Prolonged Proteasome Inhibition Cyclically Upregulates Oct3/4 and Nanog Gene Expression, but Reduces Induced Pluripotent Stem Cell Colony Formation

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Abstract

There is ample evidence that the ubiquitin–proteasome system is an important regulator of transcription and its activity is necessary for maintaining pluripotency and promoting cellular reprogramming. Moreover, proteasome activity contributes to maintaining the open chromatin structure found in pluripotent stem cells, acting as a transcriptional inhibitor at specific gene loci generally associated with differentiation. The current study was designed to understand further the role of proteasome inhibition in reprogramming and its ability to modulate endogenous expression of pluripotency-related genes and induced pluripotent stem cells (iPSCs) colony formation. Herein, we demonstrate that acute combinatorial treatment with the proteasome inhibitors MG101 or MG132 and the histone deacetylase (HDAC) inhibitor valproic acid (VPA) increases gene expression of the pluripotency marker Oct3/4, and that MG101 alone is as effective as VPA in the induction of Oct3/4 mRNA expression in fibroblasts. Prolonged proteasome inhibition cyclically upregulates gene expression of Oct3/4 and Nanog, but reduces colony formation in the presence of the iPSC induction cocktail. In conclusion, our results demonstrate that the 26S proteasome is an essential modulator in the reprogramming process. Its inhibition enhances expression of pluripotency-related genes; however, efficient colony formation requires proteasome activity. Therefore, discovery of small molecules that increase proteasome activity might lead to more efficient cell reprogramming and generation of pluripotent cells.

Introduction

GENERATION OF INDUCED PLURIPOTENT stem cells (iPSCs) from mouse fibroblasts by retroviral transduction of four key transcription factors (Oct3/4, Sox2, c-Myc, and Klf4) has provided invaluable insight into molecular mechanisms of somatic cell reprogramming and the possibility of alternative strategies not reliant on embryos to produce pluripotent stem cells (Takahashi and Yamanaka, 2006). These iPSCs exhibited embryonic stem cell (ESC)-like morphology and proliferation capacity, endogenous pluripotent marker expression, and restored *in vitro* and *in vivo* differentiation capacity. Several laboratories rapidly reported similar results in mouse and human somatic cells

(Maherali et al., 2007; Wernig et al., 2007; Yu et al., 2007). At present, the greatest success in reprogramming is accomplished by delivery of cDNAs using retroviral or lentiviral vector systems. Future advances for applications into research and/or therapy will be dependent on the ability to produce iPSCs in the absence of genotoxic events. Several approaches to this end have proven successful and include reprogramming induced by transient transfection, non-integrating adenoviral or Sendai virus vectors, transposons, purified proteins, modified RNAs, and microRNAs (miRNAs) (for review, see Gonzalez et al., 2011). Even if the most current approaches show practical efficiency, methods that could improve the yield of fully reprogrammed iPSCs are still desired.

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The use of small-molecule modulators to probe epigenetic and/or other biochemical pathways can reveal novel insights into improving reprogramming efficiencies. For example, we have recently reported that treatment of target cells with a histone deacetylase (HDAC) inhibitor prior to reprogramming factor transfection increased ESC-like colony formation approximately two- to three-fold (Rim et al., 2012). In addition, DNA methyltransferase (DNMT) inhibition during cell culture promotes maturation of reprogrammed somatic cells, increasing the yield approximately four-fold (Rim et al., 2012). Similarly, the use of small-molecule probes to query other biological pathways may provide unique mechanistic insights regarding reprogramming.

There is ample evidence that the ubiquitin–proteasome system is an important regulator of transcription (Muratani and Tansey, 2003), and recent findings indicate that ubiquitin–proteasome system activity is necessary for maintaining pluripotency and promoting cellular reprogramming. Buckley et al. (2012) found that specific ubiquitin ligases and deubiquitylating enzymes are required for maintenance of pluripotency, pointing out the role that dynamic regulation of ubiquitin–proteasome-dependent degradation plays in determining pluripotency. Moreover, proteasome activity contributes to maintaining the open chromatin structure found in pluripotent stem cells, acting as a transcriptional inhibitor at specific gene loci generally associated with differentiation (Szutorisz et al., 2006). A role for the proteasome in determining pluripotency is also supported by evidence that gene expression of subunits of the 20S proteasome decreases as human ESCs undergo differentiation (Atkinson et al., 2012). Beneficial effects of MG132 on development of rat (Nakajima et al., 2008), murine (Gao et al., 2005), bovine (Le Bourhis et al., 2010; Tani et al., 2007), and porcine (You et al., 2010) somatic cell nuclear transfer (SCNT) embryos have also been documented.

