Effect of Inhibitor of Glycogen Synthase Kinase 3 on Self-Renewal of Human Embryonic Stem Cells

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ABSTRACT

Human embryonic stem cells (hESCs) derived from the inner cell mass of blastocysts have the ability to renew themselves and to differentiate into cell types of all lineage. The present study was carried out to investigate whether the Wnt signaling pathway is related to maintaining self-renewal of hESCs. Glycogen Synthase Kinase 3 (GSK-3) inhibitor, BIO ((2'Z,3'E)-6-Bromoindirubin-3'-oxime) was treated to Miz-hES1 line for activation of Wnt signaling pathway. BIO-nontreated hESCs (control) and BIO-treated hESCs were cultured for 5 days in the modified feeder-free system. During the culture of hESCs, differences were observed in the colony morphology between 2 groups. Controls were spread outwards whereas BIO-nontreated hESCs were clumped in the center and the differentiated cells were spreading outwards in the edges. The results of stem cell specific marker staining indicated that control were differentiated in large part whereas BIO-treated hESCs maintain self-renewal in the center of the colony. The results of lineage marker staining suggested that outer cells of the hESC colony were differentiated to the neuronal progenitor cells in both control and BIO-treated hESC. These results indicate that Wnt signaling is related to self-renewal in hESCs. In addition, control group showed higher composition of apoptotic cells (23.76%) than the BIO-treated group (5.59%). These results indicate that BIO is effective on antiapoptosis of hESCs.

(Key words : Human embryonic stem cells, Wnt signaling pathway, BIO, Self-renewal, Antiapoptosis)

INTRODUCTION

Embryonic stem cells are characterized by their abilities of self-renewal and differentiation to various cell types. They are also responsible for maintaining tissues and organs. This specific property could be used to generate specific types of tissues for cell replacement therapy to cure devastating diseases such as a Parkinson's disease (Hori et al, 2002; Kim et al., 2002).

During early mammalian embryogenesis, the first critical fate is determined between the late morula and blastocyst stages. At this stage, embryos are composed of pluripotent inner cell mass, which generates all three germ-layer tissues, and trophectoderm, which supports embryonic growth. Embryonic stem cell (ESC) lines are considered to be the immortal (Smith, 2001) and represent a powerful tool to address scientific and medical issues.

Human ESCs (hESCs) are derived from the inner cell mass of the human blastocyst. In 1998, James Thomson and his colleagues reported the methods for deriving and maintaining hESCs from the inner cell mass of human blastocysts produced *in vitro* (Thomson et al, 1998). These cells have the unique ability to reproduce itself for a long period and differentiate into cells of all tissues. Therefore, hESCs can be used as a powerful biological material to explore not only specific genetic programs but also essential mechanisms underlying embryogenesis. Most importantly, the use of hESCs for generating healthy cells or tissues has the potential to revolutionize therapies for human disease or injury. To realize possibility of the cell therapy, understanding the molecular characteristics of hESCs should be elucidated.

In mouse ESCs, it has been known that specific transcription factors are Oct-4, Nanog, Sox2, FoxD3, and Stat3. Oct-4 is expressed in the ICM and down-regulated upon differentiation (Nichols et al., 1998). Nanog, a homeobox transcription factor, plays a crucial role in the second

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embryonic cell fate specification. It is down- regulated upon differentiation into primitive endoderm (Chambers et al., 2003; Mitsui et al., 2003). Sox2 and FoxD3 are involved in the maintenance of the epiblast after implantation (Avilion et al., 2003; Hanna et al., 2002). In mouse ESCs, Stat3 activation by the cytokine leukemia inhibitory factor (LIF) is required to sustain self-renewal (Williams et al., 1988; Niwa et al., 1998; Matsuda et al., 1999), although LIF is not required for normal mouse development (Stewart et al., 1992). However, LIF/Stat-3 activation does not support self-renewal of hESCs (Sato et al., 2004).

