

ESCs have properties like pluripotency, self-renewal and continuous proliferation [2, 37–39]. For such properties, the mESCs can be maintained by the cytokine LIF, and LIF is regularly added to the culture of mESCs. Conversely, if LIF is not added, it brings the differentiation of mESCs. Because of such LIF

properties, a lot of researchers have conducted the studies related to the embryonic stem cells, focused on such LIF properties with great interest.

DMSO is generally solvent for many different kinds of organic substance, and it is widely used for cryopreserve of cell as cryoprotectant. In particular, in case of culturing ESCs, DMSO regulates the differentiation of cell. This research came to conduct this study in order to examine the effect of solvent like DMSO on in vitro development [40]. Especially, this study was really designed to investigate the effect of DMSO on the E14 mESCs and mouse embryonic body mEBs. The deduced result in this study showed the deeper understanding of the effect of DMSO on the early embryonic development. The treatment of DMSO increased the expression of pluripotency markers at mRNA level of E14 mESCs and decreased the expression of germ layer markers under mEBs condition. Overall, according to our data, this study has found out that DMSO played an important role in regulating the pluripotency of mESCs and differentiation of mEBs through the regulation of expression for pluripotency markers (Oct4, Sox2, Lin28, and Nanog) related to the pluripotency [41, 42].

It is important to understand the molecular mechanisms that control pluripotency and self-renewal in stem cells for application in the fields of developmental biology, regenerative medicine, and cancer biology. Pluripotency is sustained by a synergistic interaction between extrinsic stimulus and intrinsic circuitry, which allow for sustentation of an undifferentiated and self-renewing state. Notwithstanding, despite recent study, the precise mechanisms regulating differentiation and self-renewal remain unclear. LIF is widely used for culturing the mESCs and IPSs and inhibiting the differentiation of cell by paracrine signal while especially LIF stimulates the ESCs self renewal at mESCs [43–45]. The use of feeder cells is replaceable by adding LIF to culture solution, and serum is replaceable with BMP. Since such discovery, the condition of ESCs culture has greatly improved [46]. However, it is still difficult situation in the mechanism that DMSO affects mESCs, but this study has shown that DMSO could maintain pluripotency at mESCs without LIF.

Oct4, Sox2, Lin28, and Nanog are master modulators of pluripotency. Latest studies have suggested that Oct4, Sox2, Lin28, and Nanog form feed forward loops that maintain pluripotency of mESCs [47, 48]. The expression of Oct4 governs the fate of ES cells, and regulation of this protein induces divergent developmental programs [49]. We report here that DMSO treatment up-regulates Oct4, Sox2, and Lin28 in mESCs. Additionally, DMSO specifically inhibits the expression of endodermal, mesodermal, and ectodermal lineage markers in mESCs. Our studies enhance understanding of DMSO and its effects of mouse embryonic development.

Defining the biological significance of the TET family in development has been a primary focus of recent investigations. Oct4, Sox2, and Nanog significantly raise the expression of Tet genes at an early stage of development, and the interaction of Tet with SOX2 and NANOG promotes efficient somatic cell reprogramming [50]. Interactions between TET family members and pluripotency markers (Oct4, Nanog, and Sox2) have been observed in ESCs. In fact, TET1 and TET2 proteins were detected in pluripotency factor precipitation complexes [50]. TET proteins could function with pluripotency markers in a ordinary

pathway to control cell reprogramming and maintain mESC pluripotency. We investigated whether DMSO treatment had any effect on active DNA methylation in mESCs and, specifically, on the interaction between TET proteins and pluripotency markers (Oct4, Sox2, and Lin28) in mESCs. As shown by the qRT-PCR analyses, DMSO affected mRNA expression of the TET family of pluripotency genes involved in active DNA methylation.

Conclusions

We report the upregulation of pluripotency marker genes, including Oct4, Sox2, and Lin28, in mESCs upon DMSO treatment. Additionally, DMSO treatment specifically regulated the expression of endodermal, mesodermal, and ectodermal markers and members of the TET family in mEBs. These results improve our understanding of the effects of DMSO on mouse embryonic development and methylation. We found that DMSO is sufficient to sustain the pluripotency of mESCs in the without LIF, and that mESCs can be sustained in an undifferentiated state using a combination of LIF and DMSO.

Methods

mESC culture and differentiation

E14 mESC line (provided by Jeong Mook Lim, Seoul National University, Seoul, Korea) was cultured in serum-free Advanced® DMEM (Invitrogen UK Ltd., Paisley, UK) containing 10% FBS, 1% penicillin /streptomycin, and 1000 U/ml LIF or absence LIF(Millipore, Nottingham, UK) as previously described. Cells were passaged every 3 days and plated at a concluding mass of 5000 cells/cm^2 for all culture experiments [51]. To promote differentiation, ESCs were cultured in the presence (pluripotency control) or absence (differentiation control) of LIF and with various doses of DMSO plus LIF. Thus, cells in the DMSO conditions were likened to mESCs cultured with or without LIF. Further details of the DMSO experiments are shown below.