On the basis of these reports, we hypothesized that proteasome modulation would alter pluripotent marker gene expression when combined with an HDAC inhibitor and that this approach may be useful in reprogramming somatic cells. Here we report our evaluation of this combined system and the effect of proteasome inhibition alone on induction of pluripotency. We demonstrate that acute combinatorial treatment with the proteasome inhibitor MG101 or MG132 and the HDAC inhibitor valproic acid (VPA) increases gene expression of the pluripotency marker Oct3/4, but that MG101 alone is as effective as VPA in the induction of Oct3/4 mRNA expression in fibroblasts. Prolonged proteasome inhibition with low doses of either MG101 or MG132 cyclically upregulates gene expression of Oct3/4 and Nanog, but proteasome inhibition reduces colony formation in the presence of the iPSC induction cocktail of Oct3/4, Klf4, Sox2, and c-myc. Taken together, our results support the concept that proteasome activity is essential for somatic cell reprogramming, although gene expression of pluripotent markers such as Oct3/4 and Nanog is upregulated by proteasome inhibition.

Materials and Methods

Materials

Eagle's Minimal Essential Medium (EMEM, #30-2003) and the human normal foreskin fibroblast cells (BJF, #CRL-

2522) were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The human adipose-derived precursor cells (HPAd) and Preadipocyte Growth Medium were obtained from Cell Applications (San Diego, CA, USA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (S11150). The HDAC inhibitor VPA (#P4543) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All proteasome inhibitors [MG 262 (I-120), MG 115 (I-135), MG 101 (I-160), MG 132 (I-130) and Psi (I-140)] were purchased from Boston Biochem (Boston, MA, USA). The 20S Proteasome Activity Assay Kit was purchased from Millipore (Billerica, MA, USA). The ABI High Capacity cDNA Reverse Transcription Kit, RNase Inhibitor, Universal TaqMan Mix, and all TaqMan primer/probes pairs were obtained from Applied Biosystems (Foster City, CA, USA). The RNeasy Kit was purchased from Qiagen (Valencia, CA, USA). Images were obtained using a Nikon Eclipse TS100 Microscope equipped with a Nikon Photometrics CoolSNAP Cf camera and the MetaMorph Imaging system.

Cell culture

The human BJF cells were maintained in EMEM high glucose containing 10% FBS and antibiotics (100 units/mL penicillin G and 100 µg/mL streptomycin) in a humidified chamber at 37°C and 5% CO₂. Each treatment was begun when the fibroblasts reached ~80% confluency. HPAd cells were cultured in Preadipocyte Growth Medium and maintained at 37°C with 5% CO₂. The cells underwent passaging at ~80% confluency, and the cells were reseeded at 10,000 cells/well at each passage.

Real-time RT-PCR

Total RNA was isolated from cell pellets using a RNeasy Kit (Qiagen, Valencia, CA, USA) and incubation with RNase-free DNase I according to the manufacturer's instructions. The purified RNA was quantified using a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The RNA (500 ng) was reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) containing MultiScribe Reverse Transcriptase with random primers at 37°C for 2 h. Real-time PCR was performed with TaqMan chemistry (Applied Biosystems) using the 7900HT Fast Real-Time PCR System (Applied Biosystems) and universal cycling conditions (50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 sec, and 60°C for 1 min followed by 95°C for 15 sec, 60°C for 15 sec, and 95°C for 15 sec). Results were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH), β -actin, or ubiquitin B expression controls.

VPA and proteasome inhibitor treatment of human fibroblast cells

The human BJ fibroblasts were grown to 80% confluency in six-well plates. For the experiments shown in Figure 1, the cells were treated with growth medium (control) or VPA (5 mM in growth medium) in the absence [dimethylsulfoxide (DMSO)] or presence of the proteasome inhibitors (MG101, MG115, MG132, MG262, Psi). The proteasome inhibitors were used at the indicated concentrations in Figure 1A and 2.5 µM in Figure 1, B and C. For Figure 1, A and C,

FIG. 1. Proteasome inhibition alone or combined with an HDAC inhibitor increases Oct4 gene expression. **(A)** BJ fibroblasts were incubated with VPA (5 mM) alone or in combination with one of five proteasome inhibitors as indicated. Oct3/4 mRNA levels were assayed after 24 h. **(B)** Confirmation of proteasome inhibition by MG101 (2.5 μM) and MG132 (2.5 μM) in the absence or presence of VPA. Data are reported as the mean $\pm$ standard deviation with the statistical significance relative to control (-MG101, -MG132, -VPA); a, $p < 0.01$ and b, $p < 0.05$. **(C)** Oct3/4 mRNA levels were determined at 24 h after incubation of the BJ fibroblasts with the proteasome inhibitors and VPA as indicated. Data are reported at the mean $\pm$ standard deviation and the statistical significance is relative to the control values.