It is reported that several signal transduction pathways including Wnt, TGF-β, Janus kinase (JAK)/signal transducer and activator of transcription (STAT), Hedgehog, Notch, Receptor tyrosine kinase (RTK) is related to maintain ESC pluripotency (Sato et al., 2003). During early vertebrate embryogenesis, the canonical Wnt pathway has important embryological functions including the induction of the dorsal organizer (node) through the formation of the Nieuwkoop center (Moon, 2002). Components of the Wnt pathway promote the proliferation of primitive cells in skin (Zhu and Watt, 1999; Gat et al., 1998), gut and brain (Chenn and Walsh, 2002). Wnt signaling also inhibits differentiation of ESCs (Kielman et al., 2002). Thus, Wnt signalling may be used as a general cue for self-renewal in stem and/or progenitor cells in diverse tissues. The canonical Wnt pathway is initiated upon Wnt protein binding to the Frizzled receptor at the cell surface. Signaling downstream of the receptor leads to inactivation of GSK-3 (glycogen synthase kinase 3), resulting in the nuclear accumulation of β-catenin, which activates the transcription of Wnt target genes (Cadigan et al., 1997; Polakis et al., 2002).

In order to explore function of WNT/β-catenin pathway in hESCs, the role of Wnt signaling pathway on hESCs by the inhibitors of GSK-3, BIO ((2'Z,3'E)-6-Bromoindirubin-3'-oxime) has been studied. Chemical structure of BIO is depicted in Fig. 1. BIO is purple solid, the cell-permeable bis-indolo (indirubin) compound that acts as a highly potent ATP-competitive inhibitor of GSK-3α/β (Meijer et al., 2003; Polychronopoulos et al., 2004). Inhibition of GSK-3 by BIO induces to activate Wnt signaling pathway in hESCs (Sato et al., 2004). The present study demonstrates that BIO-treated hESCs maintain partially undifferentiated state in the modified feeder-free system and that Wnt signaling is related to self-renewal in hESCs. In addition, it has been found that BIO is effective on anti-apoptosis of hESCs.

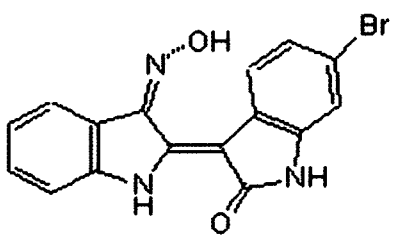

Fig. 1. Chemical structure of BIO. BIO ((2'Z,3'E)-6-Bromoindirubin-3'-oxime), initially derived from Trian purple, is the selective and potent inhibitor of GSK-3.

MATERIALS AND METHODS

Preparation and Culture of MEF Cells

In this study, hESCs were maintained undifferentiated state on MEF (mouse embryonic fibroblast) feeder layers. MEF cells were isolated from the Day 13.5 postcoitum fetuses of B6CBAF mice. Then, MEF cells were cultured in the high glucose Dulbecco modified Eagle medium (DMEM) with L-glutamine (GIBCO), 10% fetal bovine serum (FBS; GIBCO), and 1% Penicillin/Streptomycin (GIBCO). Cultured MEFs were mitotically inactivated with 0.1 mg/ml mytomycin C (Sigma) for 3 h and washed three times with PBS. Mitotically inactivated MEFs were then trypsinized with trypsin-EDTA (GibcoBRL/Invitrogen), washed twice with culture medium, placed in 1.8 ml cryovial (Nunc, Life Technologies, Denmark), frozen in -70°C freezer for 1 day and cryopreserved in LN_2 (Liquid Nitrogen). The cryopreservation medium consisted of 20% dimethyl sulfoxide (DMSO; SIGMA), 40% FBS, 40% DMEM. These frozen MEFs were thawed a day before the hESCs were plated. After rapidly thawing in a water bath at 37°C until the ice was disappeared, these cells were washed with cultured medium, counted with hematocytometer (Marienfeld) and plated at a concentration of 7.5×10^4cells/cm^2 on 0.1% gelatin-coated tissue culture dishes.

