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Research Article

Histone Deacetylase SIRT1 Facilitates OCT4 Gene Expression and Generation of Induced Pluripotent Stem Cells

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Abstract

Expression of defined sets of transcription factors can reprogram somatic cells to Induced Pluripotent Stem Cells (iPSCs). This technology holds enormous potential as iPSCs can be used as an alternative source for regenerative medicine. However, the low efficiency and associated genome instability in generating iPSCs impede the use of this method for biomedical applications. We report here that SIRT1, a member of the sirtuin family of NAD+-dependent protein deacetylases, can stimulate the transcription of the master pluripotency transcription factor OCT4 and iPSC formation. Using a luciferase reporter system in human keratinocytes, we show that sirtinol (a pan sirtuins inhibitor) inhibits OCT4 expression by 10-fold and SIRT1 overproduction enhances OCT4 expression by 3-fold. In addition, expression of OCT4 is nearly abrogated in Sirt1 knockout mouse embryonic fibroblast cells. To further investigate the role of SIRT1 on gene expression of pluripotency factors and iPSC formation, we transfected human keratinocytes with episomal plasmids expressing reprogramming factors OCT4, KLF4, SOX2, and MYC. SIRT1 overproduction enhances the transcription of all plasmid-derived reprogramming factors. However, SIRT1 only stimulates endogenous OCT4 expression, but not endogenous KLF4, SOX2, and MYC. Moreover, iPSCs are generated more efficiently when SIRT1 is overexpressed. Thus, SIRT1 enhances somatic cell reprogramming, in part, through enhancement of endogenous OCT4 expression and ectopic transcription factors. Our finding will take steps closer to generate high quality iPSCs for tissue replacement.

Introduction

Recent advances in reprogramming somatic cells into Induced Pluripotent Stem Cells (iPSCs) provide exciting opportunity for generating renewed tissue for disease modeling and therapy. It has been shown that over-expressing four key transcription factors OCT4 (also known as OCT3/4 or POU5F1), KLF4, SOX2, and c-MYC (known as OKSM) enables reprogramming of mouse and human somatic cells to iPSCs that closely resemble Embryonic Stem Cells (ESCs) [1-6]. However, several issues pertaining to their effectiveness and safety need to be addressed before the technology can be used in clinical settings [1,7]. The major hurdles in the process of developing human cell based therapy include low efficiency, use of retroviral vectors, and tumorigenic potential of the proto-oncogenes c-MYC and KLF4 to generate iPSCs. Moreover, iPSCs generated by various methods vary widely in their differentiation capacity and genomic stability. Therefore, methods to improve the process are being vigorously pursued.

Efforts have been made to improve the efficiency of iPSC generation [8,9]. Now it is possible to reprogram somatic cells with fewer transcription factors through optimization of methods and use of small molecules. Recent studies have shown that, reprogramming can be achieved by OCT4 over-expression alone in combination with certain small molecules [10,11]. Therefore, OCT4 may serve as a pluripotency determinant in reprogramming. Reprogramming can be facilitated by chromatin-remodeling components of the BAF complex [12] and inhibitors of Histone/Protein Deacetylases (HDACs) and DNA MethylTransferases (DNMTs) [13-17]. Other small molecules like ascorbic acid and

GSK3-b inhibitor have also been reported to shorten the process of iPSCs generation [18]. Several virus-free methods have been developed to generate iPSCs including using recombinant proteins [19,20], repeated administration of synthetic modified mRNA of five factors (OSKM and LIN28) [21], and episomal plasmids [6,22]. However, their induction efficiencies are still low and may be accompanied by undesirable side effects.

Histone modification and DNA methylation control epigenetic gene expression and are key factors in somatic cell reprogramming. SIRT1 is a member of sirtuins family of NAD+-dependent histone/protein deacetylases (class III HDACs)(reviewed in [23]). SIRT1 deacetylates histones, many DNA repair enzymes, DNMT1, the tumor suppressor p53, and other proteins [24-28]. Through this deacetylase activity, SIRT1 modulates gene expression, metabolism, stress response, genomic stability, and longevity [29,30]. Interestingly, SIRT1 expression is higher in stem cells than in differentiated cells and its levels decline considerably during lineage specification [31-34]. It has been shown that mouse Sirt1 can facilitate iPSC generation from Mouse Embryonic Fibroblasts (MEFs) through deacetylating p53 and Sox2, as well as enhancement of Nanog expression [32,35]. Thus, SIRT1 is important for pluripotency.

OCT4 transcription factor promotes self-renewal and inhibits differentiation of stem cells [36,37]. Because ectopic expression of OCT4 alone with small molecules can produce iPSCs [10,11], we initiated a search for factors that can stimulate OCT4 gene expression. In this study, we examined whether SIRT1 can enhance the generation of iPSCs through induction of the pluripotency transcription factors OCT4, KLF4, SOX2, and MYC. We found that SIRT1 stimulates both endogenous and ectopic OCT4 gene expression in human cells. However, SIRT1 can only stimulate plasmid-derived KLF4, SOX2, and MYC expressions, but not their endogenous genes. Moreover, SIRT1 overexpression induces more iPSC-like colonies. Thus, SIRT1 enhances reprogramming by activating pluripotency genes.

Materials and Methods

Cell Culture

Normal Human Epithelial Keratinocytes (NHEKs, purchased from Lonza) were grown in keratinocyte-SFM medium (Life Technology) with addition of 5 ng/ml epidermal growth factor 1-53 (EGF 1-53), 50 µg/ml Bovine Pituitary Extract (BPE) and 1% penicillin/streptomycin. NHEKs at 3rd passage were used to generate iPSCs. For iPSC feeder, Mouse Embryonic Fibroblast (MEF) cells (Millipore) were grown in DMEM (Dulbecco's Modified Eagle's Medium, Invitrogen) supplied with 15% Fetal Bovine Serum (FBS), 0.1 mM MEM non-essential amino acids, 2 mM L-glutamine, and 1% penicillin/streptomycinin gelatin-coated dishes to 90% confluence. The cells were then treated with 10 µg/mL mitomycin C (Roche) for 2 hour, washed 3 times with PBS, replaced with fresh medium, and seeded with transfected NHEKs. Sirt1$^{+/+}$ (wild-type) and sirt1$^{+/+}$ (knockout) MEF cells (obtained from Dr. Toren Finkel, National Institutes of Health, Bethesda, MD, U.S.A.) were maintained in DMEM supplemented with 15% FBS and 1% penicillin/streptomycin.

