Cellular and Molecular Biology

Sari-Kaplan et al., Cell Mol Biol 2017, 63:1

Research Article Open Access

The Role of Proteasome in the Cell Cycle Progression of Induced Pluripotent Stem Cells

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Abstract

Most of eukaryotic proteins are degraded by proteasomal system. Conserved activity of proteasome provides degradation of damaged proteins and regulation of signaling pathways. Mesenchymal stem cells (MSCs) are self-renewing cells with the ability of multipotency and important in stem cell research because of easy isolation and ethical considerations about embryonic stem cells (ESCs). Induced pluripotent stem cells (iPSCs) are also promising in stem cell research, which are conducted via re-programming of somatic cells. Previously, proteasome activity was shown to be high in hESCs and iPSCs. In this study, the relation between proteasome activity and cell cycle stages were compared. First of all, proteasome activity of MSCs, iPSCs, fibroblasts was measured. Then, the cell cycle stages of cells were determined and phosphorylated retinoblastoma levels were analyzed.

Proteasome activity of iPSCs was higher than MSCs and fibroblasts. Most of iPSCs were found in S and G2/M while MSCs and fibroblasts were in G0/G1 phase. Our data showed that phospho-pRb levels were about 4 times and 2 times higher in iPCSs and in MSCs compared to fibroblasts. It can be concluded that higher proteasomal activity is related with higher potency of cells and Phospho-pRb is also induced in the same way.

Keywords: Proteasomal activity; Stem cell potency; Cell cycle; Induced pluripotent stem cells; Mesenchymal stem cells

Abbreviations: ESCs: Embryonic Stem Cells; hESCs: Human Embryonic Stem Cells; GAPDH: Glyceraldehyde-3-Phosphate Dehydrogenase; iPSCs: Induced Pluripotent Stem Cells; MCA: Methyl Coumarin; MSCs: Mesenchymal Stem Cells; PI: Propidium Iodide; PVDF: Polyvinylidene Difluoride; pRb: Retinoblastoma Gene Product; S: Svedberg Unit

Introduction

Most eukaryotic proteins are degraded by the proteasomal system in an ATP-dependent and independent manner. Proteasomal system is quite conserved throughout the evolution from archaea to eukaryotes. It degrades oxidized and misfolded/unfolded proteins and regulates signaling mechanisms. Protein degradation is performed by the catalytic subunits of the 20S core particle, which have caspase-, trypsin and chymotrypsin-like protease activities [8].

Mesenchymal stem cells (MSCs) are self-renewing cells with multipotency and are easily isolated from different sources such as adipose tissue and bone marrow. MSCs have become important tools in stem cell research due to ethical limitations in using embryonic stem cells (ESCs) [1-3]. On the other hand, induced pluripotent stem cells (iPSCs) are novel stem cell research tools, which are formed via re-programming of adult somatic cells with four transcription factors, i.e., Oct4, Sox2, c-myc, and Klf4. It is believed that iPSCs will be useful in making disease specific or patient specific stem cells. However, further research is required to solve the problem of potential teratoma formation or tumorigenesis [16,17].

Proteasomal system is involved in the degradation of many target proteins that have roles in cell proliferation, cell cycle regulation and replicative senescence. Vilchez et al. have compared proteasomal activities of hESCs and iPSCs and have shown that those of hESCs and iPSCs were higher than somatic cells. It was also shown that differentiation of stem cells into somatic cells cause a decrease in their proteasomal activity.

In this study, we have focused on the retinoblastoma gene product, pRb, is a phosphoprotein which arrests cells in G1 phase and is phosphorylated or dephosphorylated during the cell cycle. Its hypophosphorylated form is accepted to be the active form that predominates in quiescent cells. On the other hand, hyperphosphorylated form of pRb is inactive and is abundant in proliferating cells. pRb forms a complex with E2F-1 which controls many lethal molecules that fall into three main categories: i) cell cycle regulation genes such as c-myc; ii) molecules that are activated in G1 phase of cell cycle such as cyclins; iii) transcriptional regulation of molecules that are necessary for S phase of cycle such as cyclin A and DNA polymerase. Fast proliferating cells contain abundant amounts of E2F-1 proteins. Like many other molecules regulating cell cycle, i.e., cyclin E and p27, E2F-1 is also degraded by the ubiquitin-proteasome system [4-8]. In its hypophosphorylated form pRb is bound to E2F-1 preventing its activation and ubiquitination. Hyperphosphorylation of pRb in late G1 leads to dissociation and proteasomal degradation of E2F-1 [6].

We have compared the proteasomal activities and cell cycle stages of MSCs, fibroblast-derived iPSCs and fibroblasts. Cell cycle stages were determined by propidium iodide (PI) staining and flow cytometry. Phosphorylated pRb level was evaluated by immunoblotting to show the cross-talk between proteasomal activity and cell cycle [9-20].