hESCs Culture

Miz-hES1 line (Park et al., 2003), which was supplied from MizMedi Hospital and cultured with MEF feeders for 135 passages, was used in these experiments. The culture medium consisted of 77.5% D-MEM/F-12 (GIBCO) supplemented with 20% serum replacement (GIBCO), 0.1 mM β-mercaptoethanol (SIGMA), 1% MEM non-essential amino acid solution (GIBCO), 4 ng/ml basic fibroblast growth factor (bFGF; GIBCO) and 0.5% Penicillin/Streptomycin (GIBCO). hESCs were cultured at 37°C, 7.5% CO_2 in air. To maintain undifferentiated state, hESCs were passaged every 4 to 5 days by mechanical dissection. Transferred hES colonies were grown on the fresh MEF feeders.

For *in vitro* experiments, the modified feeder-free system was developed. In this system, hESCs were transferred to the mitomycin C-treated MEF feeder cells coated on the dishes. hESCs were attached on MEF feeder layers for 2 days, and then only MEF feeder cells were mechanically removed by using a hand-made glass pippet. In this condition, hESCs were cultured for another 3~5 days in the nonconditioned media (control). In order to study the effect of BIO on hESCs, hESCs were cultured in nonconditioned medium with 5 nM BIO (MERCK) for 5 days (BIO-treated). During the culture of hESCs, approximately 70% of medium

volume was exchanged daily.

Immunohistochemistry

hES cells were washed with phosphate buffered saline (PBS), fixed with fresh 4% paraformaldehyde in PBS for 1 hr at 4°C, and washed 6 times with 0.1% Tween 20 (Sigma) in PBS for 10 min each. The cells were permeabilized with 0.5% Triton X 100 (Sigma) in PBS for 1 hr at room temperature and incubated with the primary antibody for 1 hr. Stage-specific embryonic antigen (SSEA) 1, 3,and 4 (Chemicon) were used as primary antibodies. Additional primary antibodies were Oct-4 (Santacuz, Sc8628), GFAP (Chemicon, Mab3402), Nestin (Chemicon, Ma b5326), GATA2 (Santacruz, Sc9008), α-FP (Santacruz, Sc8399) and β-catenin (BD science, 610153). The secondary antibody was Cy3 or conjugated antibodies. Then, the immunostaining was visualized by the complex of avidin and horseradish peroxidase (Vectastain ABC system; Vector Laboratory, Burlingame, CA), and DAB substrate (Vector company). To detect alkaline phosphate activity, the cells were stained with the AP (Alkaline phosphatase) staining kit (Sigma AP Kit).

After air-drying, stained samples were mounted on the slide. Individual samples were observed under diverse magnifications (×40, ×100, ×200, ×400) on the inverted microscope (Olympus) equipped with a camera. The images were evaluated using the Adobe Photoshop program.

Flow Cytometry

For cell cycle and apoptosis assays, individual colonies were harvested and dissociated into single cells with the dissociation buffer for 20 min at 37°C. Then, dissociated cells were washed twice with PBS, fixed with 70% ethanol at 4°C for more than 1 hr, stained with 100 mg/ml propidium iodide (Sigma) containing 100 mg/ml RNase. These cells (10,000 events) were analyzed for DNA content by the flow cytometry (Becton-Dickinson San Jose, CA). The data were analyzed by the CellQuest program (FACSCalibur, BD).

RESULTS

BIO Activates Wnt Signaling in hESCs

The central player of Wnt signaling is β-catenin, which is a transcription cofactor with T cell factor/lymphoid enhancer factor TCF/LEF in the Wnt pathway (Cadigan et al., 1997) and a structural adaptor protein linking cadherins to the actin cytoskeleton in cell-cell adhesion (Jarnora et al., 2002). In order to examine whether BIO-treatment activates Wnt signaling of hESCs, the expression of β-catenin was evaluated at the cellular level (Fig. 2). In the control group, β-catenin was distributed throughout the plasma membrane at sites of cell-cell contact, but it was not accumulated in the nucleus. Interestingly, hESCs treated with BIO showed that β-catenin was distributed throughout the plasma membrane and in the nucleus. These data indicate that BIO activates Wnt signaling pathway in hESCs.

