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High sensitivity of embryonic stem cells to proteasome inhibitors correlates with low expression of heat shock protein and decrease of pluripotent cell marker expression

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The ubiquitin-proteasome system is a major proteolytic system for nonlysosomal degradation of cellular proteins. Here, we investigated the response of mouse embryonic stem (ES) cells under proteotoxic stress. Proteasome inhibitors induced expression of heat shock protein 70 (HSP70) in a concentration- and time-dependent manner, and also induced apoptosis of ES cells. Importantly, more apoptotic cells were observed in ES cells compared with other somatic cells. To understand this phenomenon, we further investigated the expression of HSP70 and pluripotent cell markers. HSP70 expression was more significantly increased in somatic cells than in ES cells, and expression levels of pluripotent cell markers such as Oct4 and Nanog were decreased in ES cells. These results suggest that higher sensitivity of ES cells to proteotoxic stress may be related with lower capacity of HSP70 expression and decreased pluripotent cell marker expression, which is essential for the survival of ES cells. [BMB reports 2012; 45(5): 299-304]

INTRODUCTION

Embryonic stem (ES) cells are derived from remnant embryos after *in vitro* fertilization and are specifically established from the inner cell mass of blastocysts. ES cells have the ability to self-renew, maintaining their stemness on mouse embryonic fibroblast (MEF) cells (1) and have pluripotent ability of differentiation to multiple tissue lineages in certain conditions (2, 3). Understanding of the molecular mechanisms underlying self-renewal and differentiation of these pluripotent cells is critically important in basic stem cell biology and future applications, and studies on ES cells may provide general information to understand pluripotent cells (4).

The ubiquitin-proteasome system is a major proteolytic system for non-lysosomal degradation of damaged and abnormal proteins. The ubiquitin-proteasome system is also responsible for the degradation and proteolytic processing of cellular proteins essential for the regulation of basic cellular processes, such as development, differentiation, proliferation, and cell cycling (5, 6). In addition, it is known to be involved in apoptosis (7).

Heat shock proteins (HSPs) are a family of evolutionarily conserved chaperone proteins and induced by various stimuli such as infection, high temperature, free radicals, and mechanical stress (8). HSP70 is involved in the protein folding process and translocation of proteins through the intracellular membrane and in the interactions with signal transduction proteins (9-11). Another function of HSP70 is to inhibit cellular apoptosis induced by various stresses. HSP70 prevents cell death by interaction with apoptosis regulators such as cytochrome c and Apaf-1 and AIF (12, 13).

According to previous studies, a number of processes and molecular components that regulate self-renewal, proliferation, and differentiation in ES cells are regulated by the ubiquitin-proteasome system (14). ES cells have unique molecular properties compared with adult cells, including a unique transcriptional hierarchy, a poised epigenetic state, and a short cell cycle transit time (15). Furthermore, ES cells are known to be more sensitive to environmental stress than adult cells (16). In this study, we treated mouse ES cells with proteasome inhibitors and investigated HSP70 expression and survival of mouse ES cells. We also compared the sensitivity of ES cells to proteotoxic stress with other somatic cells.

RESULTS AND DISCUSSION

Expression of HSP70 by proteasome inhibitors in mouse ES cells

Proteotoxic stress inhibiting regular protein processing is known to activate the transcription factor HSF1 and induce ex-

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pression of HSPs in many cells (9). To check whether proteasome inhibitors induced a stress response in ES cells, we first assessed induction of HSP70 after treatment with proteasome inhibitors such as MG132, Lactacystin, and Epoxomicin for 24 h. As shown in Fig. 1A, all of the tested proteasome inhibitors induced expression of HSP70 at the mRNA level as well as the protein level, implying that HSP70 expression is regulated at the transcriptional level. When the expression levels of HSP70 were examined in detail in the MG132- or Epoxomicin-treated cells, it was found that HSP70 expression was upregulated in a dosage-dependent and time-dependent manner (Fig. 1B and C).