OCT4 Promoter Driven Luciferase Reporter Assay

To facilitate the measurement of OCT4 gene expression, we transfected NHEK cells with OCT4-Luciferase (OCT4-Luc) reporter plasmid (obtained from Dr. Richard Eckert, University of Maryland Medical School, MD, U.S.A.) which contains the OCT4 promoter (2601 nucleotides upstream of transcription start) upstream to the luciferase coding region. Six hours post transfection, histone deacetylase inhibitors [100 nM trichostatin A (TSA, Sigma/Aldrich) or 25 µM sirtinol (Sigma/Aldrich)] were added to the cells for 42 hr. To investigate the role of SIRT1 on OCT4 gene expression, we co-transfected SIRT1 expression plasmid (pECE-SIRT1, Addgene) with OCT4-Luc reporter plasmid into NHEK. In addition, Sirt1$^{+/+}$ (wild-type) and sirt1$^{-/-}$ (knockout) MEF cells were transfected with the OCT4-Luc reporter plasmid. Two days post-transfection, cells were collected and subjected for luciferase assay (Invitrogen) according to the manufacturer's protocol. In brief, cells were washed with 1X PBS twice and lysed with lysis reagent. Cell lysate (20 µl) was mixed with 100 µl of luciferase assay reagent and the luciferase activity was measured by a Berthold Lumat LB 9507 Luminometer.

Induced Pluripotent Stem Cells (IPSCs) Generation Using Episomal Vectors

iPSCs were generated from NHEKs by methods as described by Okita et al. [22] with some modifications (Figure 1).

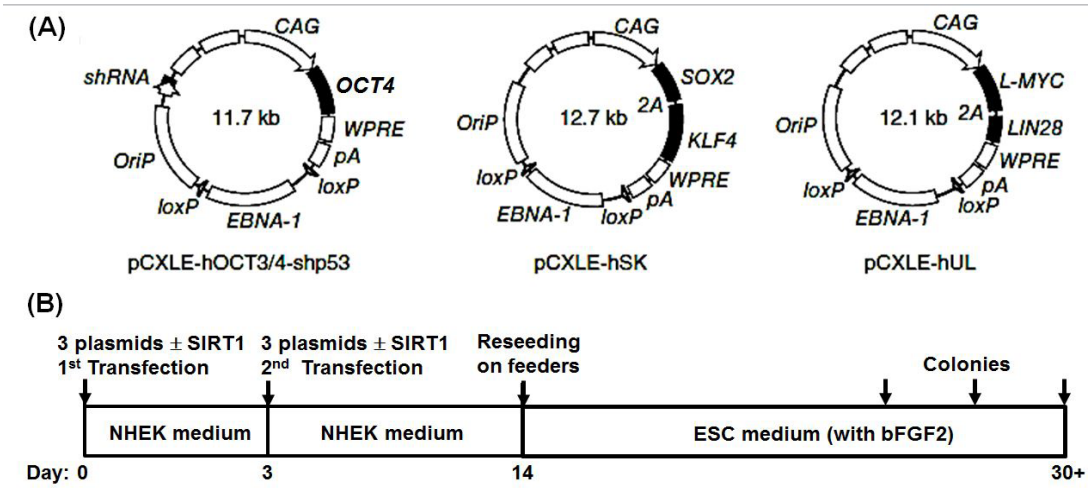

Figure 1: Protocol for iPSC generation.

(A) The three episomal plasmids to express reprogramming factors as described by Okita et al. [22]. CAG, CAG promoter; shRNA, p53 knockdown RNA; WPRE, woodchuck hepatitis post-transcriptional regulatory element; and pA, polyadenylation signal.

(B) Schematic of the iPSC induction protocol. Detailed procedures are described in the Materials and Methods section. NHEK: Normal human epithelial keratinocyte; bFGF: basic fibroblast growth factor.

NHEK cells (3rd passage, 50% confluent) were co-transfected with Okita episomal plasmid vectors (pCXLE-hOCT3/4-shp53, pCXLE-hSK, and pCXLE-hUL) [22] (referred to as Okita plasmids, Figure 1) with or without pECE-SIRT1. pCXLE-hOCT3/4-shp53 contains OCT4 gene and p53 knockdown RNA (shRNA). pCXLE-hSK contains SOX2and KLF4 genes while pCXLE-hUL contains L-MYC and LIN28 genes. These Okita plasmids contain genes encode reprogramming factors under the control of CAG promoter which is composed of sequences from the Cytomegalovirus (CMV) early enhancer element; the promoter, the first exon and the first intron of chicken β-actin gene; and the splice acceptor of the rabbit beta-globin gene [38]. Controls are no plasmid and pECE cloning vector. Three days after transfection, a second transfection was performed. Cells were maintained in NHEK medium for 2 weeks before passaging onto MEF feeders in ESC medium (DMEM-F12 with 10% KnockOut™ Serum Replacement, Thermal Fisher), 1% Glutamax (Thermal Fisher), 0.1 mM MEM non-essential amino acids, and 8 μg/ml FGF basic (bFGF2) (Life Technology). These cells were maintained and observed for iPSC formation for more than 30 days. iPSC-like colonies were monitored in a Nikon ELWD 0.3 microscope, counted, and examined for the presence of the pluripotent cell markers (see below).

Reverse Transcription Quantitative PCR (RT-qPCR)

Total RNA from transfected NHEK cells was isolated by using TRIZOL reagents (Invitrogen) according to the manufacture's protocol. RNA (100 ng) was used as a template for one-step RT-qPCR reactions using primers listed in Table 1.

Name	Sequence	Purpose
Pla-OCT4-F	CAT TCA AAC TGA GGT AAG GG	5' primer of plasmid encoded OCT4
Pla-OCT4-R	TAG CGT AAA AGG AGC AAC ATA G	3' primer of plasmid encoded OCT4
Pla-KLF4-F	CCA CCT CGC CTT ACA CAT GAA GA	5' primer of plasmid encoded KLF4
Pla-KLF4-R	TAG CGT AAA AGG AGC AAC ATA G	3' primer of plasmid encoded KLF4
Pla-SOX2-F	TTC ACA TGT CCC AGC ACT ACC AGA	5' primer of plasmid encoded SOX2
Pla-SOX2-R	TTT GTT TGA CAG GAG CGA CAA T	3' primer of plasmid encoded SOX2
Pla-L-MYC-F	GGC TGA GAA GAG GAT GGC TAC	5' primer of plasmid encoded L-MYC
Pla-L-MYC-R	TTT GTT TGA CAG GAG CGA CAA T	3' primer of plasmid encoded L-MYC
ENDO-OCT4-F	CCC CAG GGC CCC ATT TTG GTA CC	5' primer of endogenous OCT4
ENDO-OCT4-R	GGA CAG GGG GAG GGG AGG AG	3' primer of endogenous OCT4
ENDO-KLF4-F	GGA CCA CCT CGC CTT ACA CA	5' primer of endogenous KLF4
ENDO-KLF4-R	GGC TCC TTC CCT CAT CGG GAA GAC	3' primer of endogenous KLF4
ENDO-SOX2-F	TTC ACA TGT CCC AGC ACT ACC AGA	5' primer of endogenous Sox2
ENDO-SOX2-R	CAC CCC TCC CAT TTC CCT CG	3' primer of endogenous Sox2
ENDO-L-MYC-F	GGG CTG AGA AGA GGA TGG CTA CA	5' primer of endogenous L-MYC
ENDO-L-MYC-R	CCT CCC TCT CCC CTT TAG TAA TTT GCA	3' primer of endogenous L-MYC
SIRT1-F	TAGCCTTGTCAGATAAGGAAGGA	5' primer of SIRT1
SIRT1-R	ACAGCTTCACAGTCAACTTTGT	3' primer of SIRT1
Beta-ACTIN-F	ACCAACTGGGACGACATGGA	5' primer of b-act in
Beta-ACTIN-R	TACATGGCTGGGGTGTTGAA	3' primer of b-actin

Table 1: Oligonucleotides used for qPCR.