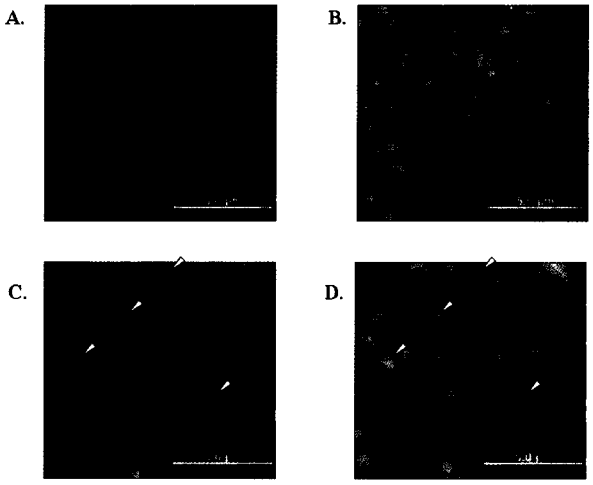

Fig. 2. BIO-treated hESC activates Wnt signaling. Immunofluorescent images of hESCs grown under control (A) and BIO-treated (C) conditions and incubated with β-catenin specific antibody. hESCs treated with BIO showed nuclear accumulation of β-catenin whereas the control did not. The arrows indicate stained β-catenin in nuclei. Cells were counterstained with DAPI; control (B), BIO-treated group (D). Scale bars, 5 μm.

The Activation of Wnt Signaling Sustains the Self-renewal of hESCs

During culture of hESCs, differences were observed in the colony morphology between control and BIO-treated groups (Fig. 3). In the control group, the cells were spread

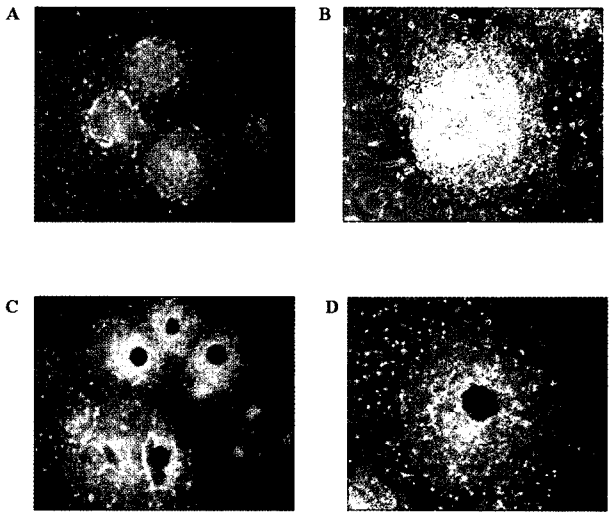

Fig. 3. Different morphologies between control and BIO-treated hESCs. In the control group, the cell was spread outwards. The gap between cells in outside is looser than that of inner cells. A: magnification ×40, B: ×100. In the BIO-treated group, the cells were clumped in the center and differentiated cells were spreading outwards in the edges. C: magnification ×40, D: ×100. Scale bars, 20 μm.

outwards. The cells outside was looser than the inner cells. In BIO-treated group, the cells were divided two parts, which the cells were clumped in the center and the differentiated cells were spreading outwards in the edges. In this study, the distinctive morphology was called "center-clumped colony". As shown in Table 1, BIO-treated group showed a higher proportion of center-clumped colony than the control ($p<0.05$).

In order to examine whether BIO-treated hESCs maintain self-renewal, hESCs were stained with stem cell-specific markers. In the control, hESCs were weakly stained with AP, SSEA (stage specific embryonic antigens)-3, -4, Oct-4 except SSEA-1. These data indicate that most hESCs in the control group were differentiated. In BIO-treated group, hESCs in the center showed AP, SSEA-3, -4, Oct-4 staining whereas outward in the edges did not. Our data demonstrated that, in BIO- treated hESCs, the self-renewal was maintained in the center, but the hESCs in outside edges were differentiated (Fig. 4).

