中 國 醫 藥 大 學

專題研究計畫成果報告

計畫名稱：結合缺氧及 N-Butylidenephthalide (BP) 建立高效率的誘導性多能幹細胞

計畫編號：CMU 99-COL -45

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中 華 民 國 101 年 6 月 1 日\

一、 前言及文獻探討：

Early-stage mammalian embryos develop in a low O_2 environment (hypoxia) (Ezashi et al., 2005). Embryonic stem (ES) cells are generally derived from the inner cell mass of blastocysts produced by in vitro fertilization techniques (Reubinoff et al., 2000; Thomson et al., 1998). However, ES cells are generally cultured under an atmosphere of 21% O_2 (normoxia). Under normoxic conditions stem cells tend to differentiate spontaneously. Such conditions may not be the most suitable, therefore, for hES cell propagation. Previous studies have shown that hES cells grew as well under hypoxic as normoxic condition (Ezashi et al., 2005). Hypoxic hES cultures showed slower trend toward spontaneous cell differentiation. (Ezashi et al., 2005). Hypoxic culture would appear to be necessary to maintain full pluripotency of hES cells (Ezashi et al., 2005).

The primary transcriptional regulators of both cellular and systemic hypoxic adaptation in mammals are hypoxia-inducible factors (HIFs) (Covello et al., 2006). Hypoxia-inducible factor-1 (HIF1) is a transcription factor found in mammalian cells cultured under reduced oxygen tension that plays an essential role in cellular and systemic homeostatic responses to hypoxia (Wang et al., 1995). HIF1 has a key role in cellular response to hypoxia, including the regulation of genes involved in energy metabolism, angiogenesis, and apoptosis. The alpha subunits of HIF are rapidly degraded by the proteasome under normoxic conditions but are stabilized by hypoxia. HIF1 activity is controlled by the oxygen-regulated expression of the HIF1a subunit. Under normoxic conditions, the HIF1a protein is subject to ubiquitination and proteasomal degradation. Sutter et al. (2000) reported that missense mutations and/or deletions involving several different regions of the HIF1A gene result in constitutive expression and transcriptional activity in nonhypoxic cells (Sutter et al., 2000). The authors demonstrated that hypoxia results in decreased ubiquitination of HIF1-alpha and that missense mutations increase HIF1-alpha expression under nonhypoxic conditions by blocking ubiquitination.

The more recent identification of HIF-2 (also known as endothelial PAS domain protein 1 [EPAS 1]) has raised important questions about the relative activity of these factors in mediating hypoxic adaptation (Covello et al., 2006). Whereas HIF-1 is broadly expressed, HIF-2 transcripts are restricted to particular cell types, including vascular endothelial cells, neural crest cell derivatives, lung type II pneumocytes, liver parenchyma, and interstitial cells in the kidney (Ema et al., 1997). HIF-1 and HIF-2 may regulate overlapping but nonidentical target genes. Previous study reported that HIF-2a, but not HIF-1a, binds to the Oct-4 promoter and induces Oct-4 expression and transcriptional activity (Ema et al., 1997).

Induced pluripotent stem (iPS) cells were a novel research field to study the therapeutic approaches on stem cells. The pluripotency of hES cells endows them with an exciting potential for use in tissue repair and replacement (Ezashi et al., 2005). iPS cells could be generated from adult human dermal fibroblasts following transfection with four genes: Oct4,

Sox2, c-Myc and Klf4 (Takahashi and Yamanaka, 2006). Human iPS cells were similar to hES in proliferation, morphology, gene expression, surface antigens, epigenetic status of pluripotent cell-specific genes, and telomerase activity. The hypothesis of this study is that HIF-1a and HIF-2a could regulate the genes expression of Oct4, Sox2, c-Myc, Klf4, Nanog and Notch to maintain the self-renewal and cell pluripotency of stem cells. Furthermore, we are also interested in the molecular mechanism of HIF-1a and HIF-2a under hypoxic condition in regulating the expression of Oct4, Sox2, c-Myc and Klf4 to generate the iPS cells.

Two issues appear to limit the utility of iPS cells (Huangfu et al., 2008): (i) the low efficiency of reprogramming primary human cells, which makes it difficult to generate patient-specific iPS cells from a small starting population of cells, and (ii) the integration of viral transgenes into the somatic genome, especially oncogenes such as c-MYC and KLF4. Indeed, reactivation of the c-Myc retrovirus contributes to tumor formation in chimeric mice derived from iPS cells (Nakagawa et al., 2008; Okita et al., 2007). Through optimization of the reprogramming method, it is now possible to reprogram both mouse and human somatic cells with non-virus system.

We have successfully generated a novel iPS cell clone, **iPS-OSH cells,** by introducing only Oct4 and Sox2 into the MEF cells withour virus system in the hypoxic condition. The generation of the iPS-OSH cells indicated that our lab has the ability to generate a novel iPS cell clone. In addition, we also demonstrated that Butylidenephthalide (BP), the extract from Angelica sinensis, could increase the Oct4 and Sox2 gene expression (Figure 1).

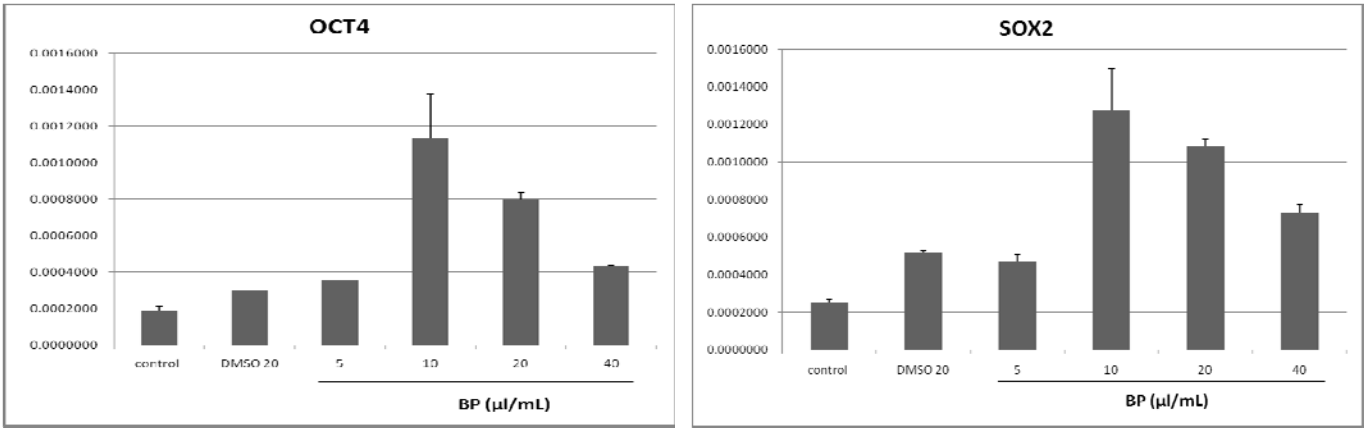

Figure 1. The gene expression levels of Oct4 and Sox2 for the MEF cells that treated with different concentration of BP by real-time PCR analysis.

