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Research Article

Histone deacetylase inhibitor valproic acid promotes the induction of pluripotency in mouse fibroblasts by suppressing reprogramming-induced senescence stress

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ABSTRACT

Histone deacetylase inhibitor valproic acid (VPA) has been used to increase the reprogramming efficiency of induced pluripotent stem cell (iPSC) from somatic cells, yet the specific molecular mechanisms underlying this effect is unknown. Here, we demonstrate that reprogramming with lentiviruses carrying the iPSC-inducing factors (Oct4-Sox2-Klf4-cMyc, OSKM) caused senescence in mouse fibroblasts, establishing a stress barrier for cell reprogramming. Administration of VPA protected cells from reprogramming-induced senescent stress. Using an *in vitro* pre-mature senescence model, we found that VPA treatment increased cell proliferation and inhibited apoptosis through the suppression of the p16/p21 pathway. In addition, VPA also inhibited the G2/M phase blockage derived from the senescence stress. These findings highlight the role of VPA in breaking the cell senescence barrier required for the induction of pluripotency.

1. Introduction

Terminally-differentiated somatic cells can be reprogrammed into pluripotent stem cells (iPSC) by inducing the expression of a combination of factors associated with pluripotency (Oct4, Sox2, Klf4, and c-Myc) [1]. The iPSCs provide a versatile and ethical alternative to create patient-specific stem cells for regenerative medicine and human disease research with a wide range of biotechnological and therapeutic applications [2]. However, generation of iPSCs from human somatic cells using defined factors is an extremely inefficient process [3,4]. Methods to improve reprogramming efficiency and to ensure genomic integrity and safety of iPSCs must be warranted before this technology can be translated into clinical application [5,6].

Numerous attempts have been made to improve the efficiency of iPSC induction, including the use of DNA methylation inhibitors [7], histone modification inhibitors [8], inhibition of tumor suppresser genes [9–11], antioxidants, vitamin C [12], and signaling pathway inhibitors [8,13–15]. Valproic acid (VPA), a well-established histone deacetylase inhibitor [16–18], has been demonstrated to have the ability of pluripotency-promoting activity [8]. In the presence of cytokine cocktails, VPA enhances long-term engraftment of hematopoietic stem cells [19] and stimulates self-renewal [20,21]. In the clinic, VPA has been used for the treatment of epilepsy, bipolar mania and migraine prophylaxis [22,23]. However, little is known about the molecular mechanisms by which VPA mediates the promotion of pluripotency.

Senescence and cellular reprogramming are deeply intertwined processes [5]. Fibroblasts cultured from old mice, which have high levels of the Ink4b/Arf/Ink4a, are less efficiently reprogrammed than are cells from young mice [11]. Lentiviral delivery of iPSC-inducing factors (OSKM) often creates cell stress, cell cycle pause, and apoptosis. Suppression of cell cycle regulator genes, like p16, p21, and p53 [9,24,25], promotes cell reprogramming. In the process of cell reprogramming [26], we noticed that supplementation with VPA significantly increased cell proliferation in OSKM lentiviruses-transfected cells, suggesting a possible anti-senescence role of VPA in cellular reprogramming. In this study, we examined whether the VPA treatment improved iPSC induction through a

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mechanism involved in the suppression of reprogramming-induced senescence stress.

2. Materials and methods

2.1. Cell lines and cell culture

MBW2 cells were fibroblast-like cells derived from culturing of M. spretus-Balb/c F1 mouse bone marrow mesenchymal stem cells [27,28]. Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 1% non-essential amino acid (NEAA) and 1% antibiotics (penicillin-streptomycin) at 37 °C in an atmosphere containing 5% CO_2.

Mouse muscle-derived fibroblasts (MDFs) and brain-derived fibroblasts (BDFs) were cultured from a CF-1 mouse fetus as previously described [27,28]. Briefly, fresh fetal tissues were minced with a razor and cultured in 6-well plates with minimum DMEM medium that just covered the tissue. Three days after culturing, fibroblast-like cells expanding around the tissue were digested with trypsin and passaged for reprogramming and senescence assays.

2.2. Promotion of mouse cell reprogramming by VPA

Mouse cell reprogramming by iPSC-inducing factors was performed as previously described [26,29]. Briefly, the Oct4-Sox2-Klf4-c-Myc (OSKM) lentiviruses were packaged and produced in 293T cells by co-transfecting the lenti-OSKM with viral packaging vectors using lipofectamine 2000 (Invitrogen, CA). The virus-containing supernatants were collected at 48 h and 72 h and concentrated with Amicon Ultra-15 Centrifugal Filter Units (Millipore, MA).

