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Effect of SP600125 on proliferation of embryonic stem cell

Yonghua Zhou, Minggui Jiang, Mei Wang, Chao Luo, Zili Wang, Sheng Wen, Wenbin Liu, Yamei Xiao*

Key Lab of Protein Chemistry and Developmental Biology of Education Ministry of China, College of Life Sciences, Hunan Normal University, Changsha, China
Email: *yameix@126.com

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ABSTRACT

SP600125 is an inhibitor of c-Jun NH_2-terminal kinase (JNK), which plays a fundamental role in regulating animal development. Using SP600125 to deal with the mouse embryonic stem cells, it is revealed that the number of the ESC colonies decreased and the size became smaller. With treatment by SP600125, the proliferation of mouse ES cells is seriously inhibited for the cycle arrested in the G2/M phase, and the effect of SP600125 on the ES cells displays correlation of dose and time. The obtained results indicate that JNK may be an important regulator in the progression of cell cycle at the G2/M cell phase for the ES cells.

Keywords: JNK; SP600125; Embryonic Stem Cell; Cell Cycle

1. INTRODUCTION

The c-Jun N-terminal kinase (JNK) is a type of main serine/threonine (Ser/Thr) protein kinases in the mitogen-activated protein kinase (MAPK) family [1,2]. It has been verified that the JNK signaling pathway is related with the apoptosis of cells, the incidence of cancer and the pathological response in mammalian [3,4]. As a synthetic polyaromatic chemical, SP600125, Anthrapyrazolone or 1,9-Pyrazoloanthrone, is a highly selective inhibitor of JNK [5,6]. Kuan [7] found that the 60-minute middle cerebral artery occlusion could increase JNK activity in the obstructed area, and SP600125 can inhibit neuronal apoptosis induced by cerebral ischemia. In the mouse preimplantation embryonic development, application of SP600125 at 10 μM slowed down the development if the embryos were cultured in a suboptimal Ham's F10 medium, while the development was improved if they were cultured in an optimized medium [8]. It suggests that the inhibition of JNK activation may cause cell cycle arrests and enhancement of apoptosis.

As a major source of generating differentiated cells for transplantation-based therapies, embryonic stem cell (ESC) holds great promise in biomedicine, in particular, for the development of new avenues to study the etiology of diseases. For more detail in this connection, one can see [9-11] and the references therein. Different from the existent results, this study is intended to further investigate the role of JNK in the cell cycle of embryonic stem cells, with employment of SP600125 to deal with the mouse embryonic stem cells.

2. MATERIAL AND METHODS

2.1. Cell Culture

The mouse embryonic stem cells (ESCs) line ES/GFP were provided by the State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences. The ESCs were cultured in two ways: with or without a feeder cell layer. With feeder cell layer of primary mouse embryo fibroblasts (MEFs), inactivated with mitomycin C (Sigma), the ESCs were cultured in the N2B27 medium, which includes DMEM/F12 (Gibco), Neurobasal medium (Gibco), N2 (Gibco), B27 (Gibco), insulin (Roche applied science), BSA (sigma), 2 mM glutamine (Gibco), 100 μM nonessential amino acids (Gibco), 0.1 mM β-mercaptoethanol (Gibco), 1000 U/ml of mouse leukemia inhibitory factor (Millipore), 100 U/ml Penicillin-streptomycin (Invitrogen), 1 μM of MEK inhibitor PD0325901 (stemgent) and 3 μM of GSK3 inhibitor CHIR99021 (stemgent). For the feeder-free way, the ESCs were cultured in the N2B27 medium, in which 5% knockout serum (Gibco) was added. The cells were cultured at 37˚C in a humid atmosphere (containing 5% CO_2).

2.2. Cell Viability Assay by the Thiazolyl Blue

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is a kind of cell viability/cytotoxicity assay

*Corresponding author.

kit (Beyotime Biotechnology, Jiangsu, China). The ES/GFP cells were inoculated at 5.0×10^4 cells/ml, cultured in medium for 24 h. Then, cells were treated with SP600125 (0, 5, 10, 15, 20, 25 μM, respectively) or vehicle (0.125% dimethyl sulfoxide (DMSO)) for 48 h. After aspirated the medium, MTT was added (final 1 mg/ml), and then cells was incubated at 37˚C for 4 h. The supernatant was replaced with DMSO to dissolve formazan production. The absorbance at wavelength 405 nm was measured using a micro ELISA reader (Bio-Rad, CA, USA). Each assay was performed in triplicate.