The hypothesis of this study is that hypoxia could up-regulated the genes expression levels of Oct4, Sox2, c-Myc and Klf4 and increase the efficiency of iPS cells generation. We will combine Oct4, Sox2, hypoxic condition and BP to find the high efficiency strategy to

generate the novel iPS cells. We will also demonstrate the molecular mechanism of HIF-1a and HIF-2a under hypoxic condition and study the epigenetic different between MEF and novel iPS cells. Finally, the novel iPS cells will be differentiated into the neural progenitor cells and transplanted into the stroke mouse model for studying the cell therapeutic approaches.

In out preliminary results, we detected that the four protein levels (Oct4, Sox2, c-Myc and Klf4) under the 3% hypoxic-stimulated condition. The results showed that all of the four genes up-regulated under the hypoxic condition (Figure 3). The results proved our hypothesis of this study.

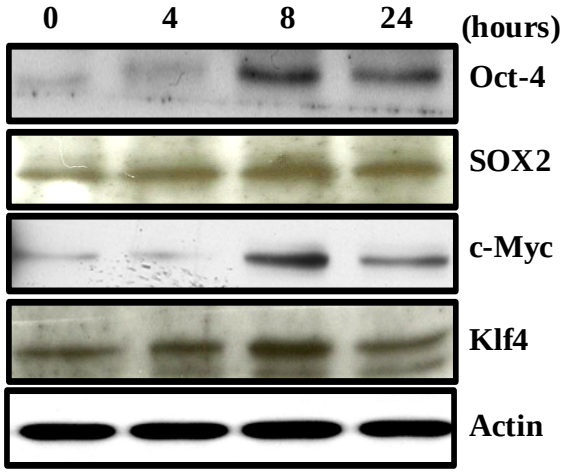

Figure 3. The protein levels of 3% hypoxic-stimulated MEF cells.

二、 研究目的：

1. Comparison the genes expression profiles of Oct4, Sox2, c-Myc, Klf4 and molecular mechanism between the following four conditions mention below and use these four conditions to generate the novel iPS cells.
   a. Overexpressing Oct4, Sox2, c-Myc, Klf4 genes in hypoxic condition.
   b. Overexpressing Oct4, Sox2, c-Myc, Klf4 and treatment with BP in hypoxic condition.
   c. Overexpressing Oct4, Sox2 genes in hypoxic condition.
   d. Overexpressing Oct4, Sox2 genes and treatment with BP in hypoxic condition.
2. Using the stem cell marker, embryonic body, methylation pattern, teratoma, gene expression profile and chimeric production to confirm the pluripotentcy of the novel iPS cells and comparison of the epigenetic difference between MEF and the novel iPS cells to study the mechanism of MEF becoming to the iPS cells.

三、 研究方法：

Pou5f1-GFP transgenic mouse model

Two male and female Pou5f1-GFP transgenic mice will obtain from The Jackson Lab. We will isolate the MEF cells from this mouse model for the iPS cells generation experiment. The detail information of this mouse model is in the website (http://jaxmice.jax.org/strain/004654.html).

Culture of mouse embryonic fibroblast cells

Primary mouse embryonic fibroblast (MEF) cells will be isolated from 13.5 days embryo of Pou5f1-GFP transgenic mice. Briefly, the embryos are retrieved by Cesarean section than free the embryos from placenta, remove internal organs and brain. The embryos are minced with fine scissors into fine tissue fragments. Then transfer the tissues into fresh dish containing Trypsin to digest the cells. The cells (MEF) are cultured in DMEM (GIBCO BRL, Grand Island, NY) with 10% heat-inactivated FBS (HyClone, Logan, Utah), penicillin (100 U per ml), streptomycin (100 ug per ml), non-essential amimo acids (0.1mM), fungizone (2.5 mg per ml) and L-glutamine (2 mM) in a humidified 37℃ incubator with 5% CO_2.

Construction of the plasmid that express Oct4, Sox2, HIF1a and HIF2a

Oct4, Sox2, HIF1a and HIF2a cDNA will be obtained from the total RNA of the mouse ES cells by RT-PCR. The PCR fragment of Oct4, Sox2, HIF1a and HIF2a will be constructed into the pGEM-T easy vector (Promega). The pGEM-T- Oct4, Sox2, HIF1a and HIF2a will be digested with EcoRI and then ligased into the pcDNA 3.1 vector. The plasmid, pcDNA-Oct4, pcDNA-Sox2, pcDNA-HIF1a and pcDNA-HIF2a will be used to the transfection experiment.

Transfection.

Plasmid DNA from plasmid constructs mentioned above is prepared and purified using a Qiagen maxi kit (Qiagen, Hilden, Germany). Cultured cells will be transfected with the plasmid DNAs using FuGene transfection system (Roche). Cells transfected with vector plasmid DNA (pcDNA 3.1) will be used as the control. Twenty-four hours after the transfection, cells will be analyzed by using real-time PCR and western blot analysis for the detection the expression levels of Oct4, Sox2, c-Myc and Klf4.

Oxygen tension incubation

To test the effects of oxygen tension on MEF, various pO2 were employed: 1%, 3%, 5% and 21% O2. Cells in culture plates/flasks were placed into hermetic chambers

(Billups-Rothenberg Inc., Del Mar, CA) into which the appropriate gas mixture was infused for three min twice a day. Oxygen tension (1%, 3%, 5% and 21% O2) was controlled by regulating the N_2 concentration. The hermetic chambers were placed into a regular incubator at 37°C.

RNA extraction and Real-time PCR.

Total RNA will be extracted from MEF cells using TRIzol (Invitrogen, Carlsbad, CA) and the concentration will be determined by spectrophotometer analysis. Complementary DNA will be produced from mRNA (5 μg) using a SuperScript II Reverse Transcriptase Kit (Invitrogen). Real-time PCR will be used to determine the gene expression levels of HIF-1a, HIF-2a, Oct4, Sox2, c-Myc, and Klf4. The procedures of real-time PCR analysis have been described (Lee et al., 2006).