Alkaline phosphatase (AP) assay

E14 mESCs, which are pluripotency and self-renewing, produce high levels of AP [35]. An Alkaline Phosphatase Detection Kit (Millipore) was used to assay AP activity following the manufacturer's instructions. Shortly, mESCs were cultured in six-well plates for 96 h, medium was eliminated, and were fixed with 4% paraformaldehyde for 1 min [51]. mESCs were washed and the AP reagent was added. After 20 min of incubation at room temperature (RT) in the dark, the reagent was removed and D-PBS was added. All colonies in the plates were counted. All experiments were performed in triplicate.

DMSO treatment

E14 mESCs were used to evaluate the dose-dependent effect of DMSO (Sigma) to assess the solvent toxicity at RT. DMSO was diluted in culture medium to final concentrations of 0.1%, 0.5%, 1.0%, and 2.0% (v/v), corresponding to low, medium, and high doses, respectively. DMSO was applied for 96 h to cells in suspension and after plating, and the effects of the various doses of DMSO compared with the controls (with and without LIF) were evaluated.

RNA isolation and quantitative real-time PCR assay

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration and quality were used a NanoDrop 2000 spectrophotometer (Thermo Scientific), and samples with an absorbance at 260/280 nm ratio of less than 1.8 were discarded [52]. Real-time PCR for the quantification of gene expression was carried out on an ABI7900 Fast Real-Time System (Applied Biosystems) according to the manufacturer's instructions. Synthesis of cDNAs for coding genes were carry out using 1 μg of total RNA according to the manufacturer's instructions (Takara). mRNA expression levels of were surveyed by real-time PCR using SYBR Green PCR Master Mix reagent kits (Takara). Internal control used GAPDH. The sequences of primers are shown in Table 1.

Statistical analysis

Values are expressed as means ± standard errors (S. E.) of the mean. Statistically significant differences between the DMSO treats(0.1%, 0.5%, 1.0%, or 2.0%) and controls were analyzed by ANOVA, followed by Dunnett's multiple comparisons test. All tests of statistical significance were two-sided, and p-values <0.05 were considered to indicate statistical significance.

Abbreviations

mESCs: Mouse embryonic stem cells

mEB: Mouse embryonic body

LIF: leukemia inhibitory factor

DMSO: Dimethyl sulfoxide

AP: alkaline phosphatase

mC: methylcytosine

hmC: hydroxymethylcytosine,

FBS: fetal bovine serum

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Funding

This research was supported by the Basic Science Research Program of the National Research Foundation of Korea (NRF), funded by the Ministry of Education (2014R1A6A3A04059193) and Science and Technology (No. 2014R1A1A2059813). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Authors' contributions

JK and JJ performed the majority of experiments, analyzed the data, and wrote the manuscript; DH and SH participated in animal experiments; ZY assisted with data analysis and edited the manuscript, MO designed coordinated the research. All authors had read and approved the manuscript, and ensure that this is the case.

Acknowledgements

Not applicable.

References

1.Jacob SW, Herschler R: *Pharmacology of DMSO. Cryobiology* 1986, *23(*1):14–27.

2.Chambers I, Smith A: *Self-renewal of teratocarcinoma and embryonic stem cells. Oncogene* 2004, *23(*43):7150–7160.

3.Wang J, Rao S, Chu J, Shen X, Levasseur DN, Theunissen TW, Orkin SH: *A protein interaction network for pluripotency of embryonic stem cells. Nature* 2006, *444(*7117):364–368.

4.Chen X, Xu B, Han X, Mao Z, Talbot P, Chen M, Du G, Chen A, Liu J, Wang X *et al: Effect of bisphenol A on pluripotency of mouse embryonic stem cells and differentiation capacity in mouse embryoid bodies. Toxicol In Vitro, 27(*8):2249–2255.

5.Moorthy PP, Kumar AA, Devaraj H: *Expression of the Gas7 gene and Oct4 in embryonic stem cells of mice. Stem Cells Dev* 2005, *14(*6):664–670.

6.Vallier L, Pedersen RA: *Human embryonic stem cells: an in vitro model to study mechanisms controlling pluripotency in early mammalian development. Stem Cell Rev* 2005, *1(*2):119–130.

7.Sroczynska P, Lancrin C, Pearson S, Kouskoff V, Lacaud G: *In vitro differentiation of mouse embryonic stem cells as a model of early hematopoietic development. Methods Mol Biol* 2009, *538:*317–334.

8.Balmer NV, Weng MK, Zimmer B, Ivanova VN, Chambers SM, Nikolaeva E, Jagtap S, Sachinidis A, Hescheler J, Waldmann T *et al: Epigenetic changes and disturbed neural development in a human embryonic stem cell-based model relating to the fetal valproate syndrome. Hum Mol Genet, 21(*18):4104–4114.

9.Youngblood BA, Alfano R, Pettit SC, Zhang D, Dallmann HG, Huang N, Macdonald CC: *Application of recombinant human leukemia inhibitory factor (LIF) produced in rice (Oryza sativa L.) for maintenance of mouse embryonic stem cells. J Biotechnol, 172:*67–72.

10.Ogawa K, Matsui H, Ohtsuka S, Niwa H: *A novel mechanism for regulating clonal propagation of mouse ES cells. Genes Cells* 2004, *9(*5):471–477.

11.Kawahara Y, Manabe T, Matsumoto M, Kajiume T, Yuge L: *LIF-free embryonic stem cell culture in simulated microgravity. PLoS One* 2009, *4(7*):e6343.