2.3. Flow Cytometry Analysis

The ES/GFP cells were collected by trypsinization, washed with ice-cold PBS, and fixed in 70% ethanol for 24 h at 4˚C. The cells were collected by centrifugation at 300 × g for 5 min and resuspended in PBS containing 10 mg/ml RNase. After incubating at 37˚C for 20 min, and ESCs were treated with 10 mg/ml PI for 30 min at 4˚C, then cells were analyzed using a FACSCalibur (Becton Dickinson, Mountain View, CA) flow cytometer. The data were analyzed using the Modi FIT program (Verity Software House, Topsham, ME).

2.4. Immuno-Fluorescence

The ES/ GFP cells which grew to 60% - 70% confluency on glass coverslips were fixed with 4% paraformaldehyde for 30 min. Then, they were washed three times in PBS for 5 min and permeabilized using 0.3% Triton X-100/PBS for 15 min. These cells were blocked for 1h in 0.3% Triton X-100 with 2% BSA, then, anti-JNK1 mouse antibody (Santa Cruz) was diluted 1:100 and incubated overnight at 4˚C. Subsequently, the obtained cells were washed three times in PBS for 5 min. These cells were treated with fluorescence-conjugated secondary antibodies cy3 (Santa Cruz, 1:200) and incubated for 1 h at the room temperature. Then, Nuclei were stained with Hoechst 33342 (Sigma) for 20 min at the room temperature.

3. RERULTS

3.1. Growth of the ES/GFP Cells *in Vitro*

The ES/GFP cells produced green fluorescence (see **Figure 1**). The colonies of ES/GFP cells on the feeder of inactivated MEFS were round, oval or spindle (**Figure 2(A)**). The number of cell colonies was greater than that of the ESCs which were cultured in the feeder-free way (**Figure 2(B)**).

3.2. Effect of SP600125 on the Growth Form of ES/GFP Cells

The growth of ESCs in the 0.125% DMSO was same as that of those in the normal control group, both for the ESCs on feeder (**Figures 3(a)** and **(d)**) and for those on feeder-free (**Figures 3(b)** and **(e)**). With treatment by SP600125, the colonies of ESCs reduced significantly and were smaller in the group of 5 μM SP600125 (**Figures 3(c)** and **(f)**).

The effect of SP600125 on the ESCs displays the correlations of dose and time. With an increasing concentration of SP600125, the number of ESCs colonies became less and less, and the size of these cells became smaller and smaller (**Figures 4(a)-(d)**). On the other hand, with an increment in the processed time of SP600125, the number of ESCs colonies also was less and less, and the size was smaller and smaller (**Figures 4(e)-(h)**). In addition, in the cytosol of the ESCs, the JNK was mainly expressed, it seems that there

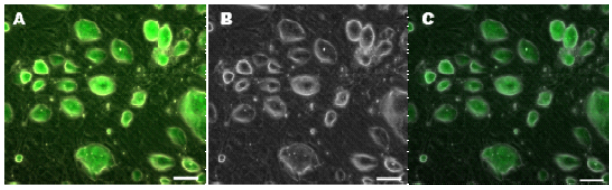

Figure 1. The cloning of ES/GFP cells cultivated with feeder for 48 h. It shows that ES/GFP cells produced green fluorescence (A). **Figure (B)** is the ES cells under the phase contrast microscope. **Figure (C)** is merged of **Figures (A)** and **(B)**. Scale bar: 100 μm.

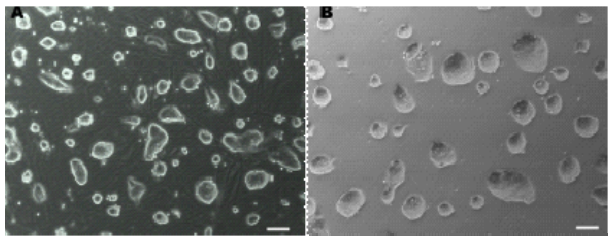

Figure 2. Characterization of ES/GFP cells cultivated with feeder (A) or feeder-free (B) for 48 h. Scale bar: 100 μm.