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Received October 25, 2017; **Accepted** December 05, 2017; **Published** December 12, 2017

Citation: Sari-Kaplan G, Mutlu-Altundag E, Kalci B, Tosun C, Karademir B, et al. (2017) The Role of Proteasome in the Cell Cycle Progression of Induced Pluripotent Stem Cells. Cell Mol Biol 63: 146.

Materials and Methods

Cell culture

Although Vilchez et al. [18] have shown that proteasome activity does not change with passage number in the same cell line; we have studied all cell lines at the same passage number. Fibroblasts (Primary Dermal Fibroblasts; Normal, Human, Adult, PCS-201-012) and mesenchymal stem cells (Adipose-Derived Mesenchymal Stem Cells; Normal, Human, PCS-500-011) were purchased from ATCC® (USA). They were cultured in Dulbecco's modified Eagle's medium supplemented with penicillin/streptomycin and fetal bovine serum in a humidified atmosphere of 5% CO_2 and 95% air at 37°C as recommended by the supplier. Fibroblast derived iPSCs were a gift from Prof. Taner Onder (KOC University, Istanbul, Turkey). The flasks and plates were coated with BD Matrigel hESC-qualified Matrix (BD Biosciences, Cat. No. 354277) at concentrations recommended by the supplier. Cells were maintained in mTeSR1 medium (Stem Cell Technologies, Cat. No. 05850) and passaged after applying dispase (Stem Cell Technologies, Cat. No. 07913).

Fluorometric analysis of proteasome activity

Cell lysates were prepared with a lysis buffer containing 1 mM dithiothreitol following freeze-thaw cycles. The lysates were centrifuged at 14,000 × g for 30 min to remove nonlysed cells, membranes, and nuclei. Supernatants were incubated in 225 mM Tris buffer (pH 7.8) containing 7.5 mM MgOAc, 7.5 mM $MgCl_2$, 45 mM KCl, and 1 mM dithiothreitol. The fluorogenic peptide succinyl-LLVY-methyl coumarin was used as substrate at a concentration of 200 μM to measure chymotrypsin-like activity of the proteasome. After 30 min of incubation at 37°C, methyl coumarin liberation was measured at 360 nm excitation/485 nm emission with a fluorescence reader (Filter Max F5 Multimode Microplate Reader). All measurements were repeated three times for three biological replicates of each study group and calculated using different concentrations of free methyl coumarin as standards.

Immunoblotting

Antibodies detecting phospho-pRb (phospho S780, ab47763, dilution 1/500) and GAPDH (ab8245, dilution 1:1000) were purchased from Abcam. For each cell line and each replication, 10^6 cells were prepared and total protein isolation was performed. There were three replicates for each group. Protein concentrations of samples were determined with Pierce™ BCA Protein Assay Kit (23225 Thermo Fisher Scientific Inc.). Proteins from cell extracts (40 μg per lane) were separated by vertical gel electrophoresis under denaturizing conditions on 7% SDS-PAGE gels. Protein bands were blotted on PVDF membranes and visualized with HRP-conjugated secondary antibodies (Cell Signaling) and Pierce™ ECL Western Blotting Substrate (32106 Thermo Fisher Scientific Inc.). Imaging was performed using ChemiDoc™ MP System (Bio-Rad Laboratories, Inc.). Lane band density was calculated with ChemiDoc™ MP System Software Image Lab Software (Bio-Rad Laboratories, Inc.).

Cell cycle analysis by flow cytometry

For each group 10^6 cells were prepared. All measurements were repeated three times for three biological replicates of each study group. After obtaining the cell pellet, cells were washed with PBS and fixed with 70% ethanol. Cells were washed twice to remove ethanol and then treated with 500 μL PI/RNase buffer (BD Pharmingen™ PI/RNase staining buffer). They were then incubated for 15 min at room temperature and analyzed with flow cytometer (BD FACS Calibur™ 4CS Flow Cytometer). Data acquisition was performed and the percentages of G1, S, and G2/M-phase cells were calculated with the CellQuest software program.

Statistical analysis

Graph-Pad Prism 7 software was used for statistical analysis. Student's t test and one-way ANOVA were performed followed by the Bonferroni's multiple comparison test. A p value of less than 0.05 was selected as the level of significance.

Results

Total proteasome activity

The results of fluorometric analysis of proteasome activity are shown in Figure 1. Our data have shown that proteasome activity was higher in iPSCs when compared to MSCs and fibroblasts ($p<0.001$, $p<0.001$). On the other hand, the proteasome activity of MSCs was higher than the proteasome activity of fibroblasts ($p<0.001$).