Western Blot Assay (WB).

The procedures of the WB have been described previously (Liu et al., 2007). In this study, mouse anti-OCT4, mouse anti-SOX2, mouse anti-c-MYC and mouse anti-Klf4 will be used in the WB.

Generation of the iPS cells.

We will transfect the plasmid pcDNA-Oct4, pcDNA-Sox2, pcDNA-HIF1a and pcDNA-HIF2a into the MEF cells every two days for 4 times. After transfecting, the MEF cell will culture in the hypoxic condition (3%) for 24 hours. On day 9, the MEF will passage on the feeder cells and observe to the ES cell-like clones. BP treatment will started on day 1 and until day 7. The strategy will be shown below (Figure 4).

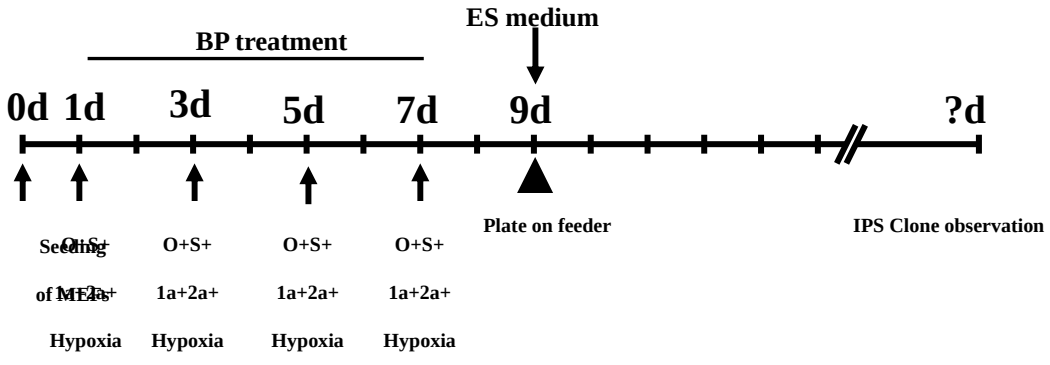

Figure 4 The strategy of generating iPS cells by introducing Oct4 and HIF2a in the hypoxic condition.

In Vitro Differentiation of iPS Cells

Cells were harvested by trypsinization and transferred to bacterial culture dishes in the ES medium without G418 or LIF. After 3 days, aggregated cells were plated onto gelatin-coated tissue culture dishes and incubated for another 3 days. The cells were stained with antia-smooth muscle actin monoclonal antibody (N1584, Dako), anti-afetoprotein polyclonal antibody (N1501, Dako) or anti-bIII tubulin monoclonal antibody (CBL412, Abcam) along with 40-6-diamidino-2-phenylindole (Sigma). Total RNA derived from plated embryoid bodies on day 6 was used for RT-PCR analysis.

Teratoma Formation and Histological Analysis

ES cells or iPS cells were suspended at 5 x 10^7 cells/ml in DMEM containing 10% FBS. Nude mice were anesthetized with diethyl ether. We injected 100 ml of the cell suspension (5 x 10^7 cells) subcutaneously into the dorsal flank. Four weeks after the injection, tumors were surgically dissected from the mice. Samples were weighed, fixed in PBS containing 4% formaldehyde, and embedded in paraffin. Sections were stained with hematoxylin and eosin.

Bisulfite Genomic Sequencing for Oct4, Sox2, Nanog and Notch promotor

Bisulfite treatment was performed using the CpGenome modification kit (Chemicon) according to the manufacturer's recommendations. Amplified products were cloned intopCR2.1-TOPO (Invitrogen). Ten randomly selected clones were sequenced with the M13 forward and M13 reverse primers for each gene.

DNA Microarray

Total RNA from ES cells, iPS cells, or MEFs were labeled with Cy3. Samples were hybridized to a Mouse Oligo Microarray (G4121B, Agilent) according to the manufacturer's protocol. Arrays were scanned with a G2565BA Microarray Scanner System (Agilent). Data were analyzed using GeneSpring GX software (Agilent).

四、 結果與討論：

MTT Assays for BP Treatment

MTT assays were used to identify MEF cell viability following BP treatment. According to data from tests using eight concentrations of BP (5, 10, 20, 40, 80, 160, 320 and 640 μg/ml), cell survival rates significantly decreased in the 80, 160, 320 and 640 μg/ml treatment groups 24 h and 72 h post-treatment (Fig. 1A). We therefore selected the 5, 10, 20 and 40 μg/ml BP concentrations for our experiments.

BP Treatment Resulted in Oct4 and Sox2 Gene Expression Up-Regulation

Our data indicated significant up-regulation of Oct4 and Sox2 gene expression levels in MEF

cells following treatment with BP, especially in the 10 μg/ml treatment group (Fig. 1B). The effect decreased as concentration increased to 20 and 40 μg/ml. Oct4 and Sox 2 expression levels were higher than in the solvent control group; in contrast, c-Myc and Klf4 gene expression levels were not.

BP Treatment Maintained ES and iPS cell Self-Renewal

After detecting Oct4 and Sox2 gene expression up-regulation following BP treatment, we performed tests to determine whether BP treatment also maintained ES and iPS cell self-renewal and pluripotency as an alternative to LIF. Alkaline phosphatase (AP), Nanog, and SSEA1 were used to identify ES and iPS cell pluripotency. According to results from our ES cell analyses, quantities of AP-positive clones in ES cells treated with 10, 20 or 40 μg/ml of BP were all significantly higher than in the controls that did not contain LIF (LIF OUT control) (Fig. 2A). However, quantities of AP-positive clones in ES cells treated with 5 μg/ml BP were lower compared to the LIF OUT cells (Fig. 2A). In addition, data for Nanog and SSEA-1 levels in ES cells indicated that those treated with 10, 20 or 40 μg/ml BP had higher quantities compared to the LIF OUT control cells (Figs 2B and C).