For reprogramming, fibroblast-like cells were seeded into 12-well plates at 3×10^4 cells per well in DMEM/F12 (Invitrogen, CA) supplemented with 20% knockout serum replacement (KSR), 0.1 mM beta-mercaptoethanol, L-Glutamine, and 1×10^{-4} M non-essential amino acids (Invitrogen, CA), and were infected with concentrated lentiviruses in the presence of polybrene (8 μg/ml). Three days after infection, the cells were digested and $3–4 \times 10^4$ cells were transferred to 100 mm dishes on mitomycin C-inactivated MEF feeder cells. The media were replaced with ES medium (DMEM/F12 supplemented with 20% KSR), 10 ng/ml Leukemia inhibitory factor (LIF, Sigma), 10 ng/ml β-FGF (PeproTech), 0.1 mM β-mercaptoethanol, L-Glutamine, and 1×10^{-4} M non-essential amino acids [30]. As previously described [8], 0.5–1.0 mM VPA was added to the medium to promote reprogramming.

2.3. Alkaline phosphatase staining

Reprogramming efficiency was measured by staining the iPSC colonies using an alkaline phosphatase (AP) kit (Millipore, MA) following the manufacturer's instruction. The cells were fixed in 4% paraformaldehyde/PBS for 1–2 min, rinsed with PBS and then incubated with staining solution in the dark at room temperature. After 15 min, colonies of cells expressing AP (red colonies) were recorded using a microscope-mounted camera [26,29].

2.4. Pluripotent marker staining

After expansion, the isolated iPSC colonies were characterized by staining pluripotent markers using the method as previously described [18,20]. Briefly, cells were fixed with 4% paraformaldehyde/PBS, permeabilized with 0.1% Triton X-100/PBS containing 3% BSA, and incubated with primary antibodies for pluripotent markers (SSEA4, Nanog, Sox2). After washing with PBS, cells were incubated with Cy3 or Alexa Fluor 488 labeled secondary antibodies. Fluorescence images were acquired with a Zeiss AxioCam Camera.

2.5. Reprogramming-induced cell senescence

Reprogramming with OSKM cocktail lentiviruses induced cell senescence, particularly in primary fibroblasts when cultured in KSR-containing mES medium. To observe cell senescence, fibroblast-like cells were transfected with OSKM lentiviruses and cell reprogramming was induced in the presence of VPA. Seven days after OSKM lentiviral infection in the presence of VPA, cells were collected and seeded into new 6-well plates to get rid of feeder cells. The lentivirus-induced senescence was analyzed by the staining of senescence-associated β-galactosidase (SA β-gal).

2.6. Induction of premature senescence

Cell reprogramming using OSKM lentiviruses is a time-consuming process and requires the support of mitomycin C-inactivated MEF feeder cells. To better study the role of VPA in cell senescence, we adopted a simple *in vitro* premature senescence cell model in the following study. In this model, premature senescence was induced in MBW2 cells and primary culture cells using a copper method as previously reported [31–34]. Copper, an essential micronutrient, plays a catalytic role in the activity of several enzymes through changes of its oxidation state (*e.g.* cytochrome c oxidase) and the generation of ROS [35]. Copper is implicated in the aging process and in age-associated disorders such as Alzheimer disease [36].

Briefly, fibroblast-like cells at 60% confluence were exposed to low doses of copper (400–600 μM $CuSO_4$). After exposure, VPA (1 mM) was added to the medium and the cells were allowed to grow for 48 h. Cell senescence was determined by SA β-gal staining.

2.7. Staining of senescence-associated β-galactosidase

Cell senescence was analyzed by staining of senescence-associated β-galactosidase (SA β-gal). The activity of SA β-gal was determined using the method as described by Beane et al. [37]. Briefly, cells were washed with PBS and fixed by 3% formaldehyde for 3–5 min at room temperature. After washing with PBS, cells were incubated at 37 °C with freshly prepared senescence-associated SA β-gal staining solution: 1 mg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal), 40 mM citric acid/sodium phosphate, pH 6.0, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$. After overnight incubation, cells demonstrating SA β-gal staining were counted using a microscope-mounted camera.

2.8. Cell Viability

Cell survival was measured using the MTT (3-(4,5-dimethylthiazol-2-yl) -2,5- diphenyltetrazolium bromide) assay [38,39]. Cells (1×10^4/well) were plated onto 96-well plates. After VPA treatment, cells were incubated with 20 μl 5 mg/ml MTT [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide](Sigma, MO) per well at 37 °C for 4 h. After removal of the media, 150 μl DMSO was added, and the cells were shaken using an orbital shaker for 10 min. The cell absorbance was measured at 490 nm using a microplate reader (Bio-TEK Instruments, USA).

Cells treated with equal volume of PBS were set the control. Cell viability (%) was calculated based on the following equation: