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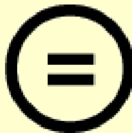

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The Role of *CIIA* gene in Cellular Reprogramming

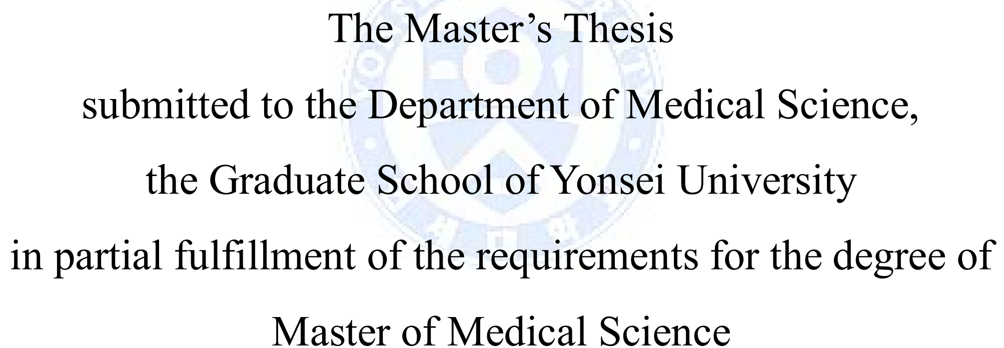
Eunhyun Ji

Department of Medical Science

The Graduate School, Yonsei University

The Role of *CIIA* gene in Cellular Reprogramming

Directed by Professor Dong-Wook Kim



The Master's Thesis
submitted to the Department of Medical Science,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of
Master of Medical Science

Eunhyun Ji

June 2015

This certifies that the Master's Thesis of Eunhyun Ji is approved

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The Graduate School
Yonsei University

June 2015

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Most importantly, I would love to thank my parents for their faith on me. My academic career would have not been possible without their pray. I especially thank my sister for all her advice and support. My sister has been my best friend all my life and I love her dearly and thank her.

And, I thank God for his love and guidance all the time.

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ABSTRACT

The role of *CIIA* gene in cellular reprogramming

Eunhyun Ji

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(Directed by Professor Dong-Wook Kim)

Terminally differentiated cells are known to be reprogrammed into induced pluripotent stem cells (iPSCs) when Yamanaka factors (Oct4, Sox2, klf4 and cmyc; OSKM) are introduced. In recent years, iPSC technology has been applied to improve the efficiency and safety of clinical applications. This study demonstrates that knockdown of the CIIA gene (a caspase-activated DNase inhibitor that interacts with ASK1) can enhance reprogramming efficiency in human fibroblasts. Short-term repression of CIIA by siRNA transfection in fibroblasts reduced epithelial-mesenchymal transition (EMT) in the early stage of reprogramming. On the other hand, prolonged suppression of CIIA through shRNA knocking-down induced epigenetic modifiers and pluripotent genes in the late stage of cellular reprogramming. At the transcriptional level, CIIA also acts as an inhibitory influence on the reprogramming process through a histone modification factor, Sirt1. In addition, fibroblasts via knocking-down of CIIA and transfection of OSKM produced greater numbers of embryonic stem cell (ESC)–like colonies, compared with those with OSKM transfection alone. Collectively, these results show that the

CIIA gene plays a role in cell fate change and provide a new mechanistic framework to better understand the effects of CIIA in cellular reprogramming.

Key words: induced pluripotent stem cells, CIIA, EMT, epigenetic modification, cellular reprogramming

The role of *CIIA* gene in cellular reprogramming

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I. INTRODUCTION

Differentiated cells can be reprogrammed into an embryonic stem cell (ESC)–like state by specific transcription factors, originally designated as Oct4, Sox2, klf4, and cMyc (OSKM).[1,2] These reprogrammed cells, called induced pluripotent stem cells (iPSCs), are promising cell sources for cellular therapy and disease modeling.[3] The reason for this is that, compared with the use of ESCs, the use of iPSCs for therapeutic applications both avoids the ethical concerns of using embryos and solves the immune rejection problem by using patient-specific iPSCs that already match the patient's own system.[4,5] However, in spite of the rapid advance of iPSC technology, the challenges that still remain are low reprogramming efficiencies and the extended time required when reprogramming.[6,7] Thus, extensive knowledge of the reprogramming mechanism has been reported in order to obtain iPSCs more efficiently. In previous studies, two critical changes have been primarily considered: a mesenchymal-to-epithelial transition (MET)[8] and an epigenetic modification.[9,10] In the early stage, an MET, the process of switching cell fate, is required for cellular reprogramming. Therefore, discovering inhibitors of EMT or MET inducers was one of previous strategies.[8,11-13] In the late stage,