12.Bird A: *DNA methylation patterns and epigenetic memory. Genes Dev* 2002, *16(*1):6–21.

13.Smith ZD, Chan MM, Mikkelsen TS, Gu H, Gnirke A, Regev A, Meissner A: *A unique regulatory phase of DNA methylation in the early mammalian embryo. Nature, 484(*7394):339–344.

14.Seisenberger S, Andrews S, Krueger F, Arand J, Walter J, Santos F, Popp C, Thienpont B, Dean W, Reik W: *The dynamics of genome-wide DNA methylation reprogramming in mouse primordial germ cells. Mol Cell, 48(*6):849–862.

15.Gu TP, Guo F, Yang H, Wu HP, Xu GF, Liu W, Xie ZG, Shi L, He X, Jin SG *et al: The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. Nature, 477(*7366):606–610.

16.Yamaguchi S, Hong K, Liu R, Shen L, Inoue A, Diep D, Zhang K, Zhang Y: *Tet1 controls meiosis by regulating meiotic gene expression. Nature, 492(*7429):443–447.

17.Hackett JA, Sengupta R, Zylicz JJ, Murakami K, Lee C, Down TA, Surani MA: *Germline DNA demethylation dynamics and imprint erasure through 5-hydroxymethylcytosine. Science, 339(*6118):448–452.

18.Tahiliani M, Koh KP, Shen Y, Pastor WA, Bandukwala H, Brudno Y, Agarwal S, Iyer LM, Liu DR, Aravind L *et al: Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. Science* 2009, *324(*5929):930–935.

19.Pastor WA, Pape UJ, Huang Y, Henderson HR, Lister R, Ko M, McLoughlin EM, Brudno Y, Mahapatra S, Kapranov P *et al: Genome-wide mapping of 5-hydroxymethylcytosine in embryonic stem cells. Nature, 473(*7347):394–397.

20.Wu H, D'Alessio AC, Ito S, Xia K, Wang Z, Cui K, Zhao K, Sun YE, Zhang Y: *Dual functions of Tet1 in transcriptional regulation in mouse embryonic stem cells. Nature, 473(*7347):389–393.

21.Williams K, Christensen J, Pedersen MT, Johansen JV, Cloos PA, Rappsilber J, Helin K: *TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. Nature, 473(*7347):343–348.

22.Ficz G, Branco MR, Seisenberger S, Santos F, Krueger F, Hore TA, Marques CJ, Andrews S, Reik W: *Dynamic regulation of 5-hydroxymethylcytosine in mouse ES cells and during differentiation. Nature, 473(*7347):398–402.

23.Hollebeeck S, Raas T, Piront N, Schneider YJ, Toussaint O, Larondelle Y, During A: *Dimethyl sulfoxide (DMSO) attenuates the inflammatory response in the in vitro intestinal Caco–2 cell model. Toxicol Lett, 206(*3):268–275.

24.Pal R, Mamidi MK, Das AK, Bhonde R: *Diverse effects of dimethyl sulfoxide (DMSO) on the differentiation potential of human embryonic stem cells. Arch Toxicol, 86(*4):651–661.

25.Thaler R, Spitzer S, Karlic H, Klaushofer K, Varga F: *DMSO is a strong inducer of DNA hydroxymethylation in pre-osteoblastic MC3T3-E1 cells. Epigenetics, 7(*6):635–651.

26.Singh MK, Singh KP, Kumar D, Shah RA, Anand T, Chauhan MS, Manik RS, Singla SK, Palta P: *Buffalo (Bubalus bubalis) ES cell-like cells are capable of in vitro skeletal myogenic differentiation. Reprod Domest Anim, 48(*2):284–291.

27.Friend C, Scher W, Holland JG, Sato T: *Hemoglobin synthesis in murine virus-induced leukemic cells in vitro: stimulation of erythroid differentiation by dimethyl sulfoxide. Proc Natl Acad Sci U S A* 1971,

*68(*2):378–382.

28.Yoshizawa T, Watanabe S, Hirose M, Miyazaki A, Sato N: *Dimethylsulfoxide maintains intercellular communication by preserving the gap junctional protein connexin32 in primary cultured hepatocyte doublets from rats. J Gastroenterol Hepatol* 1997, *12(*4):325–330.

29.Su T, Waxman DJ: *Impact of dimethyl sulfoxide on expression of nuclear receptors and drug-inducible cytochromes P450 in primary rat hepatocytes. Arch Biochem Biophys* 2004, *424(*2):226–234.

30.Hay DC, Zhao D, Fletcher J, Hewitt ZA, McLean D, Urruticoechea-Uriguen A, Black JR, Elcombe C, Ross JA, Wolf R *et al: Efficient differentiation of hepatocytes from human embryonic stem cells exhibiting markers recapitulating liver development in vivo. Stem Cells* 2008, *26(*4):894–902.

31.Iwatani M, Ikegami K, Kremenska Y, Hattori N, Tanaka S, Yagi S, Shiota K: *Dimethyl sulfoxide has an impact on epigenetic profile in mouse embryoid body. Stem Cells* 2006, *24(*11):2549–2556.