Immunoblotting

Immunoblotting was performed to detect the levels of phosphorylated pRb protein in iPSCs, MSCs and fibroblasts. Band density of phospho-pRb was compared to the housekeeping protein GAPDH for each lane. Mean values of three replicates were calculated and band density of fibroblasts was set as 100%. Percentage of signal density of iPSCs and MSCs were calculated according to the lane density of fibroblasts. Highest phosphorylated pRb level was observed in iPSCs compared to both MSCs and fibroblasts ($p<0.01$, $p<0.001$). The lanes, bands and graphs are shown in Figure 2.

Cell cycle analysis

Cell cycle phase distribution of iPSCs, MSCs and fibroblasts were analyzed by flow cytometry (Figure 3). Cell cycle stage distribution of fibroblasts was almost the same as MSCs but was quite different for iPSCs. While fibroblasts and MSCs were spending most of the cell cycle in G0/G1 phase, iPSCs were in S or G2/M phase. More than 80% of fibroblasts and more than 60% of MSCs were found to be in G0/G1 phase.

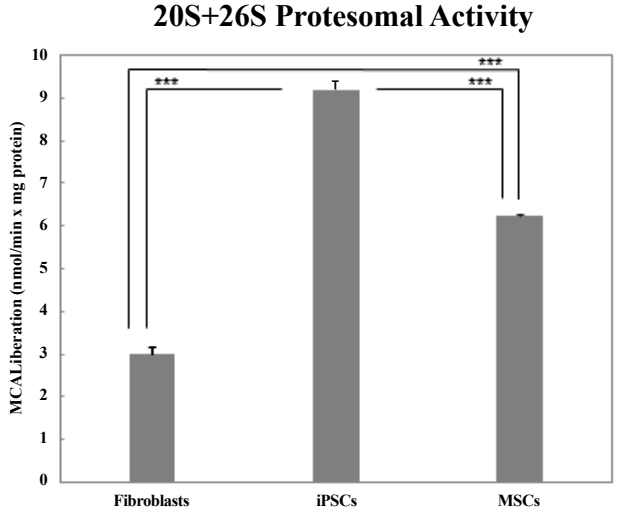

Figure 1: Total 20S+26S proteasome activity in fibroblasts, iPSCs and MSCs. Proteasome activity was measured according to the principle of MCA liberation as described in methods. Data denote mean ± SD ***p<0.001 (n=6). Results were evaluated by Student's t test.

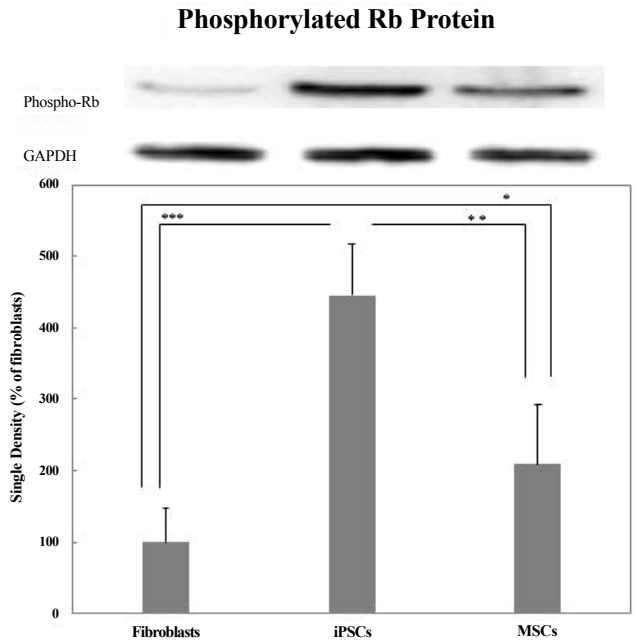

Figure 2: Cellular level of phosphorylated-pRb in fibroblasts, iPSCs and MSCs. Cell lysates were prepared and analyzed via immunoblotting as described in methods. Data denote mean ± SD *p < 0.05, ***p<0.001 (n=3). Results were evaluated by Student's t test.