Mouse iPS cells (from Nanog-GFP transgenic mice) were obtained from the laboratory of Dr. Shinya Yamanaka.[5] AP staining analysis data were similar to ES cell results, with quantities of AP-positive clones in 10, 20 and 40 μg/ml BP-treated iPS cells being significantly greater compared to the LIF OUT control cells (Fig. 3A). As shown in Fig. 3B, GFP signaling (Nanog expression) in the 10, 20 and 40 μg/ml BP-treated cells was higher than in the LIF OUT controls. The SSEA-1 stem cell marker staining showed that 40 μg/ml BP-treated cells was higher than in the LIF OUT controls (Fig. 3C). Combined, the data indicate the ability of BP to maintain ES and iPS cell self-renewal and pluripotency.

Embryoid Body Formation and Differentiation

In another test designed to determine the ability of BP to serve as a LIF substitute in ES cell cultures, embryoid body formation was used to determine ES cell pluripotency by passaging cells six times in culture containing BP (LIF replacement) medium. We observed differentiation initiated by ES cells after adding the embryoid bodies to cell culture plates. Immunofluorescent staining was used to detect cells that were positive for Tuj1 (ectoderm marker), α-smooth muscle actin (α-SMA, mesoderm marker), and Gata4 (endoderm marker). As shown in Fig. 4A, we observed ES cells differentiation in all three germ layer cell types, including neuronal cells (Tuj1), hepatocyte (Gata4) and α-smooth muscle actin (muscle cells). The LIF control differentiation in all three germ layer cell types was showed in Fig. 4B. These data also suggest that BP can serve as a substitute for LIF in ES cell cultures to maintain cell pluripotency after passaging.

Microarray Analysis of Genes Involved in Various Pathways in BP-Treated MEF Cells

Microarray analyses were performed to evaluate gene expression profiles in various pathways in MEF cells treated with 10 μg/ml BP, 40 μg/ml BP, and DMSO (control). All data were submitted to GEO system and the accession numbers are MEF (GSM886449), MEF cells treated with 10 μg/ml BP (GSM886450), and MEF cells treated with 40 μg/ml BP (GSM886451), respectively. A Babelomics web tool and KEGG pathways database were used as part of this procedure. Numbers and percentages of significantly deregulated genes with known biological functions are shown in Table 1. Higher percentages of deregulated genes post-BP treatment were observed in PPAR, ECM-receptor interaction, and Jak-Stat signaling pathways. Since the Jak-Stat signaling pathway is the most important in terms of stem cell maintenance and renewal, we selected it to study stem cell self-renewal and pluripotency maintenance mechanisms.

Phosphorylated Jak2 and Stat3 Levels in BP-Treated ES Cells

Jak2 and Stat3 are known as key Jak-Stat pathway proteins for maintaining stem cell self-renewal. We used real-time PCR and western blot assays to determine Jak2 and Stat3 gene mRNA and protein levels in BP-treated ES cells. As shown in Fig. 5A, both Jak2 and Stat3 mRNA levels significantly increased after treatment with 5 or 10 μg/ml BP. Compared to the LIF OUT controls, Jak2 and Stat3 protein levels increased in ES cells treated with 5 and 10μg/ml BP (Figs. 5B and C). In addition, the phosphorylated Jak2 and Stat3 forms (active forms) also increased following treatment with 5 and 10μg/ml BP. According to these data, BP maintains ES cell self-renewal by activating the Jak2-Stat3 signaling pathway. It was also observed that the Jak2 inhibitor, AG490 suppressed the phosphorylated Jak2 and phosphorylated Jak2 expression in ES cells treated with BP (Fig. 4D).

Cytokine Regulation in BP-Treated ES Cells

To understand why BP treatment resulted in Jak2-Stat3 signaling up-regulation, we used real-time PCR analysis to determine the mRNA levels of cytokine genes involved in the Jak2-Stat3 pathway: LIF, EGF, EPO, IL-5, IL-11 and OSM. As shown in Fig. 6A, significant up-regulation following treatment with 10 μg/ml BP was observed for LIF, EGF, EPO, IL-5, IL-11 and OSM. These data match those showing the highest levels of phosphorylated Jak2 and Stat3 following treatment with 10 μg/ml BP. Combined, the results suggest that BP is capable of increasing Jak2-Stat3-related cytokine levels, thus activating Jak2 and Stat3 proteins to maintain stem cell pluripotency.

Improved iPS Cell Generation Efficiency

One of our primary goals was to determine if BP can be used to enhance the efficiency of iPS cell generation. We therefore used MEF obtained from Pou5f1-GFP mice to introduce plasmids containing Oct4, Sox2, c-Myc and KlF4 genes once every two days (4 times total) to generate iPS cells. As part of this process, 10 μg/ml of BP-containing medium was used to

culture MEF cells 6 h post-transfection. We established two groups after seeding feeder cells on day 9: BP (T), meaning that treatment was limited to BP-only following transfection, and BP (T+P), meaning that BP treatment occurred both after transfection and after passaging feeder cells. The data show that regardless of treatment/no treatment with 10 μg/ml BP after seeding, the efficiency of iPS cell generation (GFP positive clones) significantly increased in the BP treatment group (Fig. 6B). In summary, we found evidence that BP treatment enhances iPS cell generation efficiency.

Characterizations of the novel iPS cells generated by adding BP (iPS-BP)

iPS-BP cells generated by the method mentioned above (BP[T+P]) were determined the pluripotentcy of these novel cells. We used immunofluorescent staining to determine stem cell marker expression in iPS-BP cells. iPS-BP cells were stained with mouse antibodies against Nanog, SSEA1 and alkaline phosphotase. As shown in Fig. 6C, iPS-BP cells tested positive for all four stem cell markers. In addition, we used embryoid body formation to determine iPS-BP cell pluripotency. The results indicated that iPS-OSH cells formed embryoid bodies in low-attached plates. We observed differentiation initiated by the iPS-BP cells after placing the embryoid bodies in cell culture plates. Next, we used immunofluorescent staining to detect cells that were positive for Gata4 (endoderm marker), α-smooth muscle actin (mesoderm marker), and Tuj1 (ectoderm marker). As shown in Figure 6D, we observed iPS-BP cells differentiation in all three germ layer cells, including endoderm (Gata4), mesoderm (α-smooth muscle actin), and ectoderm (Tuj1).