RT-PCR was performed using the Light Cycler 480 II Detection System (Roche) with iScript™ SYBR® Green One-Step Kit (BioRad, 1708892). The reactions were carried out with an initial step of reverse transcription at 50°C for 30 min and 95°C for 5 min followed by PCR cycles (95°C for 15 sec and 60°C for 1 min). The mRNA levels of SIRT1 and transcription factors were calculated relative to that of β-actin as ΔCt which is the difference between the number of cycles required to go above background in respective factors and β-action samples. The fold difference of mRNA levels of Transfected Cells (T) relative to no plasmid control (C) cells was calculated according to the formula $2^{\Delta Ct(C)-\Delta Ct(T)}$. The reactions were carried out in duplicate and data are averaged from three independent transfection experiments.

Immunofluorescent Staining with Stem Cell Markers OCT4 and TRA-1-60

iPSCs were grown on 12-well culture plate on top of MEF feeders. Cells were fixed with 4% par formaldehyde at 37°C for 15 min and permeabilized in 0.25% Triton-X100 at room temperature for 10 minutes. After being washed with PBS and incubated in blocking buffer (0.5% NP-40 and 5% bovine serum albumin in PBS) for 30 min, the cells were incubated with anti-OCT3/4 antibody (Santa Cruz Biotechnology) in blocking buffer at 4°C overnight. Next, the cells were washed three times with PBS and stained with Alexa Fluor 488 goat anti-mouse IgG antibody (Invitrogen) and 5 μg/ml Alexa Fluor 594 anti-human TRA-1-60-R antibody (Biolegend) for 2 hour. After being washed three times with PBS, nuclear DNA was counter stained with 500 nM DAPI (4',6'-diamidino-2-phenyllindole, Vector Laboratory) in PBS for 10 minute. Images were captured with a Nikon PCM 2000 confocal microscope scanning system.

Results

Sirtinol suppresses and SIRT1 Enhances OCT4 Expression.

Reprogramming somatic cells into iPSCs erases somatic epigenetic signatures (DNA methylation and histone modification) and establishes alternative epigenetic marks of ESCs [40]. Histone acetylation induces an open chromatin configuration associated with gene activation [41]. The level of histone acetylation is controlled by Histone/Protein Acetyltransferases (HATs) and Histone/Protein Deacetylases (HDACs) [42,43]. To test whether HDACs regulate iPSC generation from NHEK cells, we first tested whether their inhibition regulates OCT4 expression. We employed

two HDAC inhibitors: Trichostatin A (TSA), a pan inhibitor targeting class I/II HDACs and sirtinol, a pan inhibitor for class III HDACs [43]. We transfected NHEK cells with the OCT4-Luc reporter plasmid which containsthe OCT4 promoter upstream to the luciferase coding region to facilitate the measurement of OCT4 gene expression. Six hours post transfection, we treated the cell with the HDAC inhibitors for 42 hr, isolated the cell extracts, and then assayed the luciferase activity. As shown in Figure 2A, TSA slightly enhanced luciferase activity by 1.2-fold (but not statistically significant), while sirtinol significantly inhibited OCT4-Luc expression by 12.5-fold. These data indicate that sirtuins, but not class I/II HDACs, stimulate OCT4 expression.

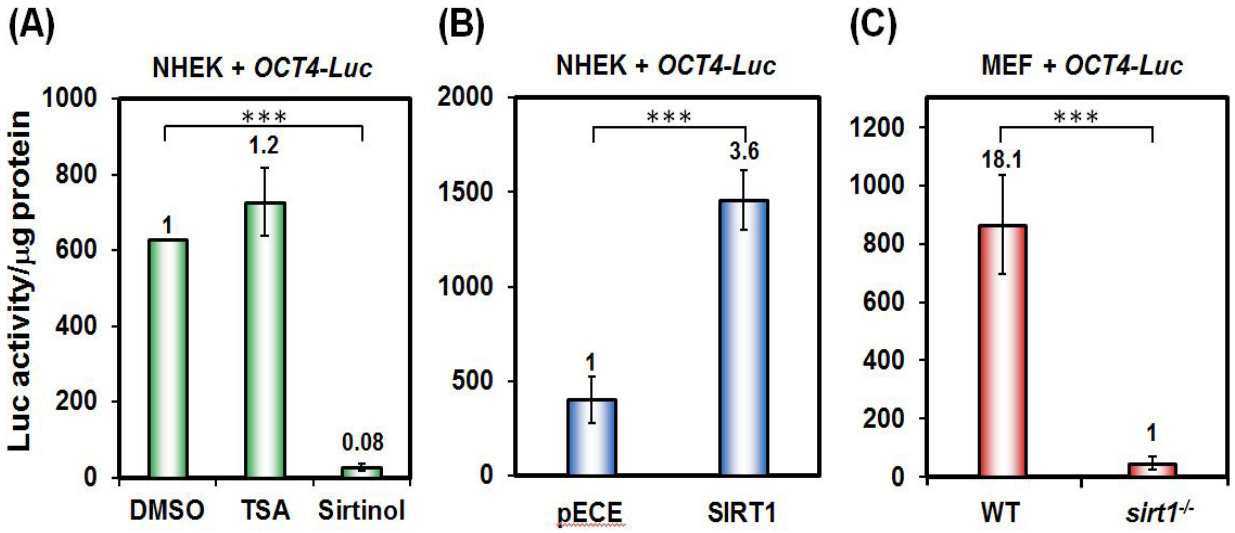

Figure 2: Effects of HDAC inhibitors and SIRT1 on OCT4 gene expression.

(A) OCT4 gene expression is regulated by HDAC inhibitors. OCT4-Luciferase (OCT4-Luc) reporter plasmid was transfected into human keratinocytes (NHEKs). Six hours post-transfection, cells were treated with trichostatin A (TSA), sirtinol, or DMSO for 42 hr. Relative luciferase activity per μg protein was on the Y-axis and the averages of fold of activity change were shown above each bar.