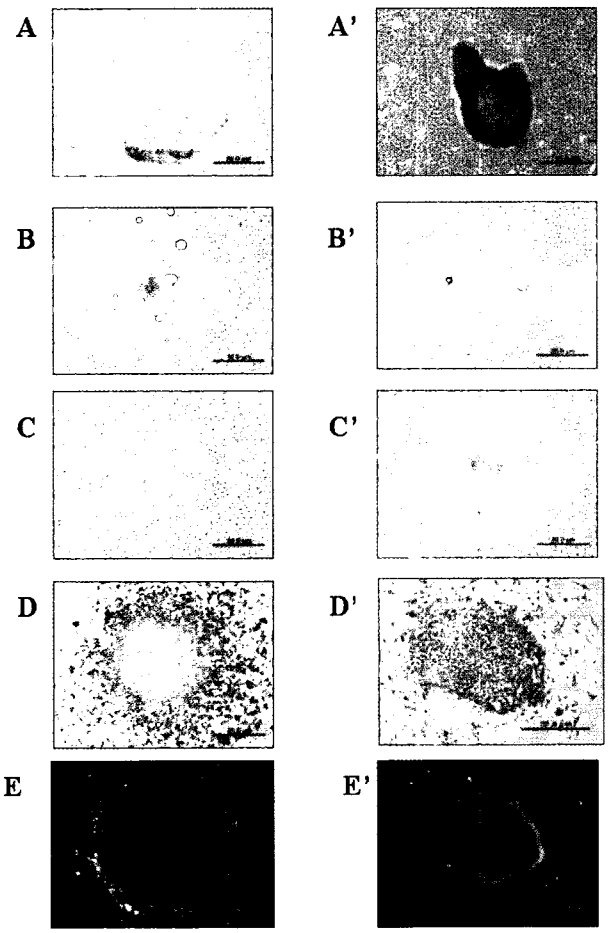

Fig. 4. Expression of stem cell specific markers in the BIO-treated hESCs. Control (A-E) and BIO-treated (A'-E') hESCs were stained with stem cell surface marker AP (A, A'), SSEA-1 (B, B'), SSEA-3 (C, C'), SSEA-4 (D, D') and Oct-4 (E, E') antibodies. These data suggest that, in BIO-treated hESCs, the self-renewal is maintained in the center. Oct-4 was counterstained with DAPI (E, E'). Scale bars, 20 μm.

Table 1. Efficiency of center-clumped colony formation by BIO-treatment.

Group	No. of colonies examined	No. of center-clumped colony	Efficiency (%)
BIO-treated	611	496	81.2[a]
Control	690	40	5.8[b]

a vs b: $p<0.05$.

Spontaneous Differentiation of the Margin Cells of hESC Colonies without MEF

When MEF were removed during culturing hESCs, it was found that the marginal cells of hESC colonies were spontaneously differentiated. In order to determine the cell fate of differentiated cells, Nestin (marker of neuronal progenitor cell), GFAP (marker of Astrocytes and Bergman glial cell), GATA2 (mesoderm marker), and α-FP (endoderm marker) antibodies were used. In the control group, cells were stained Nestin, but not GFAP, GATA2, α - FP (Fig. 5). In BIO-treated group, cells in the outside were stained Nestin, but not GFAP, GATA2, α - FP, suggesting that outer