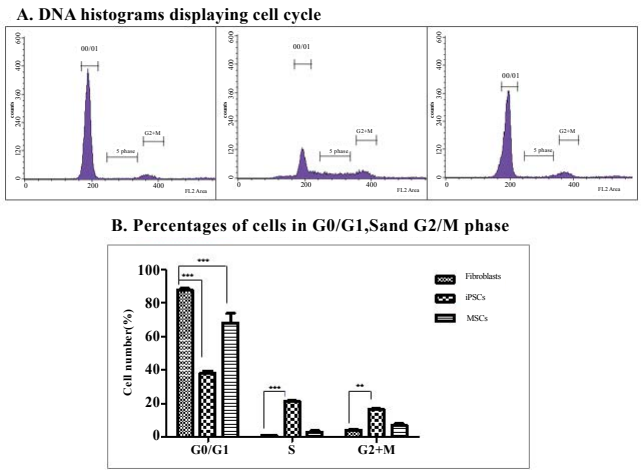

Figure 3: Cell cycle analyses in fibroblasts, iPSCs and MSCs. Cell cycle stage analysis was performed via PI staining with flow cytometer as described in methods. (A) Top panel: DNA histograms displaying cell cycle stages of fibroblasts, iPSCs and MSCs, respectively. (B) Bottom panel: percentage of cells in G0/G1, S, and G2/M phase of the cell cycle, respectively. Results were gated to exclude cellular debris and sub-G0 population. Data represent mean ± SD, *p<0.05, **p<0.01, ***p<0.001 (n=3). Results were evaluated with one-way ANOVA followed by Bonferroni's multiple comparison test.

Discussion

Proteasomal system plays many crucial roles in cells. It is composed of 20S catalytic unit and different regulators. The 20S proteasome, by itself, degrades oxidized proteins in an ATP- and ubiquitin-independent manner. Oxidized proteins are produced by energy metabolism, natural aging process, cell signaling or environmental effects [10]. Another crucial complex of the proteasomal system is the 26S proteasome, which is composed of the 20S proteasome and 19S regulators in both sides. The 26S proteasome degrades its substrates in an ATP- and ubiquitin-dependent manner. The main substrates of 26S proteasome are signaling molecules that are involved in cell cycle, apoptosis, proliferation and differentiation. Unfolded or misfolded proteins such as the ER-resident proteins that are retro-translocated to the cytosol as well as cytosolic unfolded proteins which suffer from environmental triggering factors or mutations are also degraded by the 26S proteasome [9].

ESCs are undifferentiated cells, which are immortal and capable of differentiating into all other cell types of organisms. ESCs are so-called pluripotent stem cells [14]. The potential of ESCs in therapeutic medicine is significant but inter-personal genetic material differences limit stem cell or stem cell-derived somatic cell transplantations. In addition, ethical problems on using ESCs limit their applications. Accordingly, MSCs gained importance in regenerative medicine because of their multipotency and easy isolation and culturing procedures [2,3]. Moreover, iPSCs introduced disease-specific individual pluripotent stem cells in regenerative medicine studies. Since iPSCs are produced via reprogramming of somatic cells [16,17], limitations caused by ethical considerations are prevented.

Previously, it was shown that higher proteasomal activity maintains proper homeostasis and prevents replicative senescence in ESCs and iPSCs. In the same study, proteasomal activity was reported to be decreased following the differentiation of ESCs and iPSCs into somatic cells such as neurons [18]. In the present work, total proteasomal activity was measured in fibroblasts, fibroblast-derived iPSCs and MSCs via MCA liberation method. According to our data, proteasome activity of iPSCs was found to be approximately 3 times and 1.5 times higher than proteasome activities of fibroblasts and MSCs, respectively. It can be speculated that the higher proteasomal activity is related to the higher potency of cells. When stem cells are differentiated into less potent somatic cells, proteasomal activity decreases in proportion to their potency.

pRb is an important regulator protein in cell cycle. Increased phospho-Rb leads to lower amount of E2F1 due to proteasome degradation [1]. We have revealed phosphorylated pRb levels in different cell lines and showed that phosphorylated pRb levels were about 4 times higher in iPSCs and 2 times higher in MSCs when compared to fibroblasts. Since pRb phosphorylation is reported to be higher in proliferative cells [19,20], these results suggest that iPSCs have higher proliferation rate than MSCs and fibroblasts. In accordance, somatic cells have lower proteasome activity and less pRb phosphorylation.

Conclusion

Because proteasomal activity and phospho-pRb results are correlated with each other for each cell line studied, we have also compared the cell cycle stage distributions. The number of cells in G0/G1 phase was significantly lower ($p<0.001$) and the number of cells in S and G2/M phases were higher ($p<0.001$ and $p<0.01$, respectively) in iPSCs when compared to fibroblasts. In addition, MSCs had fewer cells in G0/G1 phase and more cells were in S and G2/M phases when compared to fibroblasts. These results let us speculate that cells with higher potency such as pluripotent stem cells (iPSCs) spend less time in G0/G1 compared to multipotent stem cells (MSCs) and somatic cells (fibroblasts). Combined with the increased levels of phospho-Rb, we speculate that E2F1 levels are accordingly lower in iPSCs as compared to the relative levels in fibroblasts. The relation between proteasome activity and cell potency seems to be a dual-talk and phospho-pRb is one of the many messengers involved in their communication.

Acknowledgement

iPSCs were a gift from Assist. Prof. Dr. Tamer Onder (KOC University Medicine School), and on behalf of the co-authors, we want to thank him. "This study was supported by Marmara University Research Fund with the project number SAG-A-130511-0126."

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