RNA was harvested at 24 h after the addition of VPA and the proteasome inhibitors. Each experimental condition was assayed in triplicate, and images of cell morphology were obtained of each experiment. Reprogramming potential was assayed as induction of gene expression of the transcription factor, Oct3/4.

Proteasome activity

Proteasome activity was measured in triplicate according to the manufacturer's instructions for the 20S Proteasome Assay Kit. Briefly, the cell lysates were harvested in 50 mM Tris-Cl (pH 7.4) with 25 mM KCl, 2 mM MgCl_2 , 0.1% Triton X-100, 2 mM adenosine triphosphate (ATP), 2 mM phenylmethylsulfonyl fluoride (PMSF). MgATP was included in the lysis buffer to maintain 26S proteasome activity. Proteasome activity was measured by incubating 20 μg of each lysate with a fluorophore 7-amino-4-methylcoumarin (AMC)-labeled peptide substrate LLVY-AMC at 37°C for 60 min, and the free AMC released by proteasome activity was quantified using a 380/460-nm filter set (Molecular Devices, Sunnyvale, CA, USA). Proteasome activity is reported as relative fluorescence units (RFU)/ μg protein/h.

Each sample was measured both in the presence and in the absence of epoxomicin (0.1 μM), a highly specific 26S proteasome inhibitor (BostonBiochem, Cambridge, MA, USA) to account for any nonproteasomal degradation of the substrate.

Chronic proteasome inhibition

The HPAd cells were maintained under control (DMSO) conditions or in the presence of MG101 (1, 3, or 10 μM) or MG132 (62.5, 125, or 250 nM) for 30 days. At each passage, the cells were allowed to recover for 48 h prior to adding the proteasome inhibitors MG101 and MG132. Cells were harvested for isolation of RNA at 2, 4, 12, 19, 25, and 30 days. The effect of low-dose proteasome inhibition on reprogramming was assayed as induction of Oct3/4 and Nanog gene expression.

Measurement of apoptosis

Dual staining with Annexin V and propidium iodide (PI) allows discrimination between unaffected cells (Annexin V-negative and PI-negative), early apoptotic cells (Annexin

V-positive and PI-negative), and late apoptotic cells (Annexin V-positive and PI-positive). The human foreskin fibroblasts were maintained under control (DMSO) conditions or in the presence of MG101 (1, 3, or 10 μ M) or MG132 (62.5, 125, or 250 nM) for up to 9 days. After 1, 2, 4, and 9 days of exposure to the inhibitors, both floating and trypsinized adherent cells were stained with fluorescein isothiocyanate (FITC), Annexin V, and PI as recommended by the manufacturer (BD Biosciences, San Jose, CA, USA). The assay was performed using a flow cytometer (Becton Dickinson, San Jose, CA, USA), and data were analyzed with a Macintosh G5 workstation (Apple Computer, Cupertino, CA, USA) and Cellquest graphics software (Becton Dickinson). The total number of cells counted for each sample was at least 10,000.

Lentiviral transduction and generating iPSCs

Human foreskin fibroblasts (Millipore, Billerica, MA, USA) were plated at density of 1×10^4 cells/well in complete growth medium into six-well plates (day 0). The next day, the cells were transduced with EF1 α -STEMCCA (OKSM [Oct3/4, Klf4, SOX2, c-Myc,]) lentivirus at a multiplicity of infection (MOI) of 75 in medium supplemented with polybrene (final concentration 5 μ g/mL). Virus infection was repeated next day. Once the cells reached 90–95% confluency (day 6), virus-infected cells were replated onto an inactivated mouse embryo fibroblast (MEF) feeder layer and grown in mTeSR1 medium (STEMCELL Technologies, Vancouver, Canada). The growth medium was supplemented with either DMSO (control) or MG101 (final concentration either 1 μ M or 3 μ M) throughout the experiment, beginning on the day of cell transduction. The growth medium was exchanged every 24 h, and cell growth and morphology were monitored daily.

Alkaline phosphatase staining

An Alkaline Phosphatase Staining Kit (Stemgent, Cambridge, MA, USA) was used for alkaline phosphatase (AP) expression to indicate reprogrammed cells. Briefly, the fixed cells were stained in the dark at room temperature for 15–20 min. The colonies were observed under an inverted microscope Nikon Eclipse TS100 (Nikon Instruments, Melville, NY, USA) to confirm AP activity.

Immunofluorescent staining for Sox-2

The cells were fixed with an ethanol–acidic acid–water mix (7:2:1) for 10 min at room temperature followed by permeabilization with 0.5% saponin in 0.05% Tween-20 in phosphate-buffered saline (PBS). Nonspecific binding sites were blocked with 10% normal goat serum for 30 min at room temperature. Rabbit polyclonal primary antibodies against Sox-2 (1:200 dilution; Abcam, Cambridge, MA, USA) were applied at 4°C overnight. After washing with PBS, the cells were incubated with goat anti-rabbit immunoglobulin G (IgG) (H+L) (DyLight 594) polyclonal antibodies (Thermo Fisher Scientific, Waltham, MA, USA) for 1.5 h at room temperature. After staining, the cells were washed with PBS and observed under a fluorescence microscope (Nikon Eclipse TS100). Images were captured using NIS-Elements Br Microscope Imaging Software (Nikon Instruments, Melville, NY, USA).