Severe apoptosis induced by proeasome inhibitors in mouse ES cells

Proteasome inhibitors are known to induce apoptosis in various cell types (7). We treated mouse ES cells with proteasome inhibitors at the indicated concentrations for 24 h and observed morphological change that was presumed to indicate apoptotic cell death (Fig. 2A). To confirm apoptosis in mouse ES cells, we performed annexin V staining assays after treatment with MG132, Expoxomicin, and Lactacystin. As shown in Fig. 2B, the mean fluorescence intensity was significantly increased in the cells treated with proteasome inhibitors. This supports the notion that proteasome function is important for the survival of ES cells, as previously reported in other cells (6, 7).

It is widely known that ES cells are very sensitive to culture conditions. This means that ES cells can be more susceptible to environmental stresses including proteotoxic stress than other cells. It has also been noted that proteasome inhibitors exert distinct effects depending on cell types (7, 14, 15, 17-19). To compare the sensitivity of ES cells and somatic cells, we measured apoptosis of mouse stromal cells (STO), mouse embryonic fibroblasts (MEF), and human embryonic kidney cell line HEK293 after treatment with proteasome inhibitors. As shown in Fig. 2C, mouse ES cells showed much severer apoptosis compared with STO, HEK293, and MEF cells.

Lower expression level of HSP70 in ES cells compared with other somatic cells

HSP70 is a member of stress proteins which are involved in the protection of cells from cell death. To explain the differential sensitivity of ES cells and somatic cells to proteasome inhibitors, we evaluated HSP70 expression levels after treatment with proteasome inhibitors in mouse ES cells, STO, MEF, and HEK293 cells. As shown in Fig. 3, HSP70 is barely expressed in mouse ES cells and the basal level of HSP70 protein was higher in STO, MEF, and HEK293 cells. Furthermore, the expression of HSP70 protein was more significantly induced by proteasome inhibitors in STO, MEF, and HEK293 cells compared with mouse ES cells. These results reveal that the difference of the apoptotic response between ES cells and somatic cells correlates with the expression level of HSP70.

Decrease of pluripotent cell marker expression induced by proteasome inhibitors in mouse ES cells

To directly examine the effect of proteasome inhibition on stem cell properties of mouse ES cells, we treated ES cells with proteasome inhibitors such as MG132, Epoxomicin, and Lactacystin and examined expression levels of pluripotency markers such as Oct4 and Nanog using a Western blot