reprogramming intermediates undergo widespread epigenetic changes, including DNA methylation and histone modification, that are essential for the conversion of somatic cells to fully reprogrammed iPSCs.[9,10,14,15] Researchers also continue to uncover epigenetic modifiers that induce cellular reprogramming.[16-19]

Despite these efforts, few somatic cells form bona fide iPSC colonies: [14,17,18,20] the rest of the cells, on the other hand, fail to be fully reprogrammed because barriers interfere with the overall flow of reprogramming, including those two major cellular modifications. Therefore, this study has aimed to identify a factor that regulates those two changes during cellular reprogramming. In examining cellular and molecular changes during reprogramming, I noticed that this is extremely similar to the circumstance of down-regulation of CIIA expression in somatic cells. CIIA was initially discovered as an anti-apoptotic protein[21] that proved to be identical to VPS28. Aside from these initial studies on the role of CIIA, previous studies have reported that caspase activity is regulated by the expression of CIIA.[22] Furthermore, subsequent studies have shown that CIIA induces EMT via down-regulation of claudin-1 and is involved in TGF-β-induced migration, which is a representative EMT feature.[23] Given earlier studies about the function of CIIA, I hypothesized that CIIA potentially plays a significant role in cell fate determination as well as cell survival.

This study discovered that CIIA is a barrier to the conversion of *bona fide* iPSCs. Suppressed CIIA expression affects reprogramming to iPSCs, showing the change of EMT genes and epigenetic modifiers. Moreover, Sirt1, as one of the epigenetic modifiers, which was known as a regulator of histone modification or DNA methylation, was especially observed with increased expression in CIIA down-regulated human fibroblasts. Combining OSKM induction with CIIA inhibition in human fibroblasts revealed enhanced reprogramming efficiency. In addition to this, this study identified a molecular mechanism underlying the CIIA-Sirt axis in the early and late stages of reprogramming. This mechanism also opened

epigenetic marks of key reprogramming factors. These results suggest that the CIIA-Sirt regulatory axis is a specific point of concern during reprogramming.

II. MATERIALS AND METHODS

1. Culture of human ES cells

Human ES cell line, H9 (p37-49, WiCell Inc., Madison, Wisconsin, USA) was routinely cultured in Knockout-Serum Replacement (KSR) medium containing Dulbecco's modified Eagle's medium (DMEM)/F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 20% KSR (Invitrogen), 1X nonessential amino acid (Invitrogen), 0.1 mM beta-mercaptoethanol (Sigma, St. Louis, MO, USA), and 4 ng/ml of basic fibroblast growth factor (bFGF) (Invitrogen) on the layer of mitomycin-C (Sigma) treated mouse STO feeder cells (ATCC, Manassas, VA, USA). Human ES cell colonies were transferred onto the fresh feeder cells weekly by mechanical passaging.

2. Culture of fibroblasts

BJ human fibroblasts (ATCC, Manassas, VA, USA) were cultured in MEM with 10% FBS and 1% Glutamax. 293T cells (Invitrogen) were cultured in DMEM supplemented with FBS (10% vol/vol) and antibiotics (1%).

3. siRNA transfection

Two days prior to electroporation BJ human fibroblasts or human ES cells were plated onto 60mm culture plates. The next day cells were electroporated with the Neon™ transfection system (Invitrogen) according to the manufacturer's instructions. In each electroporation reaction, cells were electroporated with 100uM control siRNA or CIIA siRNA (sense CCU GGG AAC AAG CCG GAG CUG UAU GAG GAU U, antisense UCC UCA UAC AGC UCC GGC UUG UUC CCA GGU U). The conditions used for electroporation were 1150 V pulse voltage, 20 ms pulse width and three pulses for BJ fibroblasts and 1100 V pulse voltage, 30

ms pulse width and one pulse for hESCs. After the electroporation, each reaction was plated onto new 60mm culture plates containing BJ fibroblast or hESC medium.