32.Marks PA, Breslow R: *Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. Nat Biotechnol* 2007, *25(*1):84–90.

33.Cheung WM, Ng WW, Kung AW: *Dimethyl sulfoxide as an inducer of differentiation in preosteoblast MC3T3-E1 cells. FEBS Lett* 2006, *580(*1):121–126.

34.Ivanova N, Dobrin R, Lu R, Kotenko I, Levorse J, DeCoste C, Schafer X, Lun Y, Lemischka IR: *Dissecting self-renewal in stem cells with RNA interference. Nature* 2006, *442(*7102):533–538.

35.O'Connor MD, Kardel MD, Eaves CJ: *Functional assays for human embryonic stem cell pluripotency. Methods Mol Biol, 690*:67–80.

36.O'Connor MD, Kardel MD, Iosfina I, Youssef D, Lu M, Li MM, Vercauteren S, Nagy A, Eaves CJ: *Alkaline phosphatase-positive colony formation is a sensitive, specific, and quantitative indicator of undifferentiated human embryonic stem cells. Stem Cells* 2008, *26(*5):1109–1116.

37.Smith AG: *Embryo-derived stem cells: of mice and men. Annu Rev Cell Dev Biol* 2001, *17:*435–462.

38.Pera MF, Tam PP: *Extrinsic regulation of pluripotent stem cells. Nature, 465(*7299):713–720.

39.Nichols J, Smith A: *The origin and identity of embryonic stem cells. Development, 138(*1):3–8.

40.Jasmin, Spray DC, Campos de Carvalho AC, Mendez-Otero R: *Chemical induction of cardiac differentiation in p19 embryonal carcinoma stem cells. Stem Cells Dev, 19(*3):403–412.

41.Tay YM, Tam WL, Ang YS, Gaughwin PM, Yang H, Wang W, Liu R, George J, Ng HH, Perera RJ *et al: MicroRNA–134 modulates the differentiation of mouse embryonic stem cells, where it causes post-transcriptional attenuation of Nanog and LRH1. Stem Cells* 2008, *26(*1):17–29.

42.Tay Y, Zhang J, Thomson AM, Lim B, Rigoutsos I: *MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature* 2008, *455(*7216):1124–1128.

43.Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M, Rogers D: *Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature* 1988, *336(*6200):688–690.

44.Gearing DP, Gough NM, King JA, Hilton DJ, Nicola NA, Simpson RJ, Nice EC, Kelso A, Metcalf D: *Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF). EMBO J* 1987, *6(*13):3995–4002.

45.Williams RL, Hilton DJ, Pease S, Willson TA, Stewart CL, Gearing DP, Wagner EF, Metcalf D, Nicola NA, Gough NM: *Myeloid leukaemia inhibitory factor maintains the developmental potential of embryonic stem cells. Nature* 1988, *336(*6200):684–687.

46.Ying QL, Smith AG: *Defined conditions for neural commitment and differentiation. Methods Enzymol* 2003, *365*:327–341.

47.Niwa H: *How is pluripotency determined and maintained? Development* 2007, *134(*4):635–646.

48.Chen X, Xu H, Yuan P, Fang F, Huss M, Vega VB, Wong E, Orlov YL, Zhang W, Jiang J *et al: Integration of external signaling pathways with the core transcriptional network in embryonic stem cells. Cell* 2008, *133(*6):1106–1117.

49.Niwa H, Miyazaki J, Smith AG: *Quantitative expression of Oct–3/4 defines differentiation, dedifferentiation or self-renewal of ES cells. Nat Genet* 2000, *24(*4):372–376.

50.Zhu G, Li Y, Zhu F, Wang T, Jin W, Mu W, Lin W, Tan W, Li W, Street RC *et al: Coordination of engineered factors with TET1/2 promotes early-stage epigenetic modification during somatic cell reprogramming. Stem Cell Reports, 2(*3):253–261.

51.Rodrigues AS, Pereira SL, Correia M, Gomes A, Perestrelo T, Ramalho-Santos J: *Differentiate or Die: 3-Bromopyruvate and Pluripotency in Mouse Embryonic Stem Cells. PLoS One, 10(*8):e0135617.

52.Varum S, Momcilovic O, Castro C, Ben-Yehudah A, Ramalho-Santos J, Navara CS: *Enhancement of human embryonic stem cell pluripotency through inhibition of the mitochondrial respiratory chain. Stem Cell Res* 2009, *3(*2–3):142–156.

Table 1

Due to technical limitations, tables are only available as a download in the supplemental files section