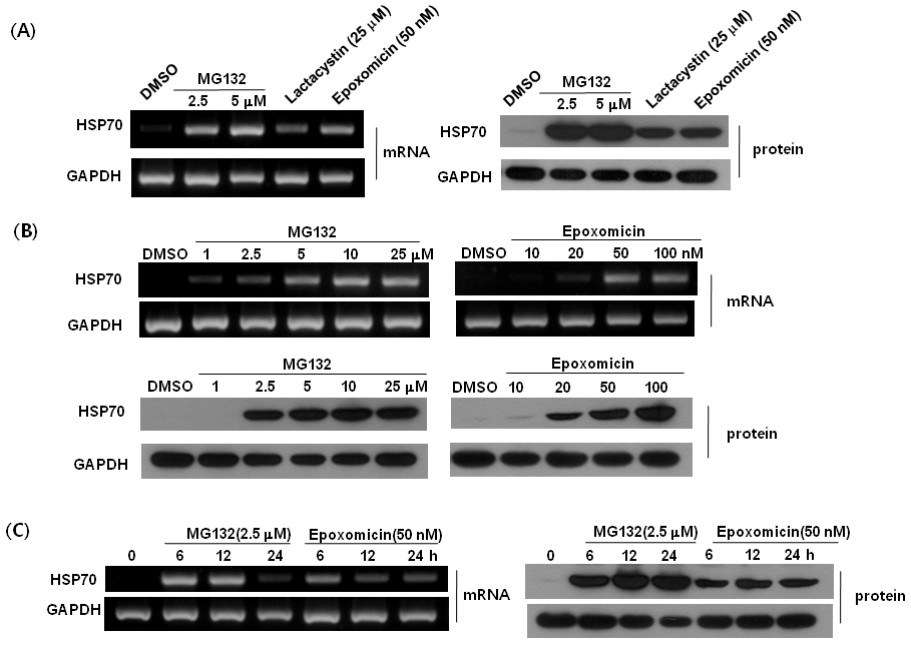

Fig. 1. Expression of HSP70 induced by proteasome inhibitors in mouse ES cells. Expression levels of HSP70 mRNA and protein were examined. (A) ES cells were treated with MG132, Lactacystin, and Epoxomicin for 24 h. (B) ES cells were treated with MG132 and Epoxomicin at the indicated concentrations for 24 h. (C) ES cells were treated with MG132 (2.5 μM) and Epoxomicin (50 nM) for the indicated periods. These results are representative of three experiments that yielded similar results.

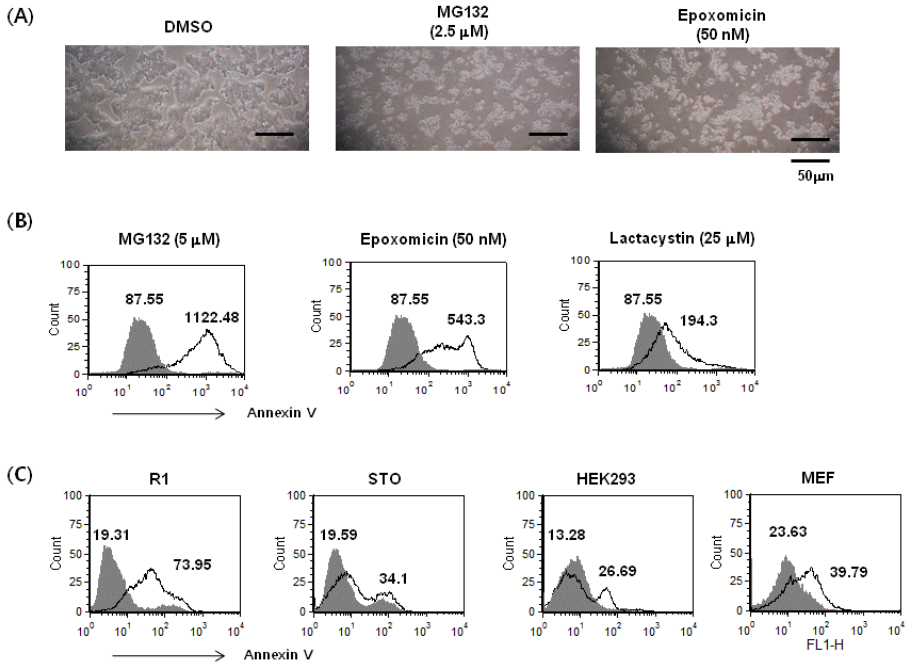

Fig. 2. Morphological change and apoptosis induced by proeasome inhibitors in mouse ES cells. (A) ES cells were treated with MG132 and Epoxomicin at the indicated concentrations for 24 h and the change of the cell shape was monitored. The scale bar represents 50 μm. (B) ES cells were treated with proteasome inhibitors for 24 h. Annexin V-stained cells were analyzed by flow cytometry. Control cells were treated with DMSO (grey). (C) STO, MEF, and HEK293 cells were treated with proteasome inhibitors for 24 h and apoptosis was compared with ES cells. Mean florescence intensities of DMSO vehicle control cells (grey) and treated cells are indicated. These results are representative of three experiments that yielded similar results.