4. Lentiviral production and shRNA transduction

shRNA fragments were obtained from Origene (Plasmid TL300557: pGFP-C-Lenti puro shCIIA, Rockville, MD, USA). shRNA fragments were obtained from Origene (Plasmid TL300557: pGFP-C-Lenti puro shCIIA, Rockville, MD, USA). Vectors were produced by transient transfection in 293FT cells. The day before transfection, plate 293FT cells in a 10 mm culture plate so that they would be 70–75% confluent on the day of transfection. On the day of transfection, remove the culture medium from the 293FT cells and replace with 7ml of growth medium (without antibiotics). For each transfection sample, 5 μg of the virus construct were mixed with 1 μg of the VSV-G envelope plasmid (pLP/VSVG, Invitrogen) and 4 μg of the packaging plasmid (pLP1 and pLP2, Invitrogen). The solution was adjusted to 200 μl with Opti-MEM® (Invitrogen) and mixed with 10 μl of 1 mg/ml polyethyleneimine (PEI, Sigma). The mixture of DNA and solution incubate for 15 minutes at room temperature. Add the mixture dropwise to each plate of cells. Mix gently by rocking the plate back and forth. Incubate the cells overnight at 37 °C in a humidified 5 % CO_2 incubator. The next day, remove the medium containing the DNA and PEI solution and replace with 10 ml complete culture medium without antibiotics. Incubate at 37 °C in a humidified 5 % CO_2 incubator. Harvest virus-containing supernatant 72–96 hours post-transfection into a 15 ml conical tube, centrifuge briefly (3000rpm for 10 min) and filter through a 0.45 μm filter. Next, clarified supernatant transfer to a sterile container and combine 1 volume of 40% PEG solution with 3 volumes of clarified supernatant and mix by gentle inversion. The mixed supernatants incubate at 4 °C for overnight. The samples centrifuge at 24,000 rpm for 1h 30minutes at 4 °C. After centrifugation, carefully remove supernatant. At last, gently resuspend the pellet in proper volume using phosphate-

buffered saline (PBS) at 4 °C for overnight. Lentivirus containing KD constructs was transduced and stable cell lines were selected by addition of Puro at 1ug/ml on day 2 following transduction, continuing for at least 1 week.

5. Lentiviral overexpression

pLVX-tight Puro-GFP based vectors expressing CIIA, or a control empty vector were used to generate virus, and transduced as above.

6. Semi-quantitative reverse transcription- and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) assay

Total RNA was extracted using an Easy-Spin® total RNA purification kit (iNtRON Biotechnology, Seoul, Korea) according to the manufacturer's instructions. One microgram of RNA was reverse transcribed to cDNA with iScript cDNA Synthesis Kit (Bio-Rad). qRT-PCR was performed using SYBR® Premix Ex Taq™ (TAKARA BIO Inc., Otsu, Japan) on the CFX96 Real-Time System (Bio-Rad) under the following conditions; 1 minute at 95 °C; 32 cycles of 15 seconds at 95 °C, 20 seconds at 62 °C, 20 seconds at 72 °C; and 15 seconds extension at 95 °C. β-actin transcript was used as an endogenous reference to calculate Ct values and relative expression level (value of $2^{-\Delta\Delta Ct}$) of target genes according to Bio-Rad's instruction. All treated samples are represented as the expression level of the gene relative to their corresponding untreated control (control value equals to one). For semi-quantitative RT-PCR, PCR reactions were carried out with 2X EmeraldAmp® GT PCR Master Mix (TAKARA BIO Inc.) and 10 pM of each primer. Samples were amplified in a GeneAmp PCR System 2700 (Applied Biosystems, Carlsbad, CA, USA) under the following conditions: denaturing step at 95 °C for 30 seconds, annealing step at 60 °C for 30 seconds, and amplification step at 72 °C for 30 seconds for 20–35 cycles. cDNA templates were normalized based on the GAPDH-specific signal. The primer sequences are shown in Table1.