(B) SIRT1 enhances OCT4 expression. NHEK cells were co-transfected with OCT4-Luc reporter plasmid and the SIRT1 expression plasmid pECE-SIRT1 or pECE vector. Relative luciferase activity was assayed at 2 days after transfection.

(C) SIRT1 deficiency abrogates OCT4 expression. $Sirt1^{+/+}$ (wild-type) and $sirt1^{-/-}$ (knockout) mouse embryonic fibroblast (MEF) cells were transfected with the OCT4-Luc reporter plasmid for 2 days. Relative luciferase activity was indicated. Error bars indicate SD; $n \geq 3$. Three stars indicate that the p-values are smaller than 0.001.

To investigate a direct involvement of sirtuins in OCT4 gene expression, we co-transfected SIRT1 expression plasmid pECE-SIRT1 with OCT4-Luc reporter plasmid into NHEK cells for two days. As shown in Figure 2B, SIRT1 overexpression can enhance OCT4-Luc expression by 3.6-fold. To further confirm the role of SIRT1 on OCT4 expression, we compared OCT4-Luc expression in $Sirt1^{+/+}$ (wild-type) and $sirt1^{-/-}$(knockout) MEF cells. The expression of OCT4-Luc was nearly abrogated in $sirt1^{-/-}$ MEF as compared to wildtype cells. In the absence of SIRT1, luciferase activity was reduced by 18-fold. Our results indicate that SIRT1 is required for OCT4 gene transcription.

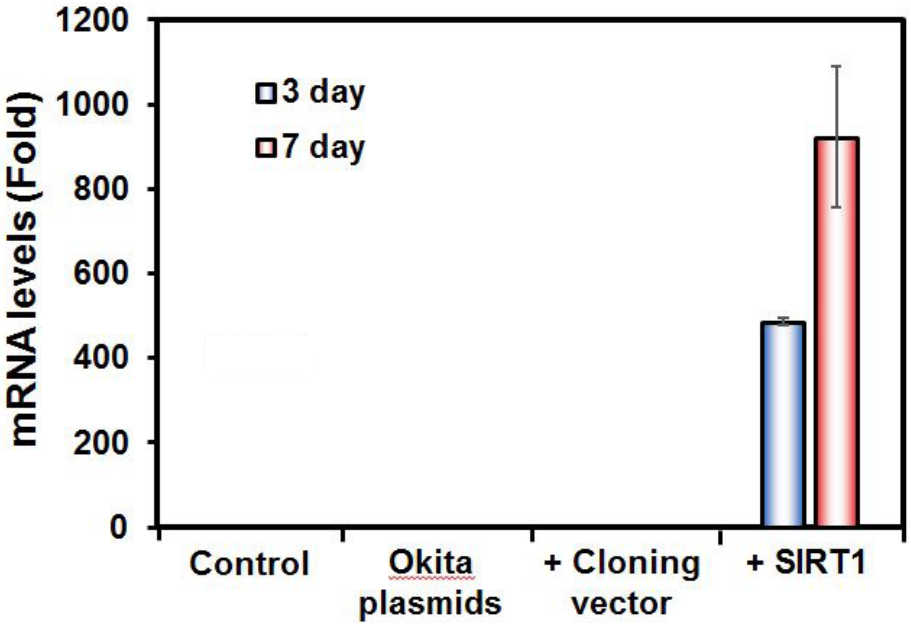

Figure 3: SIRT1 mRNA expression after pECE-SIRT1 plasmid transfection during iPSC generation.

NHEKs were co-transfected with Okita plasmids with or without SIRT1 expression vector pECE-SIRT1 (or PECE cloning vector) as outlined in Figure 1. Controls are cells without any plasmid transfection. RNA samples were isolated on 3 or 7 days post transfection. RNA (20 ng) was then subjected for RT-qPCR analyses with SIRT1 and β-actin primers as list in Table 1. The mRNA levels of SIRT1 were calculated relative to that of β-actin as ΔCt. The fold difference of mRNA levels of pECE-SIRT1 transfected cells (T) relative to no plasmid control (C) cells was calculated according to the formula $2^{\Delta Ct(C)-\Delta Ct(T)}$. Error bars indicate SD; n = 3.

SIRT1 Up-Regulates Plasmid-Derived and Endogenous OCT4 Pluripotency Regulators

Several integration-free methods have been used to generate iPSCs to avoid virus-integration induced mutagenesis. However, reprogramming efficiency using these methods is low in most cases. We elected to use the episomal plasmids developed by Okita, et al. [22] that contain pCXLE-hOCT3/4-shp53, pCXLE-hSK, and pCXLE-hUL (Figure1). With these plasmids, iPSC generation is markedly enhanced by p53 suppression and the potent L-Myc [22]. We used human keratinocytes for iPSC generation because they are easy to obtain and their reprogramming efficiency is higher as compared to the human fibroblasts [44]. We also modified the reprogramming protocols shown in Figure 1. An extra transfection with Okita plasmids with or without pECE-SIRT1 was incorporated three days after the first transfection. After the second transfection, cells were maintained in NHEK medium for additional 11 days and then passaged into the top of mitomycin-treated MEF feeders. The SIRT1 expression plasmid pECE-SIRT1 was co-transfected with the Okita plasmids into NHEK to examine the role of SIRT1 on reprogramming.

To confirm the expression of factors derived from the plasmids in the transfected cells, we monitored their mRNA levels using sets of plasmid-specific PCR primers as listed in Table 1. mRNA samples were isolated from transfected cells on days 3 and

7 after transfection. In Okita plasmids + pECE-SIRT1 transfected NHEKs, SIRT1 mRNA increased ~300 and ~900 fold on day 3 and day 7 after transfection, respectively, as compared to cells transfected with Okita plasmids + pECE cloning vector (Figure 3, Tables 2 and 3). The expression levels of OCT4, KLF4, SOX2, and L-MYC genes from cells transfected with Okita plasmids alone varied widely (Figures 4A, 4B and Tables 2 and 3, compare Okita plasmids vs. controls). On day 3 after transfection with Okita plasmids, the plasmid-encoded OCT4, KLF4, SOX2, and L-MYC were induced by 96, 261, 2678, and 499 folds, respectively, as compared to no plasmid controls (Table 2). On day 7 after transfection with Okita plasmids, the plasmid-encoded OCT4, KLF4, SOX2, and L-MYC were induced by 33, 402, 9548, and 314 folds, respectively, as compared to no plasmid controls (Table 3). Thus, the induction of OCT4 expression is the lowest and the induction of SOX2 expression is the highest. The expression of OCT4 and L-MYC declined from day 3 to day 7 while the expression of KLF4 and SOX2 kept increasing within the same time interval. The reason for these differences is unclear given they are all under the same strong CAG promoter control [38] (Figure 1).