Figures

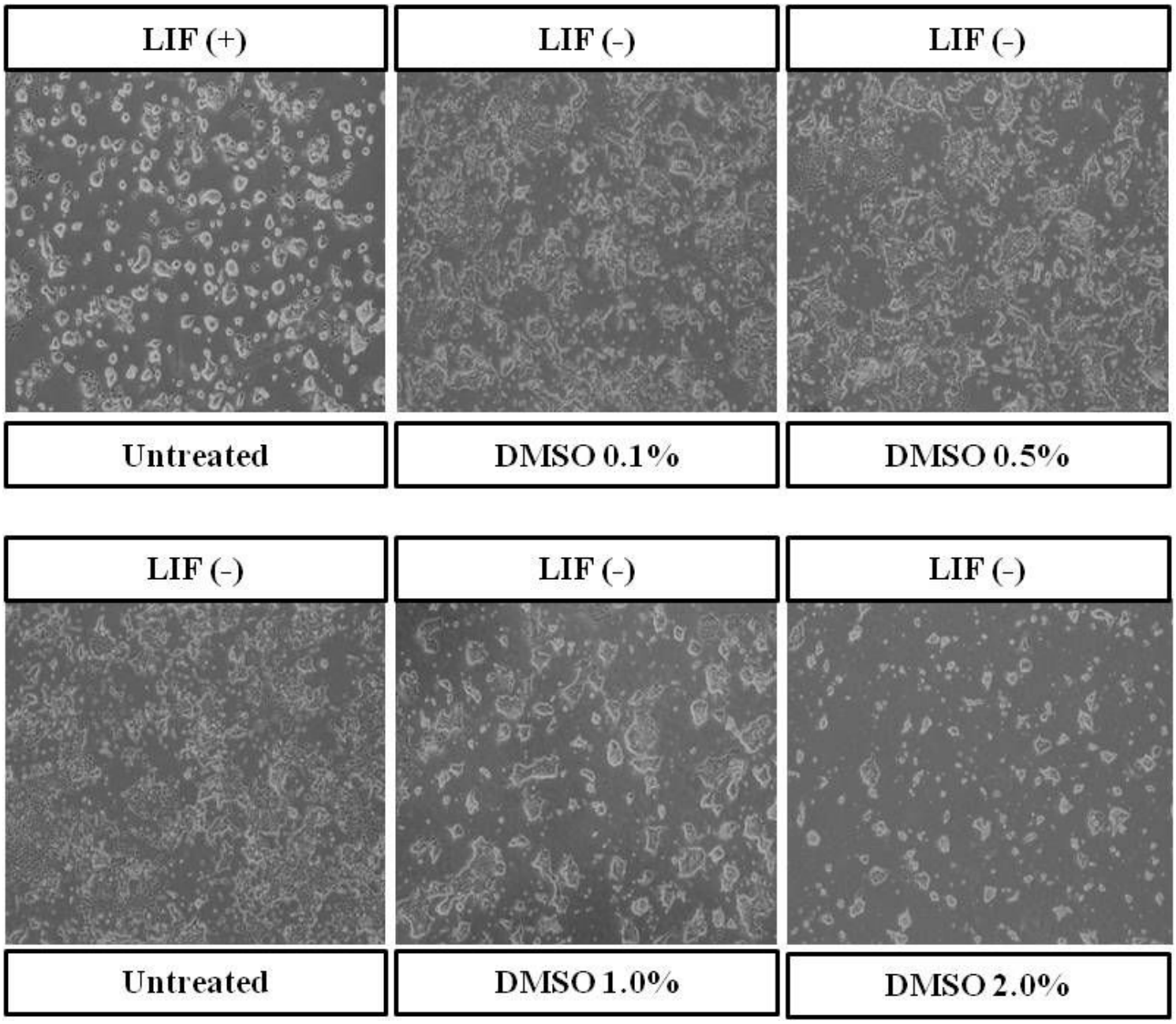

Figure 1

Effects of DMSO on mESC morphology. E14 mESCs were exposed to DMSO, and cell morphologies were observed. Magnification, 40×. Cells were cultured with various concentrations of DMSO (0.1%, 0.5%, 1.0%, or 2.0%) and for controls (with LIF or without LIF) for 96 h.