Statistical analysis

All experiments were assayed in triplicate, and statistical analysis was carried out using analysis of variance (ANOVA). Statistical significance was determined using a two-tailed *t*-test. All statistical analysis was carried out using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Variability is expressed as the mean $\pm$ standard deviation.

Results

Acute effect of proteasome inhibition on Oct3/4 gene expression

We first used a panel of proteasome inhibitors to investigate the short-term effects of proteasome inhibition in combination with VPA on Oct3/4 gene expression in human somatic cells. The BJ fibroblasts were incubated with VPA (5 mM) alone or VPA combined with one of five proteasome inhibitors—MG101, MG115, MG132, MG262 or PSI. Each proteasome inhibitor was used at 0.5, 1, and 2.5 μ M. The cells were harvested at 24 and 48 h, and Oct3/4 gene expression was assayed via real-time RT-PCR (Fig. 1A). Cellular stress was observed at 48 h for MG115, MG132, MG262, and PSI at 0.5–2.5 μ M, whereas the fibroblasts incubated in the presence of MG101 showed no signs of cytotoxicity at 0.5–2.5 μ M MG101 at 48 h. MG101 was as effective as MG132 in increasing Oct3/4 gene expression significantly when present in combination with VPA compared to VPA alone. Therefore, all subsequent experiments were carried out using MG101 or MG132 as the proteasome inhibitor.

MG101 (as known as ALLN) was first described as a calpain inhibitor (Tsubuki et al., 1996) and is less potent and less selective as a proteasome inhibitor than MG132 (Kisselev and Goldberg, 2001), although both are peptide aldehyde inhibitors of the chymotrypsin-like activity of the 20S proteasome (Kisselev and Goldberg, 2001). To determine if MG101- and MG132-mediated proteasome inhibition are comparable, we assayed the chymotrypsin-like activity of the 26S proteasome under control conditions, in the presence of MG101 or MG132 alone or MG101 and MG132 in the presence of VPA (5 mM). As shown in Figure 1B, there was no difference in the efficacy of MG101 and MG132 when present at 2.5 μ M. Interestingly, VPA alone diminished proteasome activity, but to a lesser extent than either MG101 or MG132.

In Figure 1C, we also demonstrate significant up-regulation ($p < 0.05$) of the pluripotent gene Oct3/4 with VPA treatment alone, MG132 treatment alone, and MG132 combined with VPA compared to control cells (Fig. 1C). MG101 alone is as effective in regulating Oct3/4 gene expression as VPA alone or the combination of VAP and MG101.

Long-term effect of low-dose MG101 and 132 on Oct3/4 and Nanog expression

The apparent effect of proteasome inhibition on Oct3/4 gene expression prompted us to assay the long-term effect of proteasome inhibition on expression of Oct3/4 and Nanog as genes that are required for cellular reprogramming. For this experiment, we used adipose-derived precursor cells that have proved to be multipotent (Bunnell et al., 2008; Guilak et al., 2006) and showed higher reprogramming efficiency

into iPSCs (Rim et al., 2012; Sun et al., 2009). As shown in Figure 2A, chronic treatment with a low-dose range of either proteasome inhibitor is associated with a biphasic dose-dependent response in the gene expression of Oct3/4 or Nanog. Prolonged exposure to MG132 at concentrations greater than 2.5 μ M led to cell death, prompting the use of MG132 in the nanomolar range, given an half-maximal inhibitory concentration (IC_{50}) of 100 nM for MG132 (Tsubuki et al., 1996). In general, Oct3/4 gene expression is increased by proteasome inhibition at 48 h and at days 19–25, whereas

proteasome inhibition reduces Oct3/4 expression at 96 h or at 30 days of exposure to either MG101 or MG132. Although the effect of MG101 and MG132 on Oct3/4 gene expression is comparable during the acute exposure to the inhibitors, the effect of MG101 at 19 days is substantially greater than the effect of MG132. Interestingly, the patterns of Oct3/4 and Nanog expression with proteasome inhibition are inversely related. In addition, the length of time in culture independently increases the expression of Oct3/4, beginning at day 19 and continuing until day 30 in culture (Fig. 2B).