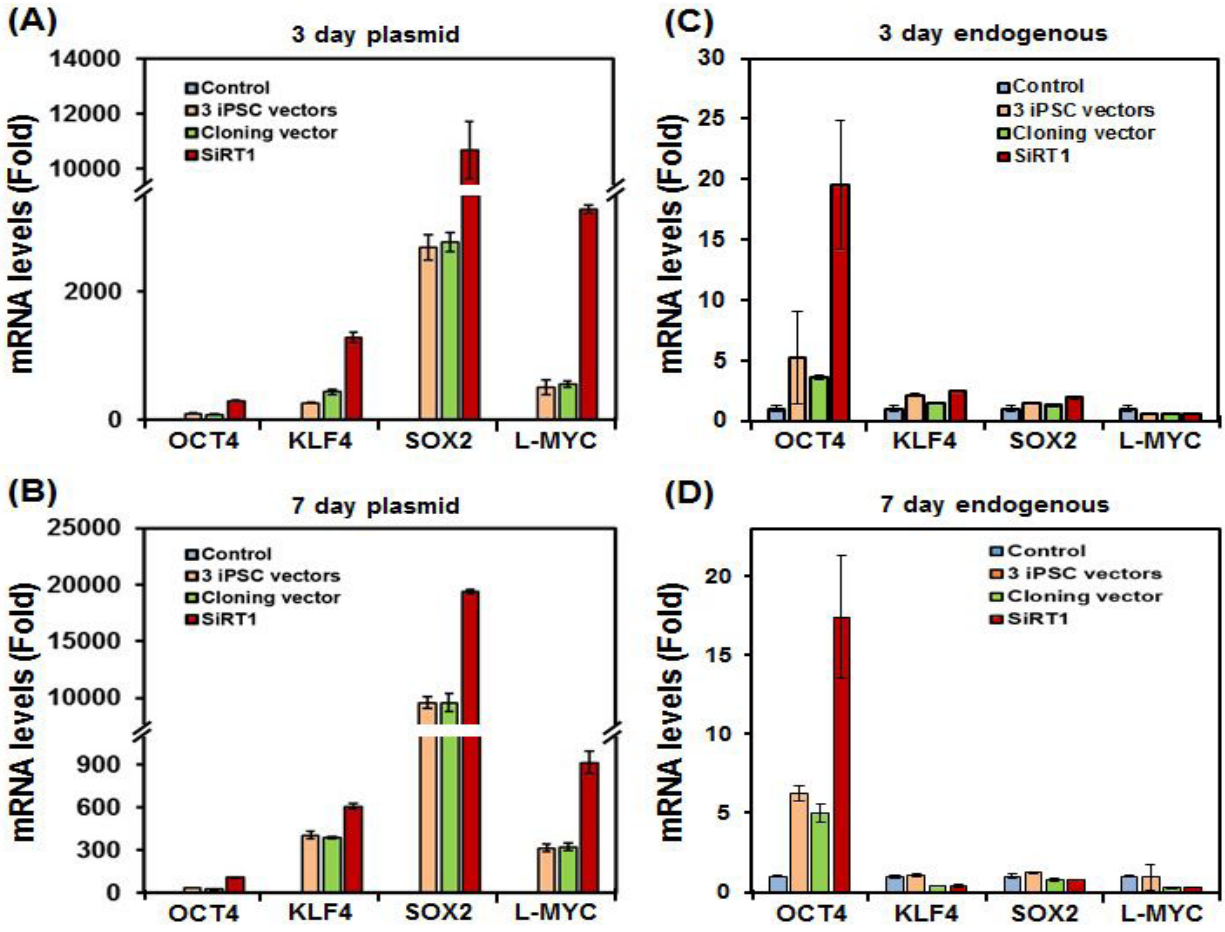

Figure 4: Gene expression of OCT4, KLF4, Sox2, and L-MYC in Okita-plasmid transfected NHEKs.

NHEKs were transfected with Okita plasmids only (orange), Okita plasmids with pECE (green), or Okita plasmids with pECE-SIRT1 (red) as outlined in Figure 1. Cells without plasmid transformation are used as controls (blue). RNA samples were isolated from cells on 3 or 7 days post transfection as indicated. RT-qPCR analyses were performed with respective primers (Table 1) to detect plasmid-derived mRNA (A and B) and endogenous mRNA (C and D). mRNA fold changes of indicated factors were calculated as described in Figure 3. Error bars indicate SD; n = 3.

SIRT1		4-Oct		KLF4		SOX2		L-MYC	
		plasmid	endo	plasmid	endo	plasmid	endo	plasmid	endo
Control	1	1	1	1	1	1	1	1	1
Okitaplasmids	0.7 ± 0	96 ± 14	5.2 ± 3.9	261 ± 13	2.1 ± 0.1	2678 ± 197	1.4 ± 0.1	499 ± 112	0.5 ± 0
Okitaplasmids + pECE	1.6 ± 0.1	78 ± 12	3.6 ± 0.2	427 ± 38	1.5 ± 0	2771 ± 149	1.3 ± 0.1	552 ± 49	0.5 ± 0
	(1)*	(1)*	(1)*	(1)*	(1)*	(1)*	(1)*	(1)*	(1)*
Okitaplasmids + ECE-SIRT1	486 ± 10	293 ± 10	20 ± 5	1276 ± 87	2.5 ± 0.1	10686 ± 1046	2.0 ± 0.1	3270 ± 64	0.6 ± 0
	(304)*	(3.8)*	(5.6)*	(3.0)*	(1.7)*	(3.9)*	(1.5)*	(5.9)*	(1.2)*
*The values in parenthesis represent mRNA fold changes of indicated factors when NHEK cells were transfected with Okita plasmids + pECE-SIRT1 as compared to Okita plasmids + pECE vector.									

Table 2: Relative gene expression on day 3 after transfection as shown in Figure 4A and 4C.

SIRT1		4-Oct		KLF4		SOX2		L-MYC	
		plasmid	endo	plasmid	endo	plasmid	endo	plasmid	endo
Control	1	1	1	1	1	1	1	1	1
Okitaplasmids	0.7 ± 0	33 ± 1	6.2 ± 0.5	402 ± 26	1.0 ± 0.1	9548 ± 515	1.2 ± 0.1	314 ± 26	0.9 ± 0.1
Okitaplasmids + pECE	1.0 ± 0	21 ± 2	5.0 ± 0.6	385 ± 6	0.4 ± 0	9558 ± 796	0.8 ± 0.1	318 ± 27	0.3 ± 0
	(1)*	(1)*	(1)*	(1)*	(1)*	(1)*	(1)*	(1)*	(1)*
Okitaplasmids + pECE-SIRT1	924 ± 167	104 ± 6	17 ± 4	607 ± 18	0.4 ± 0.1	19417 ± 190	0.8 ± 0	915 ± 81	0.3 ± 0
	(924)*	(5.0)*	(3.4)*	(1.6)*	(1)*	(2.0)*	(1)*	(2.9)*	(1)*
*The values in parenthesis represent mRNA fold changes of indicated factors when NHEK cells were transfected with Okita plasmids + pECE-SIRT1 as compared to Okita plasmids +pECE vector.									