TABLE 1. Primer sequence used in this study

Name		Sequence
CIIA	F	ATG TCG GCG TCA GAT GAG
	R	CGG TTG AAG GCG TTG TAG
Oct4	F	CCT CAC TTC ACT GCA CTG TA
	R	CAG GTT TTC TTT CCC TAG CT
Sox2	F	CCC AGC AGA CTT CAC ATG T
	R	CCT CCC ATT TCC CTC GTT TT
Nanog	F	AGC CCC AGC TCC AGT TTC AGC
	R	AAT GAT CGT CAC ATA TCT TCA GGC
c-Myc	F	TAT TCT GCC CAT TTG GGG ACA
	R	TTG GTG AAG CTA ACG TTG AGG
N-cadherin	F	CCC TGC TTC AGG CGT CTG TA
	R	TGC TTG CAT AAT GCG ATT TCA CC
ZEB1	F	ATG CAC AAC CAA GTG CAG AAG AGC
	R	TTG CCT GGT TCA GGA GAA GAT GGT
Snail	F	GGA AGC CTA ACT ACA GCG AGC T
	R	GCT GGA AGG TAA ACT CTG GAT TAG
TGFβR2	F	TGT TGA GCT CTT CAA GCA GAC CGA
	R	ACT TCT CCC ACT GCA TTA CAG CGA
Sirt1	F	TTG GTG AAG CTA ACG TTG AGG
	R	TAC AGC AAG GCG AGC ATA
TET1	F	GAA CCA TTG GAT TCA CTC AGC TTA
	R	TCA CCG TTA ACT GTA CCT GAG AAT
BRG1(SMACA4)	F	CCT AAC CCA CCC AAC CTC
	R	ACT GCT GCT GTC CTT GTA
BAF155(SMARCC1)	F	TGA TAA AGC ACA AGA TGG AGA AA
	R	TTG GTA TCC TCA CTC ACT TCA
BAF60A(SMRACD1)	F	GAG CGG GAG TTT GTC ATC
	R	CTG GTG GCA TAA GCA AGG
Actin	F	TTG CCG ACA GGA TGC AGA AGG A
	R	AGG TGG ACA GCG AGG CCAGGA T

7. *Alkaline Phosphatase staining and Immunocytochemistry*

Alkaline phosphatase staining was performed following the manufacturer's recommended procedure (Sigma-Aldrich). Cells were fixed with 4 % paraformaldehyde in PBS for 15 minutes, washed with PBS, and perforated with PBS containing 0.1 % Triton X-100 for 10 minutes. Then samples were incubated with blocking buffer [PBS containing 2 % bovine serum albumin (BSA)] for 1 hour. Cells were incubated at 4 °C with primary antibodies diluted in PBS containing 2 % BSA for overnight. The following primary antibodies were used: rabbit anti-Oct3/4 (1:200, Santa-Cruz Biotechnology, Santa-Cruz, CA, USA), mouse anti-VPS28 (1:250, Abcam, Cambridge, UK). The samples washed 3 times for 5 minutes with PBS and then incubated fluorescent-labeled secondary antibodies [Alexa Fluor 488 (green) or Alexa fluor 594 (red)-labeled donkey/goat IgG (1:1000; Molecular Probes, Invitrogen) in PBS with 2 % BSA for 1 hr at room temperature. The coverslips were rinsed for 3 times for 10 minutes each in PBS and mounted onto slides using VECTASHIELD Hardset mounting medium with DAPI (Vector laboratories, Buringame, CA, USA). Images were obtained under a fluorescence microscope ECLIPSE, Ti-U (Nikon, Tokyo, Japan).

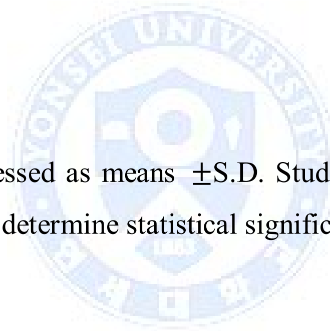
8. Chromatin Immunoprecipitation (ChIP) assay

For ChIP assays, 293T cells (Invitrogen) were cultured in DMEM supplemented with FBS (10% vol/vol) and antibiotics (1%), at which point the cells were fixed with formaldehyde for 10 min, and then 0.125 M glycine was added to stop the reaction. The cells were washed twice with PBS. The ChIP assays were performed with a ChIP kit commercially obtained from R&D systems according to the manufacturer's instructions. Briefly, the fixed cells were lysed to shear the DNA to 200~500 bp. The cross-linked complexes (DNA-protein) were immunoprecipitated with anti-H3K4me3 (Active Motif), and normal goat IgG. ChIP-enriched DNA was quantified by qRT-PCR, and primer pairs were Oct4

promoter region.