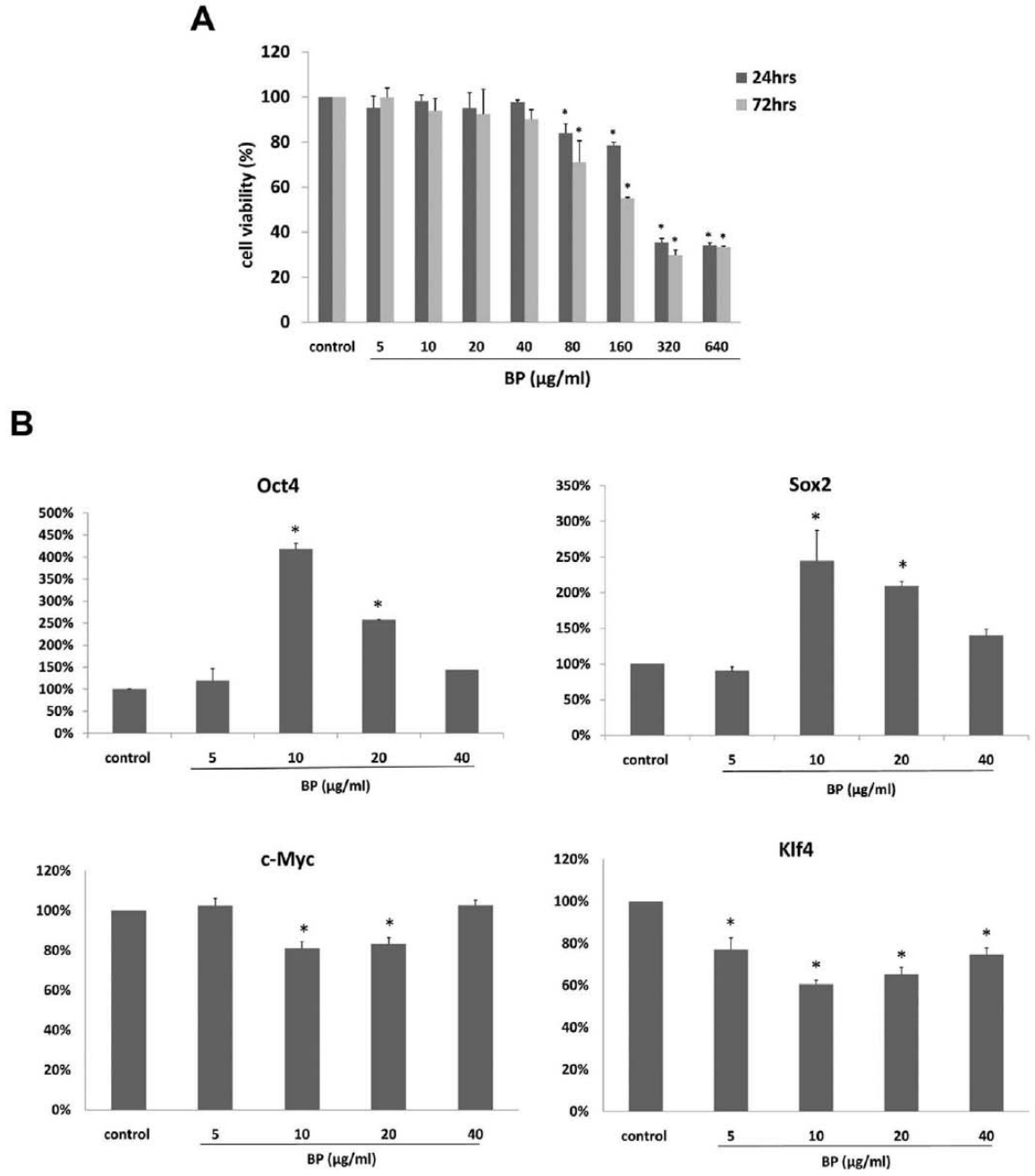

Figure 1. Cell viability and iPS cell related gene expression levels in MEF cells treated with BP. (A) MTT assay of MEF cells treated with various concentrations of BP. (B) Oct4, Sox2, c-Myc and Klf4 gene expression levels in MEF cells treated with various concentrations of BP (real-time PCR).