Table 3: Relative gene expression on day 7 after transfection as shown in Figure 4B and 4C.

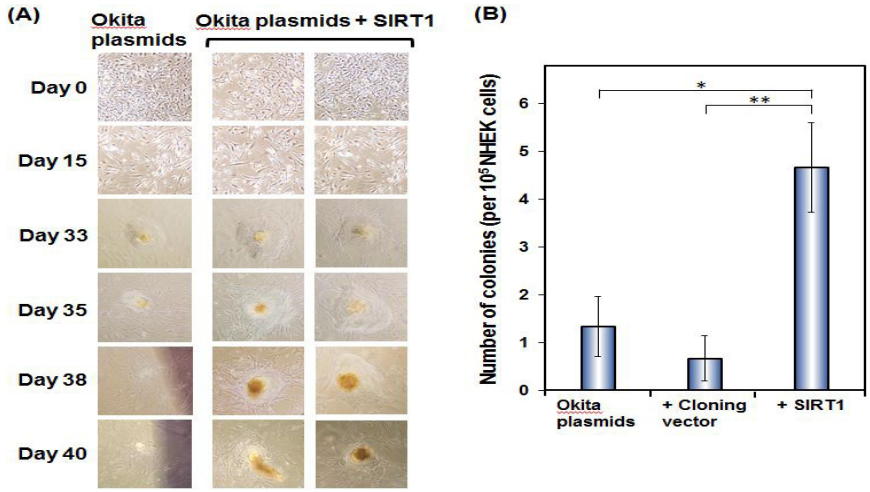

Figure 5: SIRT1 Enhances iPSC Generation.

NHEKs were transfected with Okita plasmids only, Okita plasmids + pECE, or Okita plasmids + pECE-SIRT1 as outlined in Figure 1.

(A) Images of one iPSC-like colony from Okita plasmids only and two iPSC-like colonies from Okita plasmids + pECE-SIRT1. Images of NHEKs at days 0 and 15 and iPSC colonies days 33-40 after transfection were shown.

(B) Numbers of iPSC-like colonies obtained per 105 NHEK cells seeded. Colony number was scored on day 30 after plasmid transfection. No colonies were observed from cells without Okita plasmid DNA transfection. Error bars indicate SD; n = 3. One and two stars indicate that the p-values are smaller than 0.05 and 0.01, respectively.

When SIRT1 was co-transfected with Okita plasmids in NHEK, the mRNAs of plasmid-derived OCT4, KLF4, SOX2, and L-MYC were further increased by 3.8, 3, 3.9, and 5.9 folds on day 3 after transfection (Figure 4 and Table 2, compare Okita plasmids + pECE-SIRT1 vs. Okitaplasmids + pECE). On day 7 after plasmid transfection, the plasmid-derived OCT4, KLF4, SOX2, and L-MYC were still induced by 5, 1.6, 2, and 2.9 folds in SIRT1 overexpressed cells (Figure 4 and Table 3). We also examined the endogenous gene expression of these four pluripotency regulators. Interestingly, the endogenous OCT4 was induced by 5.6 and 3.4 folds on days 3 and 7, respectively, while the other three endogenous gene expressions were not enhanced by SIRT1 overexpression (Figure 4C, 4D and Tables 2 and 3). These results indicate that SIRT1 can stimulate transcription of endogenous OCT4 gene and genes under the CAG promoter control.

SIRT1 Enhances iPSC Generation

NHEK cells transfected with the Okita plasmids were grown on top of mitomycin-treated MEF feeders for more than 30 days to generate iPSCs. At days 1 and 15, NHEK cells did not exhibit any change in morphology before and after transfection (Figure 5A). iPSC-like colonies (Figure 5A) were observed in Okita plasmid transfected cells but not in controls without plasmids. The number of iPSC-like colonies increased by7-fold from NHEK cells co-transfected with pECE-SIRT1 plasmid plus Okita plasmids as compared with that from cells co-transfected with cloning vector plus Okita plasmids (Figure 5B).

The iPSC-like colonies derived from pECE-SIRT1 plus Okita plasmids last longer than those derived from Okita plasmids alone (Figure 5A). To confirm that these colonies are bona fide iPSCs, we checked whether they express the key stem cell markers TRA-1-60 [39] and OCT4 [10,11]. The colonies were stained with Alexa Fluor 594 anti-human TRA-1-60-R antibody and OCT4 antibody followed by Alexa Fluor 488 goat anti-mouse IgG antibody. As observed under a fluorescence microscope, iPSC-like colonies express TRA-1-60-R (red fluorescence) and OCT4 (green fluorescence) (Figure 6), indicating they are iPSCs. Both MEF feeder cells and iPSC-like colonies are stained with DAPI, however, MEF feeder cells do not express TRA-1-60-R and OCT4.

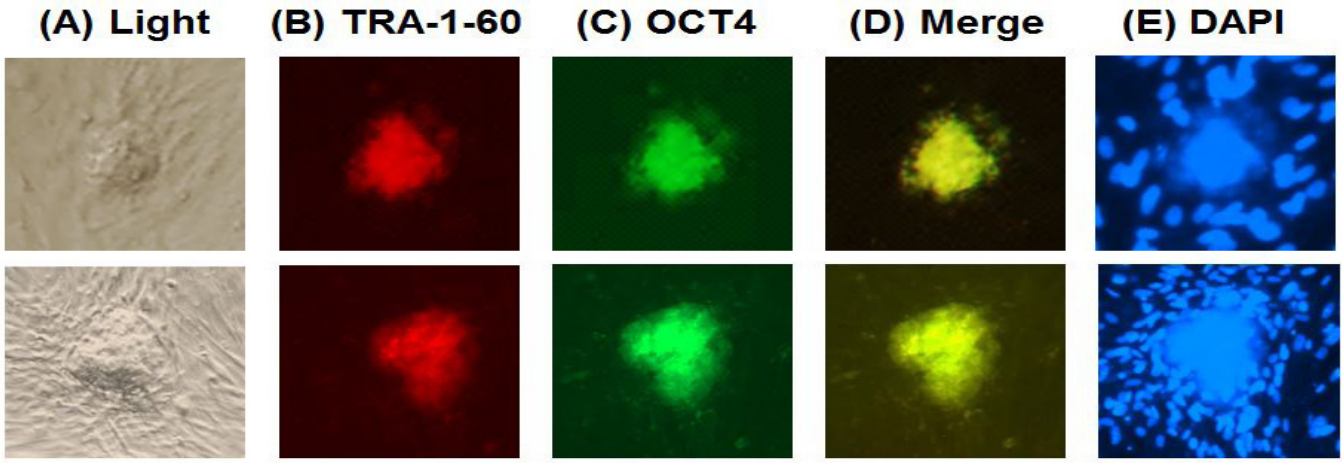

Figure 6: iPSC-like colonies express the key pluripotent stem cell markers.