9. Generation and Maintenance of iPSCs

Episomal vectors encoding defined reprogramming factors were used as reported.[24] In brief, BJ fibroblasts were electroporated by using a microporator system (Neon; Invitrogen) with episomal vector mixtures (total 3 μg) according to the manufacturer's instructions. After being pulsed three times with 1150 voltages for 20 ms, the cells were grown further in BJ medium. Seven days after transfection, cells were transferred onto a feeder layer and grown in iPSC medium. iPSC colonies that looked similar to hESCs were picked up mechanically and further cultured.



10. Statistics

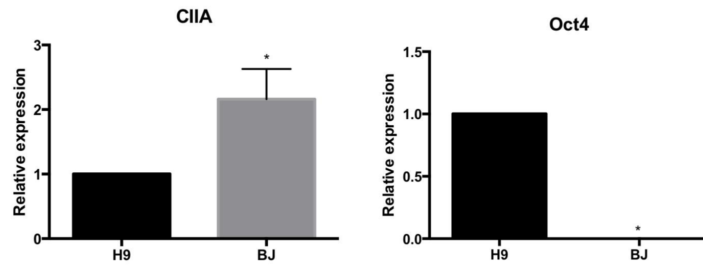
Values were expressed as means ±S.D. Student's t-test and ANOVA test using the Prism 6.0 used to determine statistical significance.

III. RESULTS

1. Difference in expression level of CIIA between undifferentiated H9 cells and differentiated BJ fibroblasts

The previous findings suggested that tight regulation of CIIA is essential for proper embryonic development as well as the growth and function of cells and tissues.[22,25] In the present study, I hypothesized that CIIA potentially plays an important role for cell fate determination. To investigate the role of CIIA in undifferentiated H9 cells and differentiated BJ fibroblasts, I performed qRT-PCR analysis targeting CIIA. The expression of CIIA was low in H9 hESCs, but BJ fibroblasts showed elevated levels of CIIA compared with H9 hESCs. In contrast, the expression level of Oct4, an important factor for pluripotency, was predominantly expressed in H9 hESCs compared with BJ fibroblasts (Fig. 1A). To further examine whether CIIA is a negative regulator in the maintenance of hESCs, I investigated its expression in hESCs inducing differentiation with retinoic acid (RA) for 4 days.[26] qRT-PCR analysis revealed that during spontaneous differentiation, CIIA mRNA time-dependently increased for 4 days, while the expression of the pluripotency-related genes Oct4, Sox2, and Nanog decreased (Fig. 1B). Consistently, immunofluorescence staining confirmed elevated expression of CIIA and downregulated expression of Oct4 during RA-induced differentiation of H9 hESCs (Fig. 1C). To further determine the role of CIIA in hESCs, CIIA-specific siRNA to knockdown CIIA mRNA expression was transfected into H9 hESCs. As shown via qRT-PCR analysis, knockdown of CIIA expression increased Oct4 expression in H9 hESCs (Fig. 1D). The results indicate that CIIA is required for the differentiation of hESCs and its suppression might play a positive role in cellular reprogramming.

A



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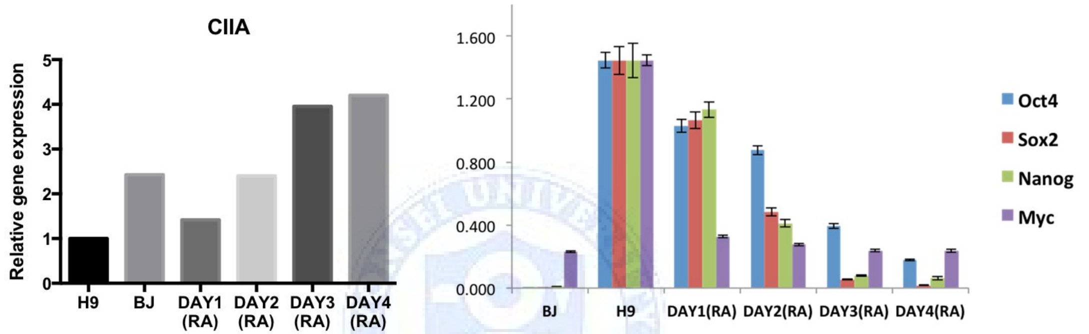