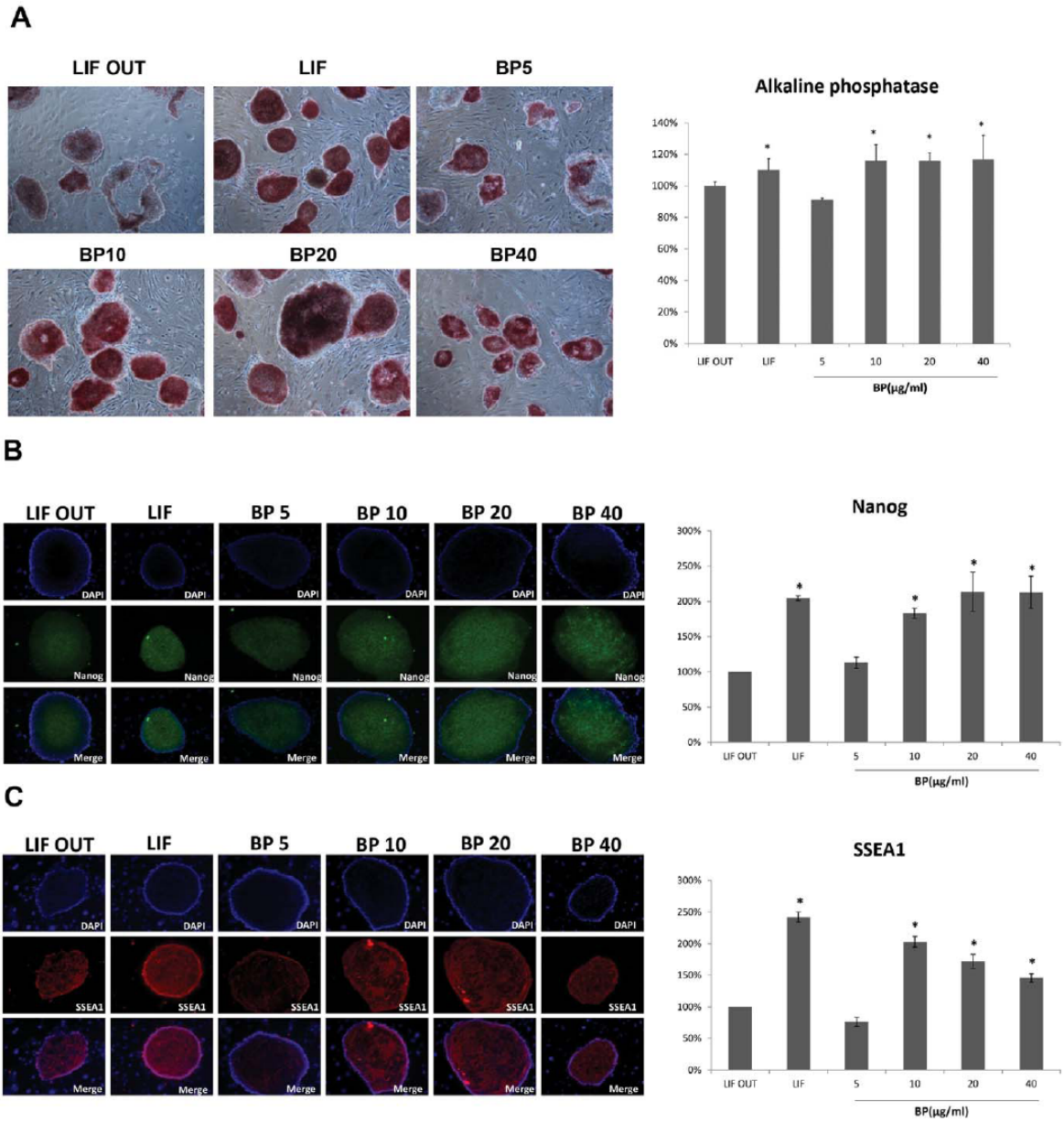

Figure 2. Stem cell markers staining in ES cells treated with BP. (A) Alkaline phosphatase staining ES cells treated with various concentrations of BP and the quantities of AP-positive clones in ES cells. (B) SSEA1 immunocytochemistry in ES cells treated with various concentrations of BP and the quantities of SSEA1 expression levels in ES cells. (C) Nanog immunocytochemistry in ES cells treated with various concentrations of BP and the quantities of SSEA1 expression levels in ES cells. *$p < 0.05$ versus control.

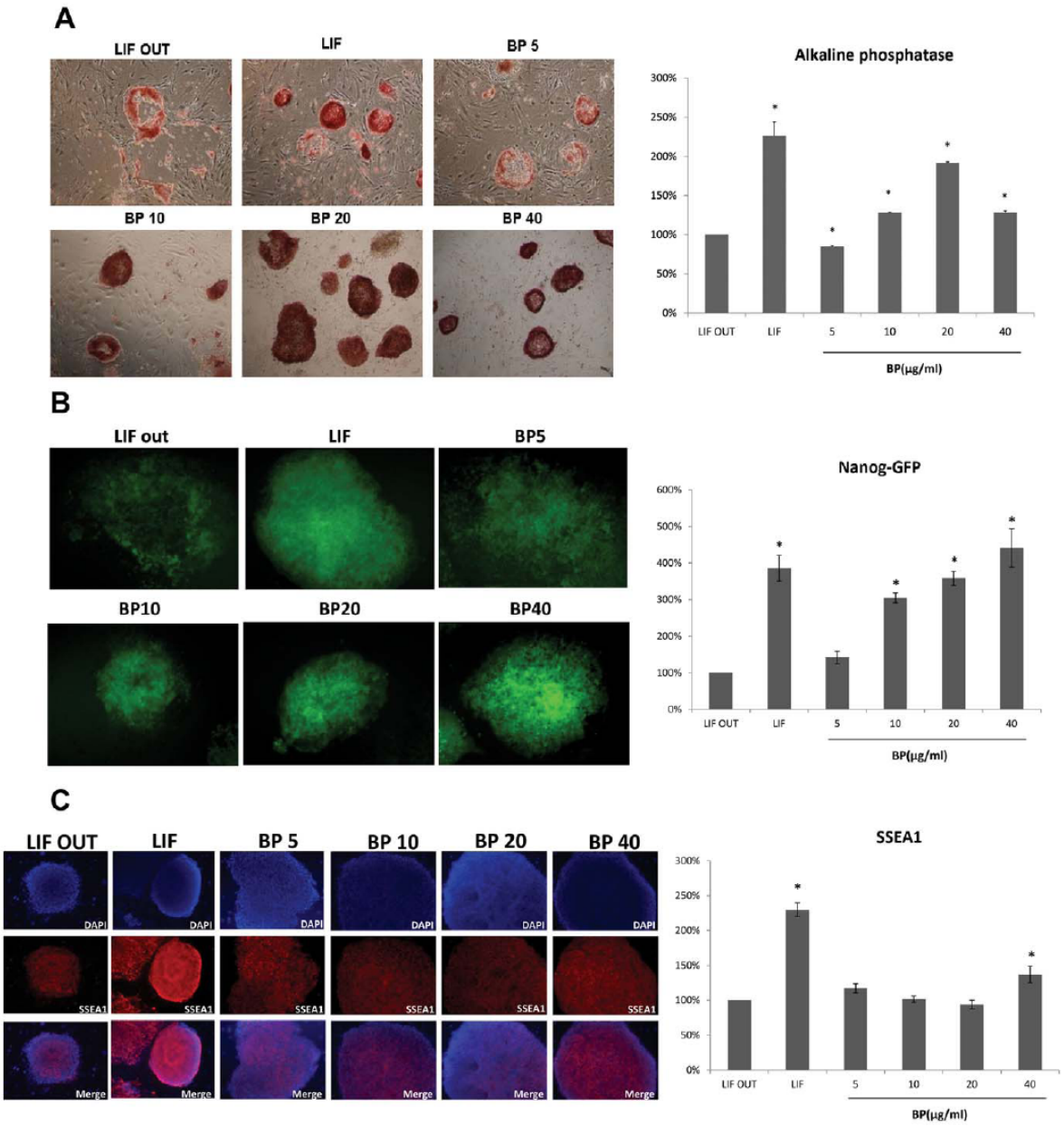

Figure 3. Stem cell markers staining in iPS cells treated with BP. (A) Alkaline phosphatase staining iPS cells treated with various concentrations of BP and the quantities of AP-positive clones in iPS cells. (B) SSEA1 immunocytochemistry in iPS cells treated with various concentrations of BP and the quantities of SSEA1 expression levels in iPS cells. (C) Nanog immunocytochemistry in iPS cells treated with various concentrations of BP and the quantities of SSEA1 expression levels in iPS cells. *$p < 0.05$ versus control.