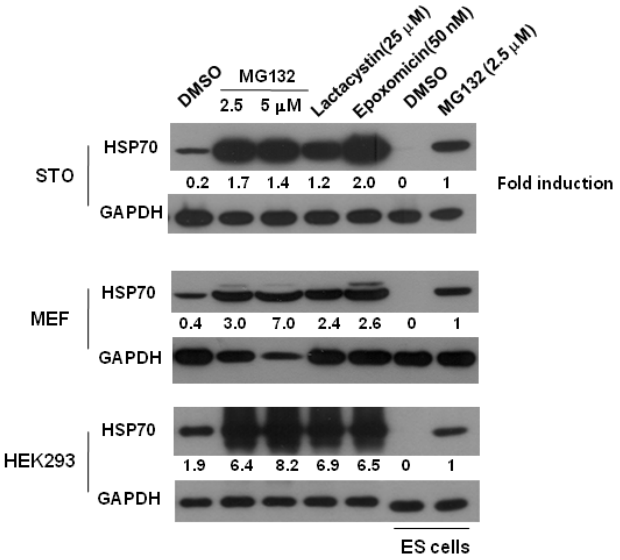

Fig. 3. Differential induction of HSP70 by proteasome inhibitors in ES cells and somatic cells. Mouse ES cells and other somatic cells such as STO, MEF, and HEK293 cells were treated with proteasome inhibitors at the indicated concentrations and expression levels of HSP70 are compared. HSP70 expression was normalized by the amount of GAPDH, and fold-induction is indicated compared with the HSP70 expression induced by MG132 (2.5 μM) in ES cells that was normalized by GAPDH. These results are representative of three experiments that yielded similar results.

analysis. As previously reported, the ubiquitin proteasome system is involved in the regulation of self-renewal, proliferation, and differentiation of ES cells (14). Oct4 is one of the major regulation factors in ES cells. It is known that Oct4 is transcriptionally regulated in ES cells. In addition, it was also reported that the abundance of Oct4 in ES cells may be tuned through its degradation by the ubquitin proteasome system (20, 21). Human Nanog was also reported to be controlled by proteasomal regulation via the PEST motif (22). Therefore, accumulation of Oct4 and Nanog was anticipated when proteasome inhibitors were treated. In contrast with our expectation, the expression of Oct4 and Nanog was markedly decreased in the presence of proteasome inhibitors for 24 h (Fig. 4A). We subsequently checked earlier time points and found that expression levels of Oct4 and Nanog were higher than those of the untreated control up to 12 h after treatment (Fig. 4B). Therefore, we can conclude that treatment of ES cells with proteasome inhibitors induces accumulation of Oct4 and Nanog as a short term effect and further incubation induces drastic decreases of Oct and Nanog. The proteasome inhibitors-induced decrease of pluripotency marker protein expression was dosage-dependent (Supplementary Fig. 1). We also assessed the expression levels of Oct4 and Nanog mRNAs by RT-PCR, and observed that there was no prominent decrease (Supplementary Fig. 2). These results suggest that the decrease of Oct4 and Nanog is mainly regulated at the post-transcriptional level when the proteasome systems are inhibited.

Recent reports on genome wide analysis suggested that Oct4 might regulate the expression of many proteasome sub-

units (23). Furthermore, a large set of genes coding for proteins involved in the ubiquitination and proteasome pathway was found to be expressed in human ES cells and to be decreased upon differentiation (24). Therefore, we propose that the ubiqutin proteasomal system in ES cells is required not only for fine tuning as a negative regulator through degradation, but also for the maintenance of Oct4 and Nanog expression as a positive regulator. The mechanism involved in the decrease of Oct4 and Nanog by proteasomal inhibitors should be defined.

Considering previous reports revealing that knockdown of Oct4 and Nanog suppressed proliferation and induced apoptosis in mouse ES cells (17, 25), decreased expression of these proteins may lead to a severe response of ES cells to proteasome inhibitors. A leukemia inhibitory factor-induced signal transducer and activator of transcription-3 (STAT3) is responsible for ES cell survival, and downregulated Oct-4 is reportedly associated with decreased phosphorylation of STAT3 (25). Therefore, we checked the phosphorylation level of STAT3 after treatment with proteasome inhibitors and found a significant decrease of phosphor-STAT3 (Fig. 4C), which could be one of the mechanisms involved in increased apoptosis in ES cells.