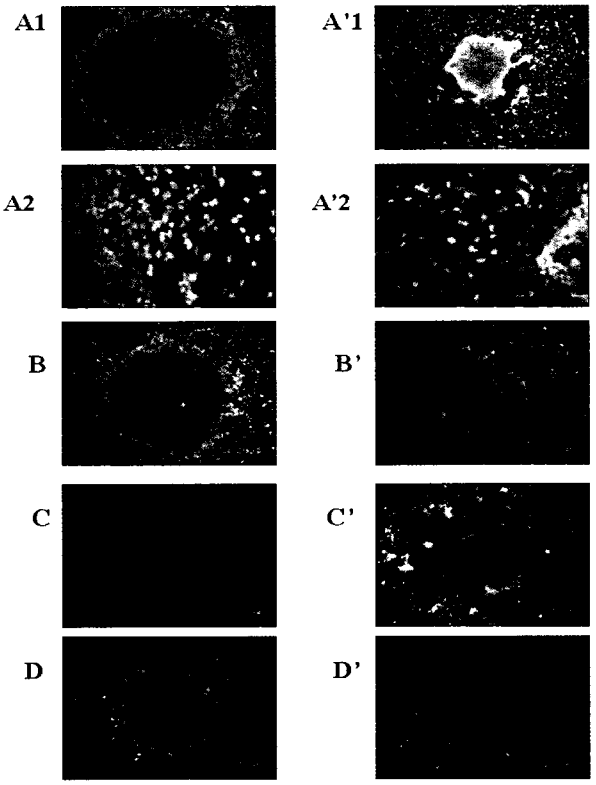

Fig. 5. Differentiation of outer hESCs to neuronal progenitor cells. Control (A-D) and BIO-treated (A'-D') hESCs were stained with antibodies against Nestin (A1A2, A'1A'2), GFAP (B, B'), GATA2 (C, C'), α-FP (D, D'), respectively. Differentiated cells were positive for Nestin antibody, but negative for GFAP, GATA2 and α-FP antibodies. Cells were counterstained with DAPI. Scale bars represent 10 μm (A2, A'2) and 20 μm (A1,A'2 and BD), respectively.

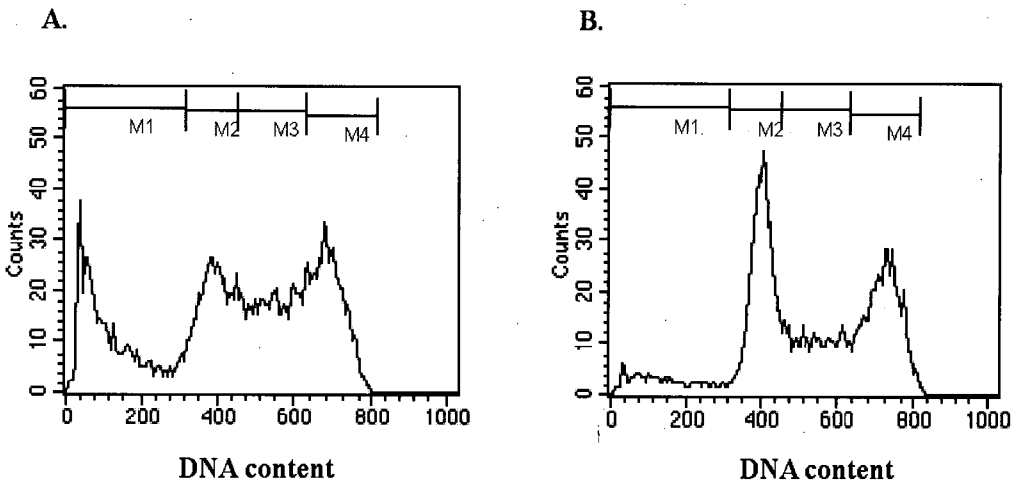

Fig. 6. Flow cytometric analysis of the BIO-treated hESC. Control group (A) and BIO-treated group (B) were fixed and stained with propidium iodide. DNA content was measured by the fluorescene- activated cell sorting analysis. The different histograms were shown between the control and BIO-treated hESCs.

Table 2. Effect of BIO-treatment on anti-apoptosis of hESCs.