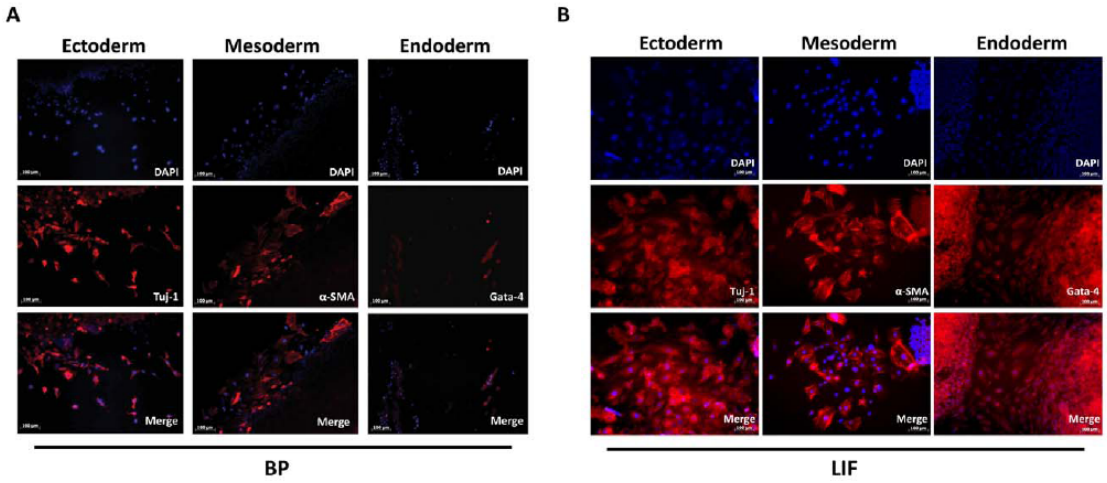

Figure 4. Embryoid body-mediated differentiation of ES cells passaged three times in (A) BP containing medium (replaced LIF) and (B) LIF containing medium as control. Immunofluorescent staining of Tuj1 (ectoderm marker), α-smooth muscle actin (α-SMA, mesoderm marker) and Gata4 (endoderm marker). Nuclei were stained with DAPI (blue).

Table 1. Numbers of significantly deregulated genes with known biological functions classified according to KEGG and Babelomics databases.

	BP10				BP40			
Function/Pathway	**I**	**D**	**No.**	**%**	**I**	**D**	**No.**	**%**
Signal transduction								
PPAR signaling pathway	14	14	28/74	37.8	12	14	27/74	36.5
ECM-receptor interaction	3	18	21/74	28.4	3	16	19/74	25.7
JAK-STAT signaling pathway	9	30	39/140	27.9	8	27	35/140	25
Calcium signaling pathway	4	28	32/146	21.9	2	30	32/146	21.9
TGF-beta signaling pathway	3	2	5/45	11.1	5	1	6/45	13.3
MAPK signaling	7	17	24/227	10.6	5	19	24/227	10.6
Wnt signaling pathway	2	5	7/66	10.6	0	6	6/66	9.1
Insulin signaling pathway	3	7	10/102	9.8	2	3	5/102	4.9
VEGF signaling pathway	2	0	2/31	6.5	0	0	0/31	0
Cell proliferation								
Cell communication	3	17	20/92	21.7	2	18	20/92	21.7
Cell cycle	0	4	4/163	2.5	0	6	6/163	3.7
Metabolism								
Lipid metabolism	3	15	18/109	16.5	2	12	14/109	12.8
Amino acid metabolism	0	1	1/23	4.3	0	2	2/23	8.7
Cell adhesion								
Cell adhesion molecules	13	20	33/150	22	13	21	34/150	22.7
Tight junction	2	7	9/66	13.6	2	9	11/66	16.7
Focal adhesion	3	16	19/173	11	2	20	22/173	12.7
Apoptosis	3	8	11/94	11.7	1	6	7/94	7.4

I: number of up-regulated genes; D: number of down-regulated genes.

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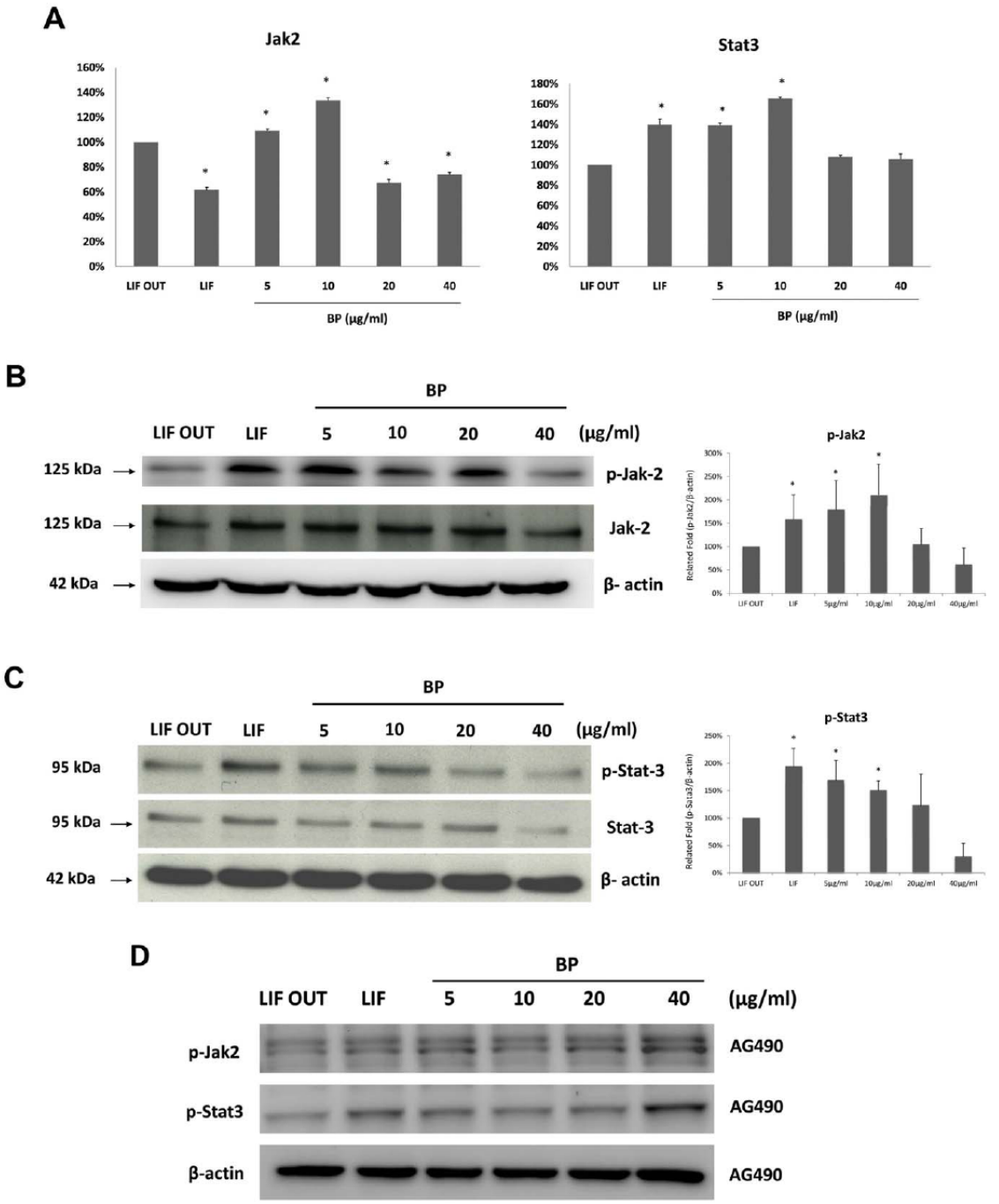