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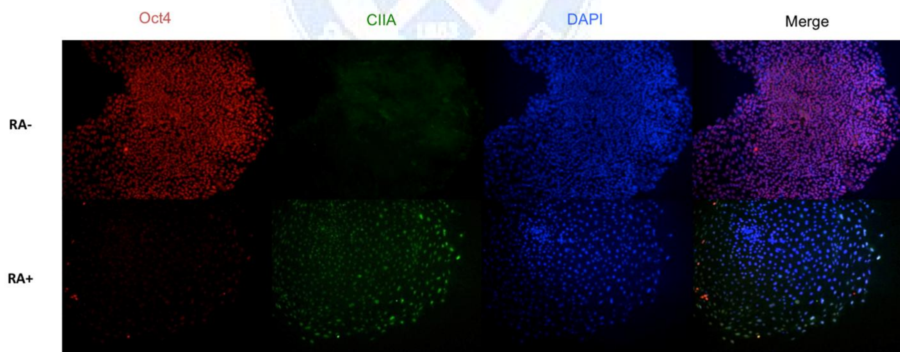

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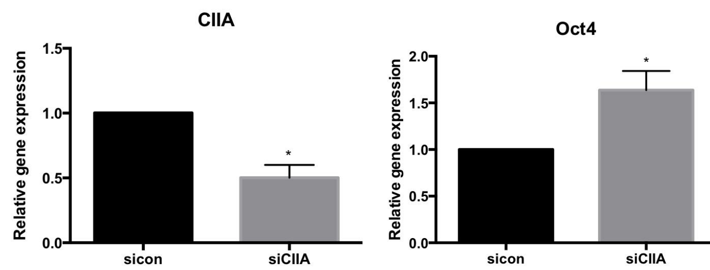


Figure 1. CIIA is required for the differentiation of hESCs *in vitro*. (A) qRT-PCR analysis targeting CIIA and Oct4 in undifferentiated H9 cells and differentiated BJ fibroblasts. (B) Time-dependent mRNA expression of pluripotent genes such as Oct4, Sox2, and Nanog, as well as CIIA in H9 hESCs after RA treatment for 4 days. (C) Immunocytochemical detection of Oct4 and CIIA in H9 cells after treatment with or without RA for 4 days. D0 (RA-) is the undifferentiated control. (D) qRT-PCR analysis targeting Oct4 and CIIA in H9 cells after transfection with control siRNA or CIIA siRNA on Day 3.

2. Effects of suppressed CIIA in human fibroblasts on reprogramming process

Previous studies have demonstrated that the first noticeable change during reprogramming of fibroblasts is their transformation into MET, the opposite of EMT.[8] It is also known that CIIA induces EMT through a reduction in the abundance of E-cadherin and claudin-1.[23] To identify whether downregulation of CIIA suppresses EMT, I performed transfection of CIIA siRNA into BJ fibroblasts and then qRT-PCR analysis targeting key transcripts related to the EMT process. It was confirmed that CIIA was knocked down initially by qRT-PCR (Fig. 2A). BJ fibroblasts transfected with CIIA siRNA showed epithelial-like morphological changes and also displayed decreased levels of EMT-related genes such as N-cadherin, ZEB1, and Snail (Fig. 2B-D). At the same time, the expression level of TGF-βR2, an EMT regulatory factor, decreased following knockdown of CIIA (Fig. 2E). Noticeably, suppression of CIIA in BJ fibroblasts elevated the expression of Klf4, which is an essential factor for reprogramming and an inducer of MET by direct activation of E-cadherin promoter. These results suggest that suppressed CIIA in human fibroblasts decreases EMT-related regulatory factors, which is the phenomenon occurring during the initial stage of reprogramming. This leads to the hypothesis that knockdown of CIIA might play a positive role on reprogramming.