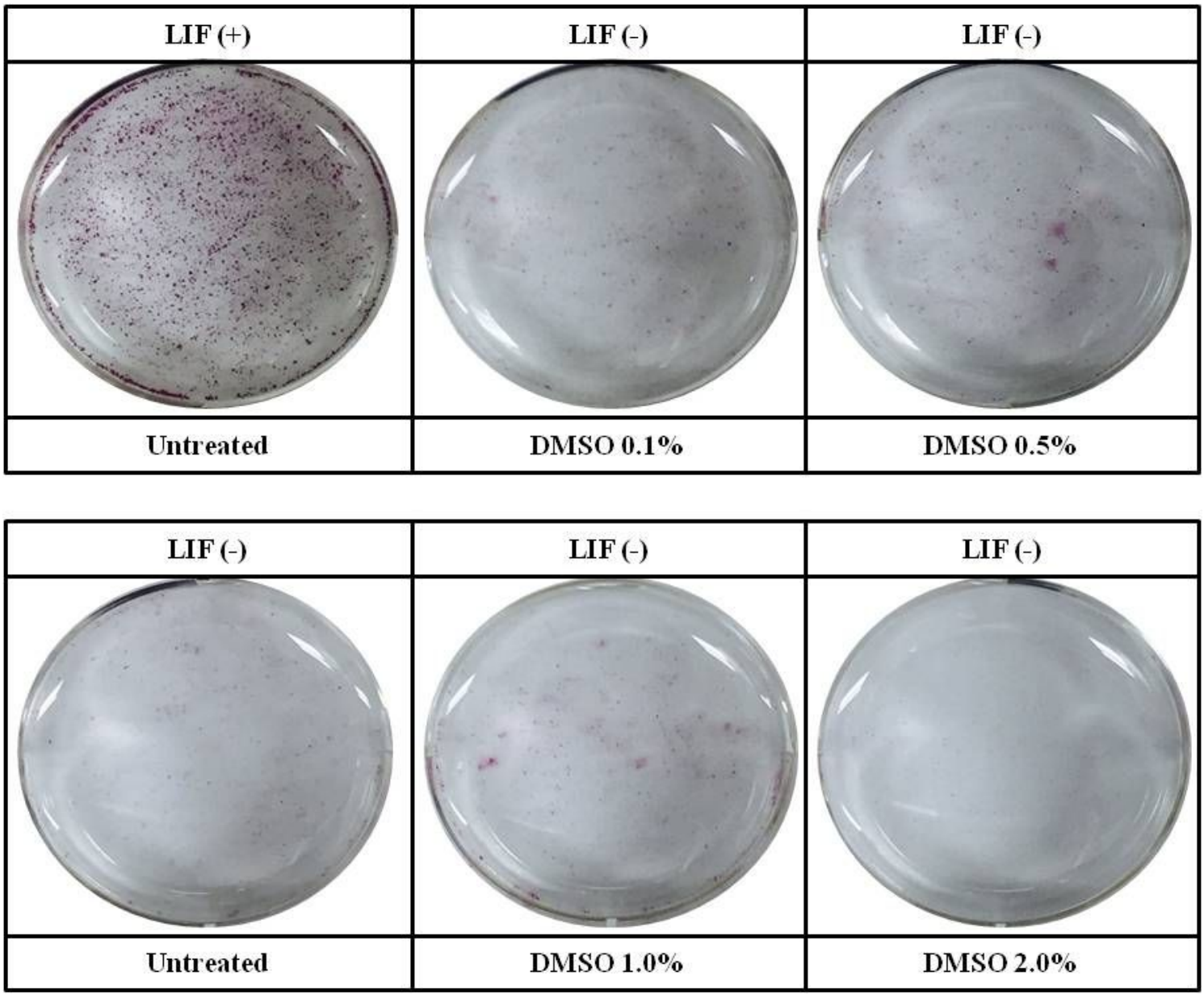

Figure 2

Effects of DMSO on alkaline phosphatase staining and activity in mESCs. Cells were cultured with various concentrations of DMSO (0.1%, 0.5%, 1.0%, or 2.0%) and for controls (with LIF or without LIF) for 96 h.