FIG. 2. Chronic proteasome inhibition results in biphasic regulation of Oct4 and Nanog mRNA expression. Expression of Oct3/4 and Nanog mRNA was analyzed in human adipose tissue stromal vascular fractions cells maintained in growth medium. (A) The cells were incubated with MG101 or MG132 at the indicated concentrations over 30 days. The cells were passaged when at ~80–90% confluency and allowed to recover for 48 h prior to reintroduction of the proteasome inhibitors. Data were normalized to control and are reported as the mean $\pm$ standard deviation. Statistical significance at each time point is relative to the control sample. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$. (B) The effect of culture conditions alone on Oct3/4 and Nanog mRNA levels over 30 days. Data are presented as mean $\pm$ standard deviation. Bars with various superscripts are significant different ($p < 0.05$).

Reprogramming human fibroblasts: Colony formation

The first emerging colonies were seen around day 9. On that day, the total number of colonies in each plate was counted on the basis of their morphology (Fig. 3A, D). OKSM transduction resulted in formation of 252 and 314 colonies (experiments 1 and 2, respectively). Treatment with the proteasome inhibitor MG101 strongly reduced the number of colonies formed: 180 and 184 when treated at concentration of 1 μ M and 93 after treatment at 3 μ M. The colonies were positively stained for AP activity (Fig. 3B, E), and for marker of pluripotency SOX-2, as revealed by immunocytochemistry (Fig. 3C, F).

Measurement of apoptosis

Using Annexin V–PI double staining followed by flow cytometric analysis, we measured the populations of dead and apoptotic cells treated with MG101 and MG132 for up to 9 days. Compared to DMSO, the populations of double-positive (necrotic/late apoptotic) and Annexin V–positive (early apoptotic) cells dramatically increased as early as after 24 h of treatment with 10 μ M MG101. Importantly, no changes were observed with MG101 treatments at the lower concentrations (Table 1). As shown in Table 2, the cells were more sensitive to MG132 exposure. Cellular apoptosis was induced with 250 nM of MG132 at all analyzed time points and after 9 days of exposure to 125 nM MG132.

Reprogramming human fibroblasts: Gene expression analysis

To further characterize the effect of proteasome inhibition on cell reprogramming, the expression of pluripotency-related genes was analyzed. qPCR analysis demonstrated robust expression of the endogenous pluripotency factors in OKSM-transduced human fibroblasts. Consistent with the colony formation, proteasome inhibition negatively effects pluripotency marker expression, as revealed by significantly lower expression of Oct3/4, Sox-2, and Nanog in the culture (Fig. 4).

Discussion

Although cellular reprogramming methods are firmly established, the molecular mechanisms underlying this process remain poorly characterized. Moreover, despite advances in the field, new methods to generate iPSCs of better quality, specificity, and efficiency are still needed. Modulating signaling pathway and epigenetic mechanisms with small molecules as an alternative to introducing exogenous transcription factors has emerged as a valuable tool to determine cell fate. Small molecules often provide reversible, temporal effects that can be finely tuned by their combination and concentrations. To date, a number of studies have identified several compounds that can enhance cell reprogramming and replace transcription factor-based reprogramming (Li and Ding, 2010; Li et al., 2012, 2013; Shi et al., 2008). These studies have used compounds that target cellular activities ranging from cytoplasmic signaling and cell cycle control to histone and DNA modification (for review, see Li et al., 2012).

There is extensive evidence that proteasome activity plays a role in many of these processes (Naujokat and Saric, 2007), including studies indicating that combinatorial use of proteasome and HDAC inhibitors acts synergistically to inhibit proliferation (Abaza et al., 2012; Heider et al., 2009). This suggests that reversible inhibition of proteasome activity by the well-characterized peptide-based proteasome inhibitors may represent an additional approach to regulate reprogramming. The current study was designed to further understand the role of proteasome inhibition in reprogramming and its ability to modulate endogenous expression of pluripotency-related genes and iPSCs colony formation.

The 26S proteasome is a large, multi-subunit complex that is composed of a 19S regulatory “lid” complex and a 20S catalytic core particle that contains three peptidase activities—a chymotrypsin-like activity, a trypsin-like activity, and a caspase-like activity (Finley, 2009). Development of peptide-based proteasome inhibitors by Goldberg and colleagues (Rock et al., 1994) demonstrated that the

FIG. 3. Characterization of established iPSC lines from human foreskin fibroblasts transduced with EF1 α -STEMCCA (OKSM) lentivirus. The growth medium was supplemented with either DMSO (A–C) or MG101 (D–E). Emerging colonies were counted based on their morphology (A and D), positively stained on AP activity (B and E), and for marker of pluripotency SOX-2 (C and F).