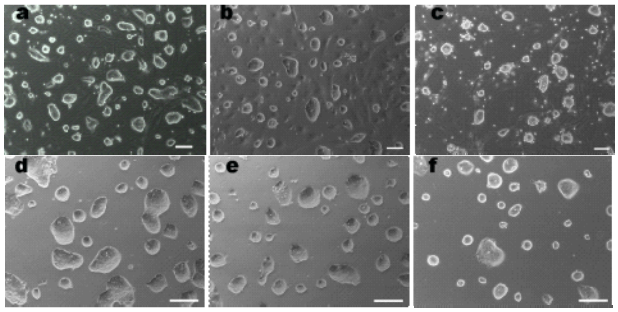

Figure 3. Characterization of the ES cells cultured in the presence of SP600125 or vehicle (0.125% DMSO) for 48 h. cells were inoculated at a concentration of 5.0×10^4 cells/ml, cultivated with feeder (a)-(c) or feeder-free (d)-(f) for 48 h. (a), (d) Normal control; (b), (e) 0.125% DMSO control group; (c), (f) Experiment group with 5 umol SP600125. Scale bar: 100 μm.

was no obvious change in the expression of JNK in the ESCs for the SP600125-treated groups (**Figure 5**).

3.3. Analysis on the Proliferation of ES/GFP Cells Treated with SP600125

The ESCs were cultured with treatment of 0, 5, 10, 15, 20, 25 μM SP600125, respectively. After 48 h, relative quantity and viability assay of ESCs were detected by MTT method. Compared with the control group, the proliferation of ESCs was obviously suppressed in the SP600125-treated groups. It also shows that less cell proliferation happened with the increment of inhibitor concentration (**Figure 6**).

The ESCs were inoculated at a concentration of 5 × 10^4 cells/ml, cultivated for 24 h, and subsequently treated with 25 μM SP600125 or vehicle (0.125% DMSO) for 12 h. After being stained with propidium iodide, the cells were detected by flow cytometer (see **Table 1**). In the control group, where the ESCs cultivated with 0.125% DMSO after 12 h, it was detected that the cells of 19.95% were at the G1 phase, the cells of 68.53% were at the S phase, and the cells of 11.52% were at the G2/M phase (**Figure 7(a)**). In the ES cells of SP600125-treated for 12 h, there were 9.09% cells at the G1 phase, 27.18% at the S phase and 63.73% at the G2/M phase (**Figure 7(b)**). These results show that SP600125 affects the cell cycle progression of ES cells, the majority of cells are at the G2/M phase with treatment of SP600125.

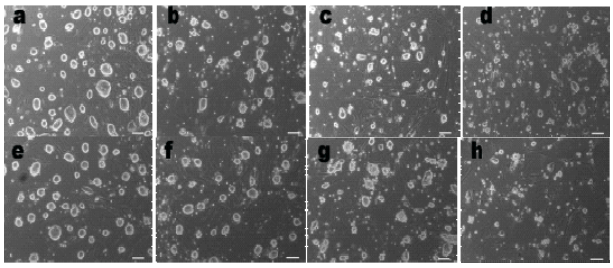

Figure 4. (a)-(h) The effect of SP600125 on the ES cells. (a)-(d): ES cells cultured with feeder were treated with 0.125% DMSO (**Figure (a)**) or 5, 20, 25 μM SP600125 respectively (**Figures (b)-(d)**) for 24 h; (e)-(f): ES cells cultured with feeder treated with 25 μM SP600125 for 12 h, 24 h, 48 h (**Figures (f)-(h)**) or vehicle (0.125% DMSO) for 48 h (**Figure (e)**). Scale bar: 100 μm.

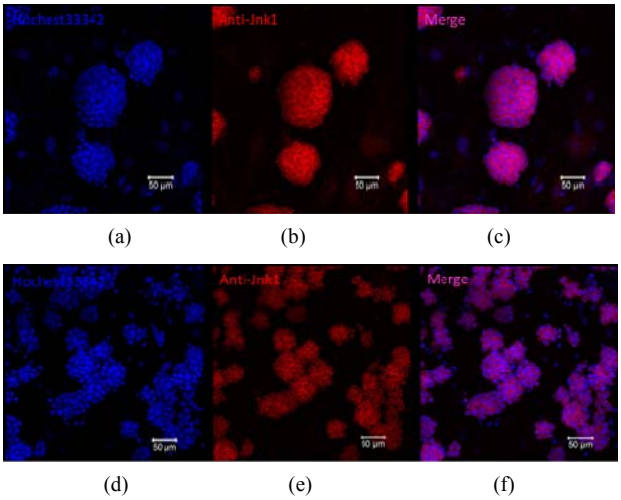

Figure 5. (a)-(f) Cell morphology of ES/GFP cells treated with SP600125 examined by immunostaining. (a)-(c): ES/GFP cultured with feeder treated with 25 μM SP600125 for 24 h; (d)-(f): The control group. JNK (red) were observed. DNA was stained by Hochest 33342 (blue). Scale bar: 50 μm.