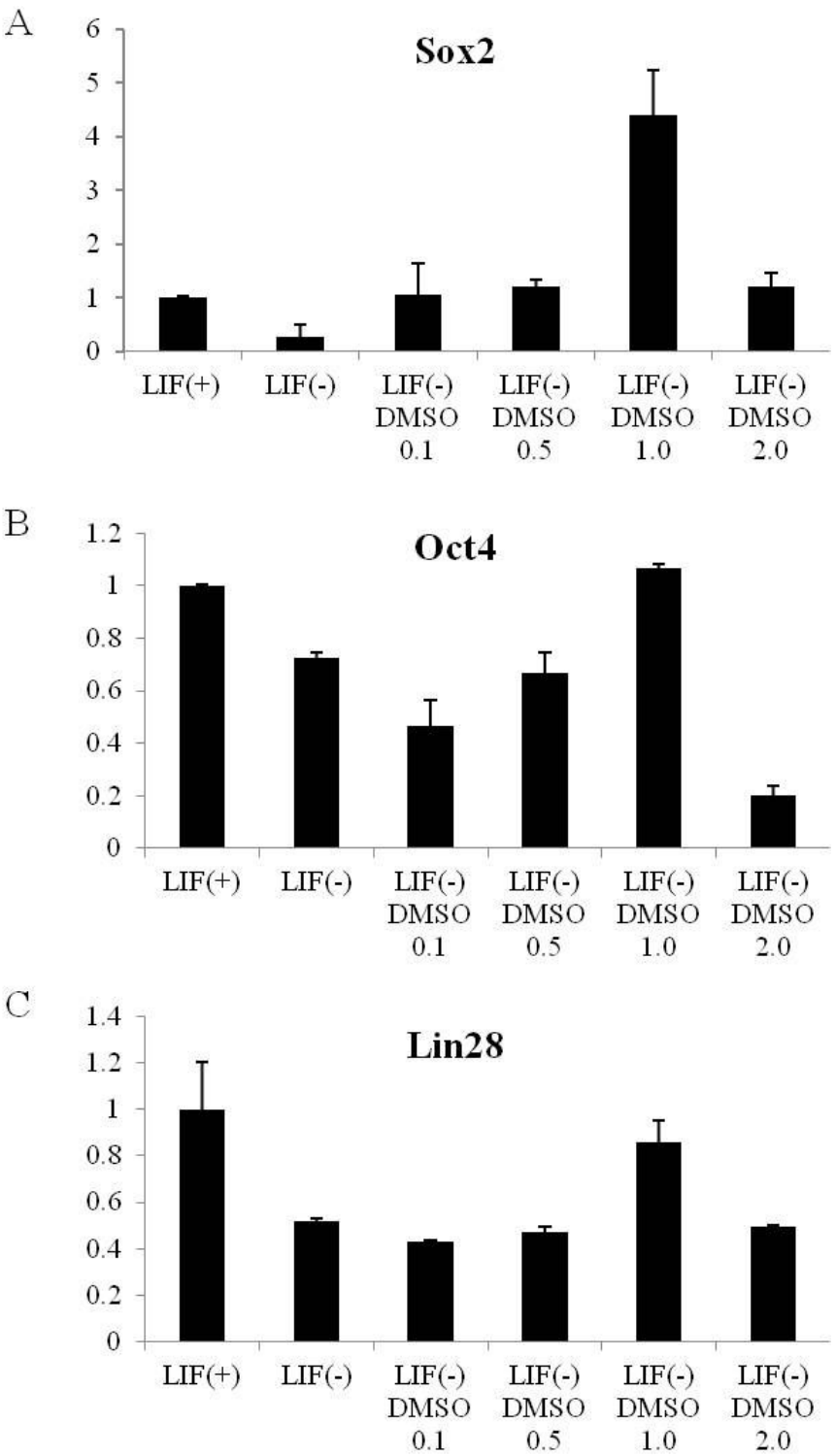

Figure 3

Effects of BPA on pluripotency and expression of Oct4, Sox2, and Lin28 in mESCs. Cells were cultured with various concentrations of DMSO (0.1%, 0.5%, 1.0%, or 2.0%) and for controls (with LIF or without LIF) for 96 h. (A–C) Oct4/Sox2/Lin28 mRNA levels were determined by quantitative real-time PCR using housekeeping gene GAPDH as an internal control. Each data point was normalized to the control (LIF),

and the means ± S.E. from three independent experiments are presented. Indicates significant difference when the values were compared to that of the control ($p < 0.05$).