TABLE 1. HUMAN FORESKIN FIBROBLASTS TREATED WITH PROTEASOME INHIBITOR MG101 WERE STAINED WITH ANNEXIN V AND PROPIDIUM IODINE FOLLOWED BY FLOW CYTOMETRY ANALYSIS

Exposure time (days)	Control				10 μ M				3 μ M				1 μ M			
	$PI^+ AV^-$	$PI^+ AV^+$	$PI^- AV^+$	$PI^- AV^-$	$PI^+ AV^-$	$PI^+ AV^+$	$PI^- AV^+$	$PI^- AV^-$	$PI^+ AV^-$	$PI^+ AV^+$	$PI^- AV^+$	$PI^- AV^-$	$PI^+ AV^-$	$PI^+ AV^+$	$PI^- AV^+$	$PI^- AV^-$
1	1.9 $\pm$ 0.4	5.6 $\pm$ 2.0	2.3 $\pm$ 1.3	5.0 $\pm$ 1.5 ^c	21.1 $\pm$ 4.6 ^c	28.8 $\pm$ 1.7 ^c	6.7 $\pm$ 2.0 ^c	1.7 $\pm$ 0.7	4.5 $\pm$ 2.5	1.9 $\pm$ 1.5	1.8 $\pm$ 0.7	1.8 $\pm$ 0.7	4.1 $\pm$ 2.4	4.1 $\pm$ 2.4	1.7 $\pm$ 1.3	1.7 $\pm$ 1.3
2	1.3 $\pm$ 0.2	3.8 $\pm$ 0.6	2.1 $\pm$ 0.3	3.7 $\pm$ 1.6	28.8 $\pm$ 1.7 ^c	19.7 $\pm$ 5.4 ^c	19.7 $\pm$ 5.4 ^c	3.4 $\pm$ 2.8	13.6 $\pm$ 10.0	10.6 $\pm$ 7.0	1.0 $\pm$ 0.2	1.0 $\pm$ 0.2	4.5 $\pm$ 2.5	4.5 $\pm$ 2.5	4.5 $\pm$ 2.5	4.5 $\pm$ 2.5
4	1.1 $\pm$ 0.2	4.6 $\pm$ 0.9	3.2 $\pm$ 0.7	1.6 $\pm$ 0.8	10.8 $\pm$ 5.0 ^b	15.8 $\pm$ 11.3 ^a	15.8 $\pm$ 11.3 ^a	0.9 $\pm$ 0.2	5.0 $\pm$ 0.9	4.9 $\pm$ 2.3	0.9 $\pm$ 0.2	0.9 $\pm$ 0.2	4.1 $\pm$ 0.5	4.1 $\pm$ 0.5	3.3 $\pm$ 1.4	3.3 $\pm$ 1.4
9	1.6 $\pm$ 1.1	6.5 $\pm$ 1.5	3.4 $\pm$ 1.4	2.9 $\pm$ 1.3	14.8 $\pm$ 6.9 ^a	4.5 $\pm$ 0.5	4.5 $\pm$ 0.5	1.8 $\pm$ 0.3	7.5 $\pm$ 0.9	4.3 $\pm$ 1.2	2.0 $\pm$ 0.4	2.0 $\pm$ 0.4	9.5 $\pm$ 0.2	9.5 $\pm$ 0.2	5.0 $\pm$ 0.1	5.0 $\pm$ 0.1

MG101 at 10 μ M concentration significantly increased apoptotic population of cells as early as after 24 h of exposure. Data are expressed as mean $\pm$ standard deviation.

^a $p < 0.05$.

^b $p < 0.01$.

^c $p < 0.001$.

TABLE 2. HUMAN FORESKIN FIBROBLASTS TREATED WITH PROTEASOME INHIBITOR MG132 WERE STAINED WITH ANNEXIN V AND PROPIDIUM IODINE FOLLOWED BY FLOW CYTOMETRY ANALYSIS