	Control (%)	BIO-treated (%)
Apoptosis	23.76	5.59
G1	23.64	37.51
S	28.04	22.18
G2/M	24.90	34.97

cells were differentiated into the neuronal progenitor cells. These data indicate that removing MEF after 2 day of culture induces outer cells of the hESC colony differentiated to the neuronal progenitor cells in both control and BIO-treated hESCs.

Anti-Apoptotic Effect of BIO in hESC

In order to test the difference of cell cycle in the control and BIO-treated group, hESCs were stained with propidium iodide and DNA content was measured by the fluorescence-activated cell sorting analysis (Fig. 6). As shown in Table 2, control group showed higher composition of apoptotic cells (23.76%) than the BIO-treated group (5.59%), whereas BIO-treated group had a higher proportion of cells with G1 and G2/M phases. The results indicate that removing MEF may make unstable in the culture condition of hESCs, thereby inducing apoptosis. Consequently, our findings suggest that BIO may act on anti-apoptosis of hESCs.

DISCUSSION

hESCs are usually established and maintained undifferentiated state on mouse embryonic fibroblast (MEF) feeders. After hESCs attached on the bottom for 2 days, removal of MEF induced hESCs to differentiate from the outside. β-catenin staining confirmed that inhibition of GSK-3 by BIO activates Wnt signaling pathway in hESCs. BIO-nontreated hESCs were differentiated in large part whereas BIO-treated hESCs maintain self-renewal in the center of the colonies. Thus, activation of Wnt signaling by BIO maintained hESCs in the partial undifferentiated state, confirming that the Wnt signaling pathway is related to self-renewal of hESCs. The differentiated cells in control and BIO-treated group were positive for Nestin, suggesting that they differentiated into the same cell fate. Removing MEF induced hESCs to differentiate to ectoderm cell fate especially, neuronal progenitor cells.

Recently, in hESCs, activation of the WNT/β-catenin pathway by 6-bromoindirubin-3'-oxime (BIO), a pharmacological inhibitor of GSK-3, maintains the undifferentiated phenotype (Sato et al., 2004). This report describes that hESCs maintain undifferentiated state. However, in this study, hESCs maintained self-renewal only in the center, but differentiated from edges because removing MEF after 2 days could make unstable culture condition for maintaining self-renewal on the edges. These hESC colonies showed distinctive center-clumped colony morphology. It was also found that BIO-treated hESCs resulted in anti-apoptosis. Removing MEF after 2 days induced apoptosis in the control group but BIO-treated hESCs resulted in anti-apoptosis compared to the control group. These results suggest that BIO may have anti-apoptotic function. Glycogen synthase kinase 3 (GSK-3) has a major role in Wnt and Hedgehog signaling pathways and regulate the cell-division cycle, apoptosis, circadian rhythm, trascription and insulin action. Many evidences support a speculation that pharmacological inhibitors of GSK-3 could be used to protect cells from

apoptosis (Meijer et al., 2004; King and Jope, 2005). BIO, which is one of pharmacological inhibitors of GSK-3, may have the same effect to hESCs.

The unexpected data found in the cell cycle results were that the proportion of S phase in BIO-treated group was lower than the control. The composition of proliferating cells was slightly higher in the control (28.04%) than in the BIO-treated group (22.18%). hESCs are unusual for cell cycle distribution since the proportion of S phase is fairly high (Lee et al., 2005). In this study, hESCs has quite high proliferation capacity. There is a report that Wnt signaling can proliferate haematopoietic stem cells (Reya T., 2003). Because BIO activates Wnt signaling and maintains partial self-renewal in hESCs, BIO may induce hESCs to elevate proliferation capacity.

This study demonstrates that Wnt signaling plays an important role in the self-renewal of hESCs and BIO, a GSK-3 inhibitor, may have anti-apoptotic function in hESCs. There are many reports that Wnt signaling is related to maintaining self-renewal in various pluripotent stem cells and progenitor cells. There is also evidence that Wnt signaling can provide an instructive signal that changes the fate of stem cells (Lee et al., 2004). Here our results also indicate that modulating Wnt signaling plays an important role on controlling the stem cell fate.

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