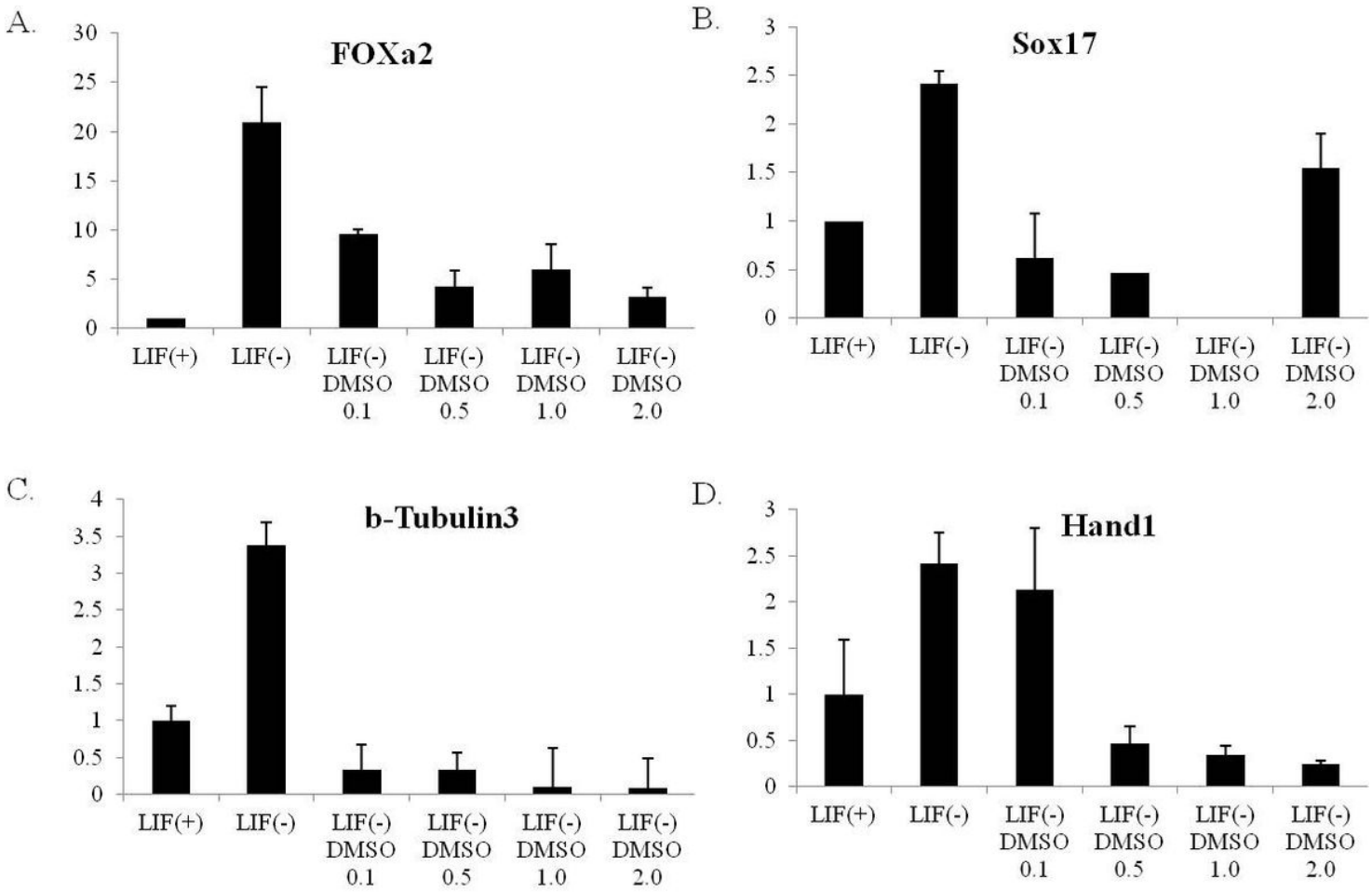

Figure 4

Effects of DMSO on differentiation capacity and expression of markers of three germ layers markers in mESCs. mRNA levels of endodermal markers (Foxa2, Sox17) and mesodermal (Hand1) and ectodermal (β-tubulin 3) markers were determined by quantitative real-time PCR using GAPDH as an internal control. Each data point was normalized to the control (with LIF), and the means ± S.E. from three independent experiments are presented. Indicates significant difference when the values were compared to that of the control ($p < 0.05$).

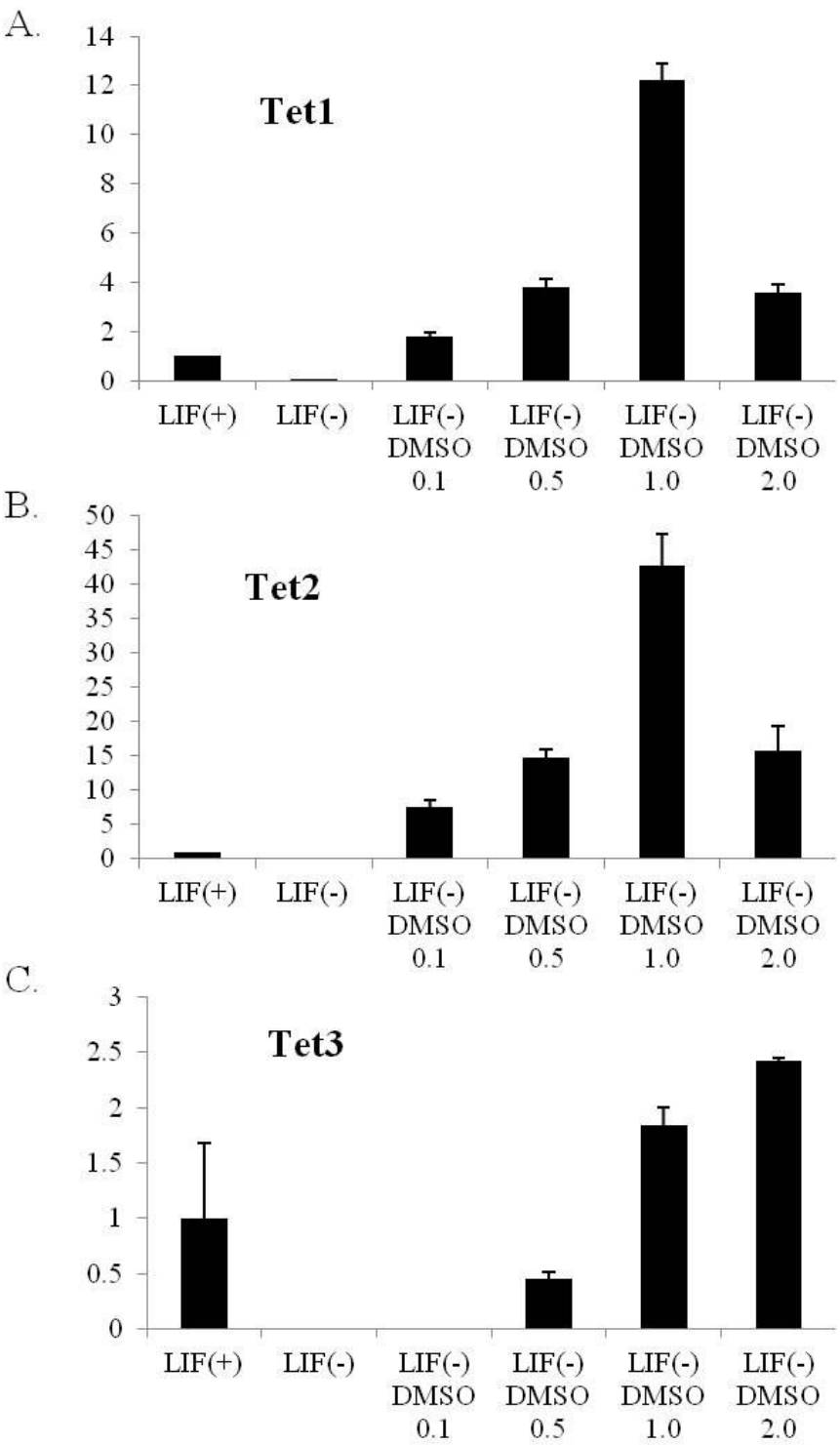

Figure 5

Effects of DMSO on DNA methylation and expression of TET oncogenes in mESCs. Tet1 (Tet oncogene 1), Tet2 (Tet oncogene2), and Tet3 (Tet oncogene3) mRNA levels were determined by quantitative real-time PCR using GAPDH as an internal control. Each data point was normalized to the control (with LIF), and the means ± S.E. from three independent experiments are presented. Indicates significant difference when the values were compared to that of the control ($p < 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.jpg