Taken together, the present results indicate that proteasome inhibitors induced apoptosis of ES cells that is severer than other somatic cells tested here. This higher susceptibility was correlated with a lower level of HSP70 expression and decreased Oct4 and Nanog expression in ES cells. ES cells are established from the inner cell mass of blastocytes, which will develop to an embryo. Therefore, the higher susceptibility of ES cells to environmental stress-induced apoptosis may be explained in the context that ES cells are designed to maintain ES cell properties only in optimal conditions and abnormal ES cells may be removed by apoptosis instead of being repaired in stress conditions. For efficient application of ES cells in future cell therapy, a stable and efficacious cell culture system is required. Therefore, understanding of the stress response and unique properties of ES cells will be helpful for improvement of ES cell culture technology.

MATERIALS AND METHODS

Maintenance of mouse ES cells

The mouse ES cell line R1 was maintained on MEF cells in Dulbecco's modified Eagle's medium (DMEM) including 15% fetal bovine serum (FBS, Hyclone Inc., Logan, UT, USA), 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin. MEF cells were harvested and irradiated with 40 Gy, and seeded at a density of ~5.5×10^4 cells/ml in MEF medium (DMEM, 10% FBS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin) one day prior to ES cell seeding.

Maintenance of STO, MEF, and HEK 293 cells

Stromal cells STO and MEF cells were maintained in DMEM including 10% FBS, 2 mM L-glutamine, 0.1 mM β-mercaptoe-

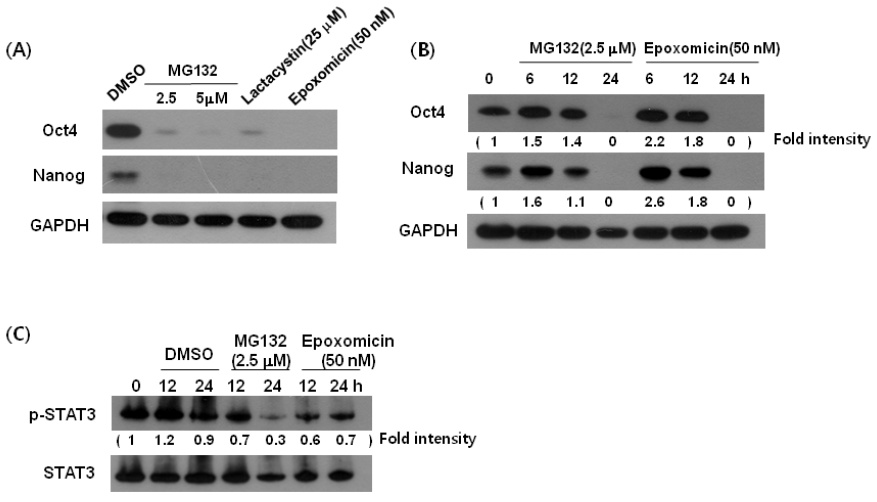

Fig. 4. Decrease of pluripotency marker gene expression and STAT3 phosphorylation by proteasome inhibitors. (A, B) Mouse ES cells were treated with proteasome inhibitors and expression levels of Oct4 and Nanog was analyzed by Western blotting. ES cells were treated with DMSO, MG132, Lactacystin, and Epoxomicin for 24 h (A). ES cells were treated with MG132 and Epoxomicin for the indicated periods (B). Expression levels of Oct4 and Nanog were normalized by the amount of GAPDH, and fold-intensity is indicated compared with Oct4 and Nanog protein levels in DMSO-treated control cells. (C) ES cells were treated with MG132 and Epoxomicin for the indicated periods, and phosphorylation levels of STAT3 were analyzed by Western blotting. Fold intensity is indicated compared with phosphor-STAT3 (p-STAT3) expression level in DMSO control cells. These results are representative of three experiments that yielded similar results.

thanol, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin. Human embryonic kidney cell line HEK293 was maintained in DMEM with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin.

