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Research Article

The effects of LSD1 inhibition on self-renewal and differentiation of human induced pluripotent stem cells

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ABSTRACT

Human induced pluripotent stem cells (hiPSCs) are capable of unlimited self-renewal and can generate nearly all cells in the body. Changes induced by different LSD1 activities on the regulation of hiPSC self-renewal and differentiation and the mechanism underlying such changes were determined. We used two different LSD1 inhibitors (phenelzine sulfate and tranylcypromine) and RNAi technique to inhibit LSD1 activity, and we obtained hiPSCs showing 71.3%, 53.28%, and 31.33% of the LSD1 activity in normal hiPSCs. The cells still maintained satisfactory self-renewal capacity when LSD1 activity was at 71.3%. The growth rate of hiPSCs decreased and cells differentiated when LSD1 activity was at approximately 53.28%. The hiPSCs were mainly arrested in the G0/G1 phase and simultaneously differentiated into endodermal tissue when LSD1 activity was at 31.33%. Teratoma experiments showed that the downregulation of LSD1 resulted in low teratoma volume. When LSD1 activity was below 50%, pluripotency of hiPSCs was impaired, and the teratomas mainly comprised endodermal and mesodermal tissues. This phenomenon was achieved by regulating the critical balance between histone methylation and demethylation at regulatory regions of several key pluripotent and developmental genes.

1. Introduction

Histone lysine specific demethylase 1(LSD1) [1] was found expressed in many stem cells [2–4], and involved in regulating proliferation and differentiation of stem cells, including embryonic stem cells [5]. LSD1 is a female parent transcription factor, and LSD1 transcripts disappear in zygotic genome activation (ZGA) process, and then recover to oocytes transcriptional level in the blastocyst stage [6]. LSD1 knockdown in drosophila embryo caused histone H3K4 higly methylating and abnormal expression of genes, resulting in the death of the embryo [7]. Recent studies have shown LSD1 participated in the maintenance of pluripotency through the regulation of bivalent domains, a chromatin environment present at the regulatory regions of developmental genes that contain both H3K4 di/trimethylation and H3K27 trimethylation marks [5]. LSD1 knockout in human ESCs caused the mRNA levels of pluripotency genes OCT4, SOX2 and NANOG reduced, but never more than 50%, while genes that control endodermal and mesodermal lineages, such as FOXA2, EOMES, BMP2 and SOX17, were strongly upregulated. LSD1 occupies the promoters of developmental genes that contain bivalent domains and are co-occupied by OCT4 and NANOG in human embryonic stem cells, where it controls the levels of H3K4 methylation through its demethylase activity. With LSD1 knockdown, H3K4 methylation levels in the promoter region of these target genes increased significantly, which promoting the activation of gene transcription, and resulting in ESCs turning to differentiating.

Human inpluripotent stem cells were obtained by introducing four defined transcription factors, Oct4, Sox2, Klf4, and c-Myc to reprogram somatic cells to iPSCs, which showed ESC-like properties in almost all aspects [8,9]. However, previous reports demonstrated difference about proteomics and global gene expression profiles between human ESCs and iPSCs [10,11]. What changes different LSD1 activity will make on regulating hiPSCs' self-renewal and differentiation and how is the mechanism? This will play a guiding role in stem cells' clinical applications. Here, we used two different LSD1 inhibitors (Tranylcypromine, phenelzine sulfate) and RNAi technique to inhibit LSD1 activity, and we gained hiPSCs cells of 71.3%, 53.28% and 31.33% LSD1 activity respectively. Notably, the expression levels of LSD1 were high in undifferentiated hiPSCs. Normal cells maintained a good self-renewal capacity, and differentiation genes were silenced. When LSD1

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activity was at about 70%, the mRNA levels of pluripotency gens were still high; when LSD1 activity was at about 50%, hiPSCs growth rate reduced and the expression levels of endodermal and mesodermal genes upregulated; when LSD1 activity was at about 30%, cells mainly arrested in the G0/G1 phase, and the mRNA level of endodermal genes upregulated simultaneously. Thus, we conclude that 50% activity of LSD1 may be the balance point of undifferentiated hiPSCs turn to differentiated cells. The teratoma experiments showed the downregulation of LSD1 resulted in the teratoma having smaller volume. This indicates that the proliferation of hiPSCs was damaged when LSD1 activity was too low. When LSD1 activity was below 50%, pluripotency of hiPSCs was impaired and teratomas were mainly composited with endoderm and mesoderm tissues, such as the digestive glands and intestinal villi, etc. This is achieved through regulating the critical balance between histone methylation and demethylation at regulatory regions of several key pluripotent and developmental genes.

2. Materials and methods

2.1. hiPSCs culture in vitro

Human iPSCs (NF1-4-iPS-C11) were supplied by Stem Core (Key Laboratory of Regenerative Biology, South China Institute for Stem Cell Biology and Regenerative Medicine, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China) under the appropriate human ethics and material transfer agreements [12]. Human iPSCs were cultured by using feeder-free culture conditions with mTeSR1 (Stem Cell Technologies) and matrigel (BD Biosciences). Culture medium was changed every day, and cells were passaged using dispase (Gibco Technologies) and manual dissection when grow cells to 80–90% confluence.