Exposure time (days)	Control				250 nM				125 nM				62.5 nM			
	$PI^+ AV^-$	$PI^+ AV^+$	$PI^- AV^+$	$PI^- AV^-$	$PI^+ AV^-$	$PI^+ AV^+$	$PI^- AV^+$	$PI^- AV^-$	$PI^+ AV^-$	$PI^+ AV^+$	$PI^- AV^+$	$PI^- AV^-$	$PI^+ AV^-$	$PI^+ AV^+$	$PI^- AV^+$	$PI^- AV^-$
1	1.8 $\pm$ 0.4	6.6 $\pm$ 1.0	3.0 $\pm$ 0.7	2.3 $\pm$ 1.0	13.0 $\pm$ 3.4 ^a	25.9 $\pm$ 7.0 ^a	3.9 $\pm$ 0.4	2.6 $\pm$ 1.4	3.3 $\pm$ 1.0	3.3 $\pm$ 1.0	1.0 $\pm$ 0.8	1.7 $\pm$ 0.7	4.1 $\pm$ 2.7	4.1 $\pm$ 2.7	2.0 $\pm$ 1.7	2.0 $\pm$ 1.7
2	1.3 $\pm$ 0.2	3.8 $\pm$ 0.6	2.1 $\pm$ 0.3	3.1 $\pm$ 1.2	25.9 $\pm$ 7.0 ^a	18.7 $\pm$ 5.7 ^a	18.7 $\pm$ 5.7 ^a	1.6 $\pm$ 1.9	6.6 $\pm$ 1.9	6.6 $\pm$ 1.9	7.2 $\pm$ 3.1	1.1 $\pm$ 0.2	4.5 $\pm$ 0.9	4.5 $\pm$ 0.9	4.4 $\pm$ 0.6	4.4 $\pm$ 0.6
4	1.1 $\pm$ 0.2	4.6 $\pm$ 0.9	3.2 $\pm$ 0.7	1.0 $\pm$ 0.7	10.0 $\pm$ 5.1	13.4 $\pm$ 10.9	13.4 $\pm$ 10.9	0.8 $\pm$ 0.3	5.4 $\pm$ 2.6	5.4 $\pm$ 2.6	10.0 $\pm$ 8.6	0.7 $\pm$ 0.3	3.6 $\pm$ 0.8	3.6 $\pm$ 0.8	3.9 $\pm$ 2.6	3.9 $\pm$ 2.6
9	1.6 $\pm$ 1.1	6.5 $\pm$ 1.5	3.4 $\pm$ 1.4	2.4 $\pm$ 1.5	34.7 $\pm$ 6.4 ^a	16.7 $\pm$ 9.5	16.7 $\pm$ 9.5	3.7 $\pm$ 2.3	33.7 $\pm$ 2.7 ^a	33.7 $\pm$ 2.7 ^a	6.6 $\pm$ 0.6	2.7 $\pm$ 1.0	12.0 $\pm$ 4.9	12.0 $\pm$ 4.9	5.8 $\pm$ 1.8	5.8 $\pm$ 1.8

The cells were more sensitive to MG132 than MG101 exposure. Data are expressed as mean $\pm$ standard deviation.

^a $p < 0.001$.

FIG. 4. Quantitative real-time PCR was performed on emerged colonies to determine expression of pluripotency-related genes. Treatment with MG101 significantly reduced expression of Oct3/4, Nanog, and Sox-2 mRNA levels. Data are presented as mean $\pm$ standard deviation. (*) $p < 0.05$; (**) $p < 0.01$.

proteasome is responsible for the degradation of most cytoplasmic and nuclear eukaryotic proteins. These peptide aldehydes were the first proteasome inhibitors developed and continue to be widely used. Among the peptide aldehyde inhibitors, MG132 (Z-leu-leu-leu-al) is a potent reversible proteasome inhibitor (K_i 2–4 nM) that is more selective than the earlier proteasome inhibitors typified by MG101 (ALLN, K_i 0.14 μ M), which was first described as a calpain inhibitor (for review, see de Bettignies and Coux, 2010; Kisselev and Goldberg, 2001).

Among reprogramming factors, the POU-family transcription factor Oct3/4 appears to be one of the most, if not the most, important pluripotency regulators. Its overexpression is sufficient to induce pluripotency in cells endogenously expressing reprogramming factors at higher levels (Kim et al., 2009a, 2009b; Tsai et al., 2011; Wu et al., 2011) or in combinatorial treatment with small molecules (Li et al., 2011; Yuan et al., 2011; Zhu et al., 2010). There is accumulating evidence that regulation of Oct3/4 by the ubiquitin–proteasome system plays a significant role in determining pluripotency. Elevated expression of the Oct3/4 protein in F9 cells treated with MG132 has been documented (Liao and Jin, 2010), and ubiquitin-dependent degradation of Oct3/4 may serve to promote pluripotency (Xu et al., 2004; Xu et al., 2009). In addition, Niwa et al. (2000) found that self-renewal depends on maintaining Oct 4 levels within a specific range. Our study of short-term treatment of human foreskin fibroblasts with either MG101 or MG132 indicates proteasome inhibition alone significantly increases Oct3/4 mRNA expression. In studies showing that other factors could replace Oct3/4 in the reprogramming process, reprogramming is still achieved by reactivating the endogenous Oct3/4 locus (Buganim et al., 2012; Gao et al., 2013; Heng et al., 2010; Yang et al., 2010; Zhang et al., 2006). Hence, our observation that proteasome inhibition increases Oct3/4 mRNA expression at the initial reprogramming stage (short-term treatment) suggests proteasome inhibition may increase the efficiency of reactivating Oct3/4 and other core pluripotency genes. However, long-term exposure to either MG132 or MG101 resulted in a biphasic increase in Oct3/4 expression that did not persist after 12 days in culture.