Treatment with proteasome inhibitors

5×10^5 -1 $\times 10^6$ of mouse ES cells were placed into gelatin-coated 6-well plates and grown overnight with a MEF-conditioned medium prepared as described previously (26). 1×10^6 of STO, MEF, and HEK293 cells were placed on 6-well plates and grown overnight. Cells were treated with proteasome inhibitors at the indicated concentration for 24 h or as indicated in individual experiments. The proteasome inhibitors used in this study are as follows: MG132 (#474790, Calbiochem, San Diego, CA), Lactacystin (L6785), and Epoxomicin (E3652, Sigma Aldrich, St. Louis, MO, USA).

Western blotting

Harvested cells were lysed in lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.15 U/ml aprotonin, 10 mM EDTA, 10 μg/ml leupeptin, 100 mM NaF, 2 mM Na_3VO_4, and 1% NP-40). Samples were resolved by SDS-polyacrylamide gel electrophoresis, and electro-transferred to polyvinylidene fluoride (PVDF) membranes (Millipore Corp, Bedford, MA, USA). The membranes were blocked with 5% dry milk and probed with an appropriate primary antibody. Immunoreactive proteins were detected by horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and an ECL reagent (iNtRon, Seongnam, Korea). The membranes were stripped and then probed with another primary antibody when necessary. Antibodies to HSP70 (W27, sc-24) and glyceraldehydes 3-phosphate dehydrogenase (GAPDH) (6C5, sc-32233) were purchased from Santa Cruz Biotechnology Inc. The antibodies to OCT3/4 (#611203) and Nanog (AF2729) were purchased from BD Biosciences and Millipore Corporation, respectively. The antibodies to STAT3 (#9132) and phosphor-STAT3 (#9138S) were purchased from Cell Signaling Technology (Beverly, MA, USA).

RT-PCR analysis

Total RNA was isolated using TRI Reagent® according to the instructions provided by the manufacturer (MRC, Cincinnati, OH, USA). 5 μg of total RNA was reverse-transcribed in the first-strand buffer containing 6 μg/ml oligo(dT) primer, 50 U M-MLV reverse transcriptase (Invitrogen), 2 mM dNTP, and 40 U RNaseOUT recombinant ribonuclease inhibitor (Invitrogen). The reaction was conducted at 42°C for 1 hr. One microliter of the cDNA synthesis was subjected to the standard PCR reaction for 20-30 cycles of denaturation for 60 sec at 95°C, annealing for 60 sec at 58°C, and elongation for 60 sec at 72°C. The primer sequences used are as follows. GAPDH, 5′-ACCA CAGTCCATGCCATCAC-3′ (sense) and 5′-TCCACCACCCTGT TGCTGTA-3′ (anti-sense) (product size 452 bp). HSP70, 5′-CA AGATCACCATCACCAACG-3′ (sense) and 5′-CTGGTACAGC CCACTGATGA-3′ (antisense) (366 bp). OCT4, 5′-CTCGAAC CACATCCTTCTCT-3′ (sense) and 5′- GGCGTTCTCTTTGGAA AGGTGTTG-3′ (antisense) (313 bp). Nanog, 5′AGGGTCTGCT ACTGAGATGCTCTG-3′ (sense) and 5′CAACCACTGGTTTTT CTGCCAC-3′ (antisense) (364 bp).

Detection of Annexin V by flow cytometry analysis

5×10^5 of mouse ES cells were placed into gelatin-coated 6-well plates and grown overnight with MEF-CM. mouse ES cell were treated with a proteasome inhibitor at the indicated concentration for 24 hr. Apoptosis was measured by annexin V (Roche Diagnostics, Mannheim, Germany) staining and analyzed by FACSCalibur from Becton Dickinson (Heidelberg, Germany). The data were analyzed with the software FCS Express 3 (De Novo Software, Los Angeles, CA, USA).

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