2.2. RNAi or LSD1 inhibitors treating hiPSCs

Five shRNA plasmids (scrambled-shRNA, shRNA-LSD1-2495, shRNA-LSD1-863, shRNA-LSD1-927, shRNA-LSD1-1086), constructed with a pGLV2-U6-Puro vector (GenePharma, China), were transduced into hiPSCs. After transduction, LSD1 expression was quantified by real time PCR. Based on the mRNA expression, shRNA-LSD1-927 showed the highest inhibition effect of LSD1. The following shRNA lentiviral particles were used: non-target control scrambled-shRNA (5′-GTCAAGTCTCACTTGCGTC-3′) and the shRNA-927 targeting LSD1 (5′-GAAGGCTCTTCTAGCAATA-3′). The lentiviral transduction was performed according to the manufacturer's instructions with slight modifications. The hiPSCs (1×10^5) were transduced with lentiviral particles and incubated for 24 h. Then the medium was changed and the transduced cells were cultured in mTeSR1 for additional 48 h before puromycin selection (1 mg/ml; Sigma).

LSD1 inhibitors Phenelzine Sulfate (Spectrum Chemicals, CA, USA) and Tranylcypromine · $1/2H_2SO_4$ (TCP, Enzolifesciences, NY, USA) were both dissolved in ddH_2O. Concentration of tranylcypromine (TCP) ranged as 0.5 μM, 2.5 μM, 5.0 μM, 7.5 μM, 10.0 μM, 20.0 μM, 30.0 μM, 40.0 μM, 80.0 μM, 120.0 μM, 160.0 μM, 200.0 μM, 250.0 μM, and 300.0 μM. Concentration of phenelzine ranged as 0.5 μM, 1.5 μM, 2.5 μM, 5.0 μM, 7.5 μM, 10.0 μM, 15.0 μM, 20.0 μM, 30.0 μM, 40.0 μM, 80.0 μM, 120.0 μM, 160.0 μM, and 200.0 μM. Different concentration of LSD1 inhibitors were added to the medium and cells were incubated at 37 °C with 5% CO_2. The medium was replaced with fresh medium containing the drug daily, continuously for 72 h.

2.3. CCK-8 cell proliferation assay

The human iPS cells were plated on 96-well plates (100 μL/well) at a density of 3000 cells/well and allowed to grow overnight. Then the medium was added with appropriate concentrations of LSD1 inhibitors or shRNA-927-LSD1 transfected as above. After treatment, 10 μL of CCK-8 solution (WST-8, Japan) were added to each well and incubated at 37 °C incubator for 4 h. The absorbance was measured at 450 nm using a microplate reader (Bio-RAD Model 680, USA).

2.4. LSD1 activity assay

Nuclear extract/LSD1 enzyme were prepared using the EpiQuik™ Nuclear Extraction Kit II (Epigentek, New York, USA) following the manufacturer's instructions. And then the experiments were divided into four groups according to the EpiQuik™ Histone Demethylase LSD1 Activity/Inhibition Assay Kit (Epigentek, New York, USA) procedure: ① negative group: add 28 μl LSD1 matter, and 2 μl LSD1 buffer into each well; ② blank group: add 30 μl of LSD1 buffer into the blank wells; ③ standard curve group: add 29 μl of LSD1 buffer into the wells, followed by adding 1 μl of LSD1 substrate at different concentrations(0.1–10ng/μL); ④ treatment groups: add 4 μl of RNAi or LSD1 inhibitors extract and 26 μl of the substrate. In this experiment, the LSD1 substrate was dimethylated H3K4. The nuclear extracts were incubated with substrate and buffer for 60 min. Anti-demethylated H3K4 antibody and fluorescent dye were added to quantitative determination protein LSD1 at $530_{EX}/590_{EM}$ nm using a microplate reader (FLx800, USA). LSD1 activity (RFU/h/μg)=(RFU of treated group-RFU of control group)/Reaction time (min) × protein amount added (mg).

2.5. Western blotting detecting LSD1 protein levels

After RNAi or LSD1 inhibitors treating hiPSCs, 1×10^6 hiPSCs lysate was collected. Nuclear proteins were fractionated on 4–12% NuPAGE Bis–Tris gels and transferred onto PVDF membranes. The membranes were first incubated with blocking buffer (TBS with 0.05% Tween 20, 10% nonfat milk) for 1 h at 4 °C, followed by adding primary antibody [mouse anti-human LSD1 monoclonal antibody (1:1000) (Santa Cruz, USA)] incubating overnight at 4 °C. The membranes were washed with TBS-T for three times, each time for 15 min. The PVDF membrane was then added the secondary antibody [goat anti-mouse IgG antibodies (1:3000)] dilutions and incubated for 1 h at 4 °C. The proteins were visualized using a SuperSignal West Femto Chemiluminescent Substrate kit (Thermo Scientific, Rockford) and quantified by densitometric analysis using Fluor Chem SP software.

2.6. FACS analysis cell cycle and apoptosis

After RNAi or LSD1 inhibitors treating hiPSCs, cells were digested into single cell suspension, which concentration was 1×10^6/mL. For cell cycle analysis, cells were fixed in 70% ethanol overnight and then stained with 2 μL propidium iodide (PI, 50 mg/ml; KeyGEN Biotech), keeping in dark place for 15 min at room temperature. The cells were finally analyzed the percentages of G0/G1, S, and G2/M-phase cells using flow cytometry (BD Biosciences, USA). Apoptosis assays were carried out using the Annexin V-FITC Apoptosis Detection Kit (KeyGEN Biotech). Cells were stained with 5 μL FITC-Annexin V and 5 μL PI, and then incubated at room temperature for 30 min. hiPSCs apoptosis rate in each group were analyzed using flow cytometry (BD Biosciences, USA).

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