4. DISCUSSION

The JNK is involved in a variety of pathways and is activated if the cell is exposed to stress, differentiation

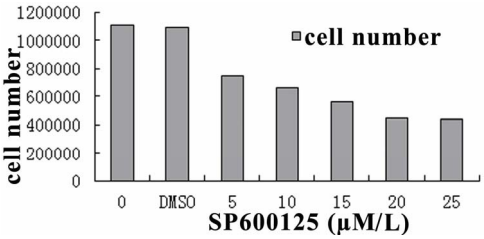

Figure 6. The detection of proliferation of ES cell by MTT. The ES cells were treated with 0, 5, 10, 15, 20, 25 μM SP600125 or vehicle (0.125% DMSO) for 48 h. Compared with control group, the proliferation of ES cells was obviously suppressed in those SP600125-treated groups.

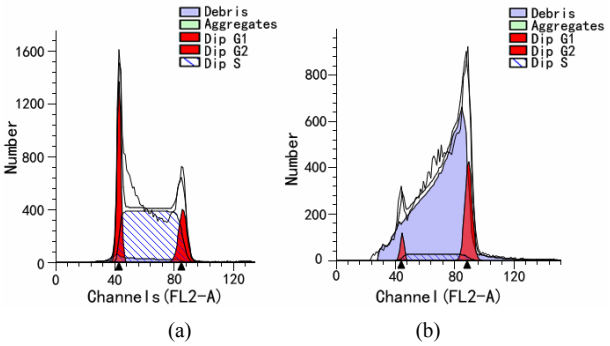

Figure 7. Cell cycle analysis of the ES cells by flow cytometer. (a) The ES cells were inoculated at a concentration of 5 × 10^4 cells/ml, cultivated for 24 h, and then treated with 0.125% DMSO for 12 h; (b) The ES cells were inoculated at a concentration of 5 × 10^4 cells/ml, cultivated for 24 h, and then treated with 25 μM SP600125 for 12 h.

Table 1. The percentages of different phase cell in the ES/GFP cells treated with SP600125 or vehicle (0.125% DMSO) for 12 h.

	percentages of different phase cell		
	G1 (%)	S (%)	G2/M (%)
0.125% DMSO	19.95	68.53	11.52
25 μM SP600125	9.09	27.18	63.73

or growth signals [4]. It has recently demonstrated that mutation in MEKK4 (upstream kinase of JNKs) renders trophoblast stem cells upregulating Slug, Twist, and MMP2 in response to FGF4 stimulation [10]. During selenium induced improvement of stem cell behavior, activation of JNK1/2 and Akt by selenium in human adipose tissue-derived stromal cells results in induction of numerous stem cell genes, including Rex-1, Nanog, and Oct-4 [11]. In addition, JNK signaling has been shown to regulate Oct-1 [12] and also likely control Sox-2 through direct action of Oct-1 [13]. SP600125, a specific inhibitor of the JNK pathway, has been shown to inhibit the JNK activation, as well as AP-1 activation in murine colitis [14]. The JNK inhibitor, SP600125, could block the L-threonine-induced expression of the stem cell marker OCT4 and several cell proliferative molecules, such as cyclin D1, cyclin E, and c-Myc [15]. This study investigated the effects of SP600125 on the viability and the cell cycle distribution of embryonic stem cells. The results show that SP600125 could reduce proliferation of the ESCs and induce G2/M-phase arrest. JNK signaling may be involved in control of stem cell differentiation. And upstream and downstream regulating factors, such as Oct-1, Oct-4 and so on, can be further investigated to find the clues of how JNK affects cell proliferation to enhance this study.

The JNK acts within a protein kinase cascade. Following its activation by phosphorylation, the JNK can phosphorylate a range of nuclear substrates, such as c-Jun, ELK-1, ATF2 and DPC4 [16]. Many of these nuclear proteins belong to transcription factors, hence, the phosphorylation by the JNKs can mediate actions via a direct link to the changes in gene expression with the exposure of cells to a range of cytokines and stress stimuli. The JNK activity may result in the development of a more malignant and chemoresistant phenotype of aneuploid cells [17,18]. It is well known that SP600125 inhibits JNK function by preventing phosphorylation of c-Jun, which prevents expression of downstream genes [16]. In our results, JNK is mainly expressed in the cytosol of ESCs, and it seems that there is no obvious change in the expression of JNK in the ESCs for SP600125-treatment. This suggests that SP600125 has no effect on the level of JNK, but affects some downstream factors through the regulation of JNK activity. The regulation of subcellular localization by JNK-mediated phosphorylation can be a critical control mechanism in the signaling downstream of JNKs [19,20].

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