Figure 5. Jak2 and Stat3 expression levels in BP-treated ES Cells. (A) Jak2 and Stat3 gene expression levels in ES cells treated with various concentrations of BP (real-time PCR). (B) Jak2 and phosphorylated-Jak2 protein levels in ES cells treated with BP (western blot). (C) Stat3 and phosphorylated-Stat3 protein levels in ES cells treated with BP (western blot). (D) phosphorylated-Jak2 and phosphorylated-Stat3 protein levels in ES cells treated with BP and Jak2 inhibitor, AG490 (western blot).

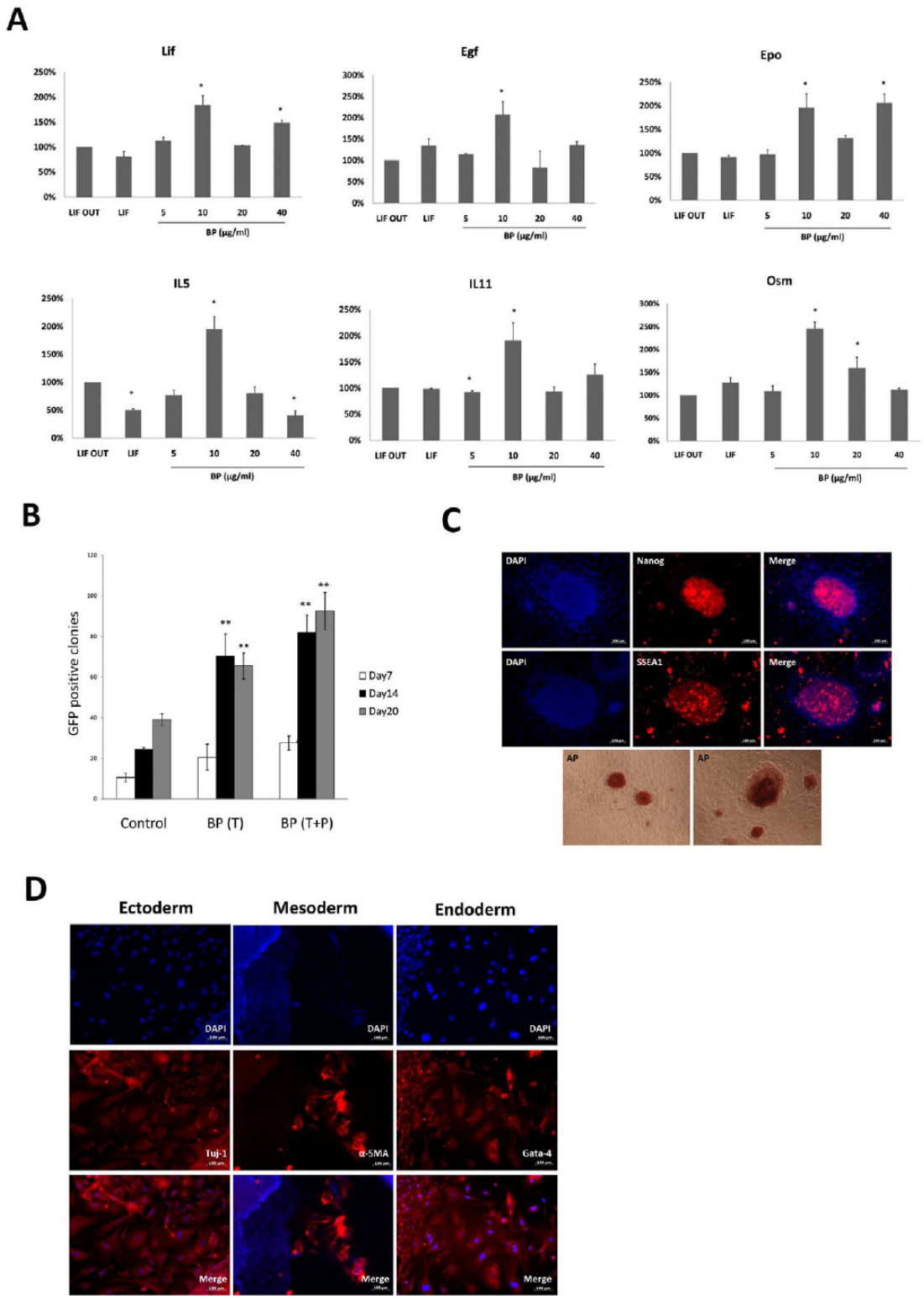

Figure 6. Cytokine regulation in BP-treated ES cells and characterizations of the novel iPS cells generated by adding BP (iPS-BP). (A) Real-time PCR results for cytokine gene expression levels associated with the Jak2-Stat3 pathway. Significant up-regulation of six cytokine genes was noted following BP treatment (10 μg/ml). (B) Pou5f1-GFP MEF cells were introduced with plasmids containing Oct4, Sox2, c-Myc and Klf4 to test iPS cell-generation efficiency. BP(T): treatment with BP post-transfection. BP(T+P): treated with BP post-transfection and following passage to feeder cells. Control group was introduced with plasmids containing Oct4, Sox2, c-Myc and Klf4 to generate non-BP treated iPS cells. (C) The stem cell markers (Nanog, SSEA1 and alkaline phosphotase) staining of iPS-BP cells. Nuclei were stained with DAPI (blue). (D) Immunofluorescent staining in iPS-BP cells: Gata4 (endoderm marker), α-smooth muscle actin (mesoderm marker), and Tuj1 (ectoderm marker). Nuclei were stained with DAPI (blue).

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