Two iPSC-like colonies were viewed under a visible light (A) and immune-stained with antibody against TRA-1-60-R (B, red fluorescence) and OCT4 (C, green fluorescence). (D) is the merged image of (B) and (C). (E) is DAPI-stained image. Both MEF feeder cells and iPSC-like colonies are stained with DAPI while only iPSC-like colonies express TRA-1-60-R and OCT4.

Discussion

The therapeutic potential of iPSCs has been hampered by low reprogram efficiency, potential mutagenesis associated with viral-mediated reprogramming, and high genome instability. It has been shown that reprogramming can be facilitated by inhibitors of classes I/II HDACs and DNMTs [13-17]. SIRT1 is a potential reprogramming factor because it regulates acetylation levels of histones and many transcription factors (reviewed in [23]. In this report, we have shown that expression of SIRT1 can enhance endogenous OCT4 gene expression in human keratinocytes. In addition, SIRT1 overproduction enhances the transcription of all plasmid-derived reprogramming factors under the CAG promoter control. Moreover, iPSC-like colonies are generated more efficiently when SIRT1 is overexpressed. Our result of SIRT1 enhancement on human iPSC generation is consistent with previous reports that mouse Sirt1 can facilitate iPSC generation from mouse embryonic fibroblasts [32,35]. Our data indicate that SIRT1 enhances somatic cell reprogramming, in part, through enhancement of gene expression of endogenous OCT4 and ectopic transcription

factors.

However, the molecular mechanism by which SIRT1 enhances the OCT4 and CAG promoter activities is unclear. As a histone deacetylase, SIRT1 would act to silence gene transcription (reviewed in [23]). This is certainly not in line with our findings that SIRT1 activates endogenous OCT4 and CAG-regulated genes. It is possible that SIRT1 activates gene expression through deacetylating or stimulating transcription factors, p53, and DNA repair enzymes [45]. One likely scenario is that SIRT1 enhances the demethylation activity of Thymine DNA Glycosylase (TDG) and thus activates gene transcription because TDG can be deacetylated and stimulated by SIRT1 [46].

Since SIRT1 stimulates the expression of OCT4 and NANOG and exerts substantial enhancement on reprogramming (this report and [32,35,47]), future development using SIRT1 activators and small molecules [10,11] may eliminate the requirement of any transcription factors for iPSC production. One potential SIRT1 activator is resveratrol (a natural phenol) [48]. Indeed, resveratrol has been shown to promote iPSC formation [49,50]. However, its direct activation of SIRT1 has been refuted several times [51-53]. Ghosh et al.[54] have provided a mechanistic explanation to this controversy. These authors have shown that resveratrol activates SIRT1 by increasing its binding with lamin A, thus facilitating SIRT1 localization to nuclear matrix. They have also shown that the rescue of adult stem cell decline by resveratrol is SIRT1-dependent. Resveratrol may be combined with Valproic Acid (VPA, a class I/II HDAC inhibitor) and/or 5-azacytidine (azaC, a DNMT inhibitor) [13-17] to achieve optimal reprogramming.

Our novel approaches will not only increase the efficiency of iPSC induction but also generate iPSCs with high genomic stability. The genomic stability of iPSCs should be stringently maintained for the safety in tissue replacement applications [8]. SIRT1 plays a pivotal role in controlling genomic stability by deacetylating DNA repair enzymes and p53 [24-28,45]. By overproducing SIRT1 or using SIRT1 activators, the derived iPSCs should be highly proliferative, tolerant to oxidative stress and DNA damage, and highly similar to embryonic stem cells. Our finding will take steps closer to generate high quality iPSCs for regenerative medicine.

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References

1. Amabile G, Meissner A (2009) Induced pluripotent stem cells: current progress and potential for regenerative medicine. Trends Mol. Med 15: 59-68.
2. Okita K, Yamanaka S (2010) Induction of pluripotency by defined factors. Exp. Cell Res 316: 2565-2570.
3. Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126: 663-676.
4. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131: 861-872.
5. Yamanaka S, Blau HM (2010) Nuclear reprogramming to a pluripotent state by three approaches. Nature 465: 704-712.
6. Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, et al. (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318: 1917-1920.
7. Okita K, Yamanaka S (2011) Induced pluripotent stem cells: opportunities and challenges. Philos. Trans. R. Soc. Lond B Biol. Sci 366: 2198-2207.
8. Maynard S, Swistowska AM, Lee JW, Liu Y, Liu ST, et al. (2008) Human embryonic stem cells have enhanced repair of multiple forms of DNA damage. Stem Cells 26: 2266-2274.
9. Nagaria P, Robert C, Rassool FV (2013) DNA double-strand break response in stem cells: mechanisms to maintain genomic integrity. Biochim. Biophys Acta 1830: 2345-2353.
10. Li Y, Zhang Q, Yin X, Yang W, Du Y, et al. (2011) Generation of iPSCs from mouse fibroblasts with a single gene, Oct4, and small molecules. Cell Res 21: 196-204.
11. Zhu S, Li W, Zhou H, Wei W, Ambasudhan R, et al. (2010) Reprogramming of human primary somatic cells by OCT4 and chemical compounds. Cell Stem Cell 7: 651-655.
12. Singhal N, Graumann J, Wu G, Arauzo-Bravo MJ, Han DW, et al. (2010) Chromatin-remodeling components of the BAF complex facilitate reprogramming. Cell 141: 943-955.
13. Gottlicher M, Minucci S, Zhu P, Kramer OH, Schimpf A, et al. (2001) Valproic acid defines a novel class of HDAC inhibitors inducing differentiation of transformed cells. EMBO J 20: 6969-6978.
14. Huangfu D, Osafune K, Maehr R, Guo W, Eijkelenboom A, et al. (2008) Induction of pluripotent stem cells from primary human fibroblasts with only Oct4 and Sox2. Nat. Biotechnol 26: 1269-1275.
15. Huangfu D, Maehr R, Guo W, Eijkelenboom A, Snitow M, et al. (2008) Induction of pluripotent stem cells by defined factors is greatly improved by small-molecule compounds. Nat. Biotechnol 26: 795-797.
16. Mikkelsen TS, Hanna J, Zhang X, Ku M, Wernig M, et al. (2008) Dissecting direct reprogramming through integrative genomic analysis. Nature 454: 49-55.
17. Tsuji-Takayama K, Inoue T, Ijiri Y, Otani T, Motoda R, et al. (2004) Demethylating agent, 5-azacytidine, reverses differentiation of embryonic stem cells. Biochem. Biophys. Res. Commun 323: 86-90.
18. Bar-Nur O, Brumbaugh J, Verheul C, Apostolou E, Pruteanu-Malinici I, et al. (2014) Small molecules facilitate rapid and synchronous iPSC generation. Nat. Methods 11: 1170-1176.
19. Kim D, Kim CH, Moon JI, Chung YG, Chang MY, et al. (2009) Generation of human induced pluripotent stem cells by direct delivery of reprogramming proteins. Cell Stem Cell 4: 472-476.