Because reprogramming is a relatively long process, the biphasic response in Oct3/4 expression might suggest that timing is an essential condition for directing cell fate in culture. Moreover, it is worth noting that long-term culture of the cells in maintenance media caused fluctuations of both Oct3/4 and Nanog expression. Because induced expression of pluripotency genes by viral transduction results in transgene integration and might cause mutagenic effects, not only identification of small molecules but also cell culture environment that can induce endogenous expression of these genes is desirable. Various elements, including culture conditions and the presence of defined and undefined supplements in culture media, could have an impact on the effectiveness of cellular reprogramming, as previously reported (Page et al., 2009). Interestingly, in our study Oct3/4 and Nanog expression was inversely affected by MG101 and MG132 at day 12 in culture, suggesting a reciprocal relationship between Oct3/4 and Nanog expression. Oct3/4 is known to suppress its own expression, which results in fluctuating transcription levels of the gene. This Oct3/4 self-suppression and fluctuating Oct4 levels regulate Nanog in a biphasic manner, as previously shown. Lower levels of Oct4 expression were reported to activate the Nanog promoter, whereas higher doses exert a negative effect in ESCs (Pan et al., 2006). A biphasic wave of Oct3/4 transcription and translation was also induced in 3T3 cells exposed to mouse ESC extract (Taranger et al., 2005). Moreover, the parallel effect of MG132 and MG101 is consistent with the proteasome, rather than calpains, as the primary target of a MG101-mediated effect on Oct3/4 and Nanog expression. Taken together, our observation of biphasic regulation of Oct3/4 and Nanog resulting from proteasome inhibition further emphasizes the dynamic relationship between Oct3/4 and Nanog and their cooperative interaction to maintain the pluripotency and self-renewing characteristics of iPSCs.

Although proteasome inhibition increases Oct3/4 expression with short-term exposure, this does not increase the efficiency of generating iPSCs. The combined administration of the four Yamanaka transcription factors (OSKM lentiviral system) and MG101 resulted in impaired generation of iPSC colonies, as demonstrated by AP counting. Thus, this study brings evidence that generating pluripotent

cells by the ectopic expression of Oct3/4, Klf4, Sox2, and c-Myc in the culture condition supplemented by a proteasome inhibitor is caused by mechanisms different than in SCNT, where MG132 improves blastocyst formation in many species (Le Bourhis et al., 2010). Moreover, colonies that emerged from the cells treated with MG101 showed lower mRNA expression levels of Oct3/4, Nanog, and Sox-2, but no change in Klf-4 or c-myc pluripotency genes when compared to nontreated cells, suggesting that Klf-4 and c-myc expression is insufficient to overcome the effect of proteasome inhibition of Oct3/4, Nanog, and Sox-2 expression. Our data clearly show that lower mRNA levels of Oct 3/4, Nanog, and Sox-2 cannot be attributed to MG101-induced apoptosis. There is evidence that increased proliferative capacity enhances cell reprogramming of human iPSCs (Ruiz et al., 2011), suggesting that reduced iPSC colony formation in the presence of MG101 is due to the anti-proliferative properties of proteasome inhibition (Legesse-Miller et al., 2012; Naujokat and Saric, 2007). However, the role of proliferation in iPSC reprogramming remains unclear, as Xu et al. (2013) reported increased proliferation during the early stage of reprogramming correlated with reduced iPSC reprogramming. Interestingly, overexpression of c-Myc was associated with increased proliferation and reduces reprogramming efficiency. This raises the possibility that the relative high expression of c-Myc in the MG101-treated cells plays a role in impaired iPSC colony generation in the presence of MG101.

Taken together, these results indicate that even if some positive effects of proteasome inhibition on reprogramming might occur, especially during the early stage of reprogramming, proteasome activity is required for more efficient colony formation. That observation is in agreement with the substantial decrease in proteasome activity that occurs upon differentiation of pluripotent stem cells (Atkinson et al., 2012) when compared to the high proteasome activity found in human (h) ESCs (Baharvand et al., 2006). Loss of pluripotency markers and a decline in the percentage of colonies positive for AP was also observed in hESCs in which the function of individual proteasome subunits was specifically inhibited (Atkinson et al., 2012). Moreover, inhibition of the PSMB8 and PSMB9 subunits of the 20S proteasome reduced iPSC colony formation with OSKM transduction (Jiang et al., 2012). The necessity of proteasome activity in reprogramming was further demonstrated by reduced cell reprogramming and iPSC colony formation with decreased expression of Psmd14, another catalytic core proteasome subunit (Buckley et al., 2012).

In conclusion, we have demonstrated that the 26S proteasome is an essential modulator in the reprogramming process. Its inhibition enhances expression of pluripotency-related genes; however, efficient colony formation requires proteasome activity. Therefore, discovery of small molecules that increase proteasome activity might lead to more efficient cell reprogramming and generation of pluripotent cells.

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