20. Stadtfeld M, Nagaya M, Utikal J, Weir G, Hochedlinger K (2008) Induced pluripotent stem cells generated without viral integration. Science 322: 945-949.

21. Warren L, Manos PD, Ahfeldt T, Loh YH, Li H, et al. (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell 7: 618-630.

22. Okita K, Matsumura Y, Sato Y, Okada A, Morizane A, et al. (2011) A more efficient method to generate integration-free human iPS cells. Nat. Methods 8: 409-412.

23. Michan S, Sinclair D (2007) Sirtuins in mammals: insights into their biological function. Biochem. J 404: 1-13.

24. Chen Y, Zhao W, Yang JS, Cheng Z, Luo H, et al. (2012) Quantitative acetylome analysis reveals the roles of SIRT1 in regulating diverse substrates and cellular pathways. Mol. Cell Proteomics 11: 1048-1062.

25. Guarente L (2011) Sirtuins, aging, and metabolism. Cold Spring Harb. Symp. Quant. Biol 76: 81-90.

26. Peng L, Yuan Z, Ling H, Fukasawa K, Robertson K, et al. (2011) SIRT1 deacetylates the DNA methyltransferase 1 (DNMT1) protein and alters its activities. Mol. Cell Biol 31: 4720-4734.

27. Yamamori T, DeRicco J, Naqvi A, Hoffman TA, Mattagajasingh I, et al. (2010) SIRT1 deacetylates APE1 and regulates cellular base excision repair. Nucleic Acids Res 38: 832-845.

28. Yuan Z, Zhang X, Sengupta N, Lane WS, Seto E (2007) SIRT1 regulates the function of the Nijmegen breakage syndrome protein. Mol. Cell 27: 149-162.

29. Kim EJ, Um SJ (2008) SIRT1: roles in aging and cancer. BMB. Rep 41: 751-756.

30. Yu J, Auwerx J (2010) Protein deacetylation by SIRT1: an emerging key post-translational modification in metabolic regulation. Pharmacol. Res 62: 35-41.

31. Calvanese V, Lara E, Suarez-Alvarez B, Abu DR, Vazquez-Chantada M, et al. (2010) Sirtuin 1 regulation of developmental genes during differentiation of stem cells. Proc. Natl. Acad. Sci. U. S. A 107: 13736-13741.

32. Lee YL, Peng Q, Fong SW, Chen AC, Lee KF, et al. (2012) Sirtuin 1 facilitates generation of induced pluripotent stem cells from mouse embryonic fibroblasts through the miR-34a and p53 pathways. PLoS. One7: e45633.

33. McBurney MW, Yang X, Jardine K, Hixon M, Boekelheide K, et al. (2003) The mammalian SIR2alpha protein has a role in embryogenesis and gametogenesis. Mol. Cell Biol 23: 38-54.

34. Saunders LR, Sharma AD, Tawney J, Nakagawa M, Okita K, et al. (2010) miRNAs regulate SIRT1 expression during mouse embryonic stem cell differentiation and in adult mouse tissues. Aging (Albany. NY) 2: 415-431.

35. Mu WL, Wang YJ, Xu P, Hao DL, Liu XZ, et al. (2015) Sox2 deacetylation by Sirt1 Is Involved in Mouse Somatic Reprogramming. Stem Cells 33: 2135-2147.

36. Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, et al. (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor. Cell 95: 379-391.

37. Radzisheuskaya A, Chia GB, dos Santos RL, Theunissen TW, Castro LF, et al. (2013) A defined Oct4 level governs cell state transitions of pluripotency entry and differentiation into all embryonic lineages. Nat. Cell Biol 15: 579-590.

38. Niwa H, Yamamura K, Miyazaki J (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108: 193-199.

39. Pera MF, Reubinoff B, Trounson A (2000) Human embryonic stem cells. J. Cell Sci 113: 5-10.

40. Buganim Y, Faddah DA, Jaenisch R (2013) Mechanisms and models of somatic cell reprogramming. Nat. Rev. Genet 14: 427-439.

41. Strahl BD, Allis CD (2000) The language of covalent histone modifications. Nature 403: 41-45.

42. Kurdistani SK, Grunstein M (2003) Histone acetylation and deacetylation in yeast. Nat. Rev. Mol. Cell Biol 4: 276-284.

43. Minucci S, Pelicci PG (2006) Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat. Rev. Cancer 6: 38-51.

44. Aasen T, Raya A, Barrero MJ, Garreta E, Consiglio A, et al. (2008) Efficient and rapid generation of induced pluripotent stem cells from human keratinocytes. Nat. Biotechnol 26: 1276-1284.

45. Alcain FJ, Villalba JM (2009) Sirtuin inhibitors. Expert. Opin. Ther. Pat 19: 283-294.

46. Madabushi A, Hwang BJ, Jin J, Lu AL (2013) Histone deacetylase SIRT1 modulates and deacetylates DNA base excision repair enzyme thymine DNA glycosylase. Biochem J 456: 89-98.

47. Han MK, Song EK, Guo Y, Ou X, Mantel C, et al. (2008) SIRT1 regulates apoptosis and Nanog expression in mouse embryonic stem cells by controlling p53 subcellular localization. Cell Stem Cell 2: 241-251.

48. Borra MT, Smith BC, Denu JM (2005) Mechanism of human SIRT1 activation by resveratrol. J. Biol. Chem 280: 17187-17195.

49. Chen T, Shen L, Yu J, Wan H, Guo A, et al. (2011) Rapamycin and other longevity-promoting compounds enhance the generation of mouse induced pluripotent stem cells. Aging Cell 10: 908-911.

50. Ding DF, Li XF, Xu H, Wang Z, Liang QQ, et al. (2013) Mechanism of resveratrol on the promotion of induced pluripotent stem cells. JIntegr Med 11: 389-396.

51. Huber JL, McBurney MW, DiStefano PS, McDonagh T (2010) SIRT1-independent mechanisms of the putative sirtuin enzyme activators SRT1720 and SRT2183. Future Med Chem 2: 1751-1759.

52. Pacholec M, Bleasdale JE, Chrunyk B, Cunningham D, Flynn D, et al. (2010) SRT1720, SRT2183, SRT1460, and resveratrol are not direct activators of SIRT1. J BiolChem 285: 8340-8351.

53. Stunkel W, Campbell RM (2011) Sirtuin 1 (SIRT1): The misunderstood HDAC. J Biomol Screen 16: 1153-1169.

54. Ghosh S, Liu B, Zhou Z (2013) Resveratrol activates SIRT1 in a Lamin A-dependent manner. Cell Cycle 12: 872-876.