To further analyze the role of CIIA, prolonged suppression of CIIA by four different CIIA shRNAs was performed in BJ fibroblasts. One week after transduction of lentivirus-producing CIIA shRNAs, it was confirmed by qRT-PCR analysis that CIIA had been knocked down (Fig. 3A). BJ fibroblasts expressing CIIA shRNAs showed decreased levels of EMT-related genes such as N-cadherin and Snail (Fig. 3B,C), and this was consistent with the result of transient knockdown experiments. Furthermore, to determine whether prolonged suppression of CIIA affects the late stage of reprogramming, I examined the expression of epigenetic modifiers involved in reprogramming. The induction of Sirt1, TET1 and BRG1 that function as epigenetic modifiers during reprogramming was observed

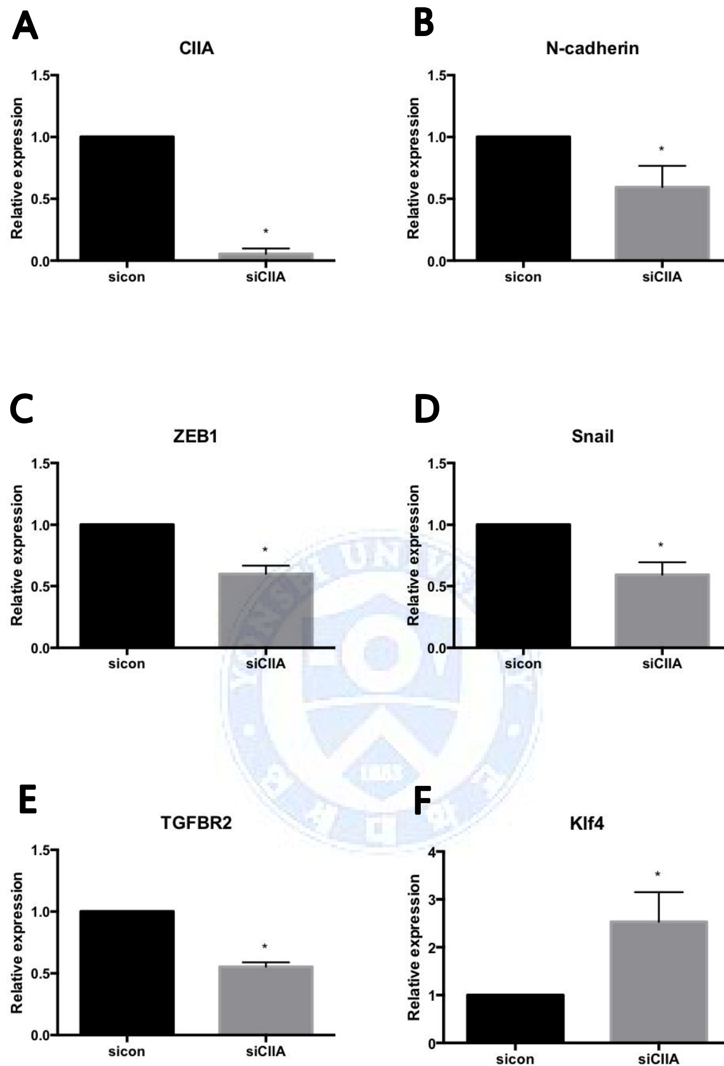
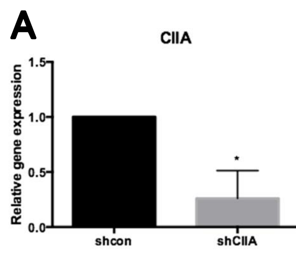


Figure 2. Downregulation of CIIA expression inhibits the expression of EMT-related genes. (A-F) Transient down-regulation of CIIA expression inhibits the levels of EMT-related genes: N-cadherin, Snail, and ZEB1. qRT-PCR analysis for the indicated genes at Day 3 after transfection of scrambled control siRNA or CIIA siRNA in BJ fibroblasts. qRT-PCR chart values indicate expression of the specific gene normalized with β-actin.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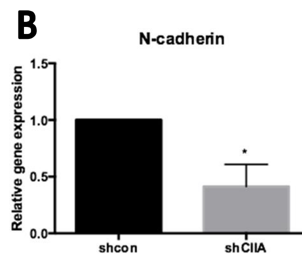
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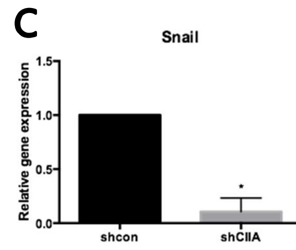
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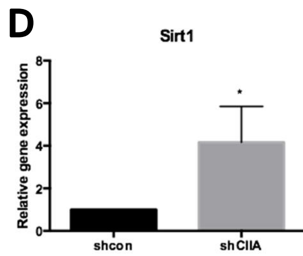
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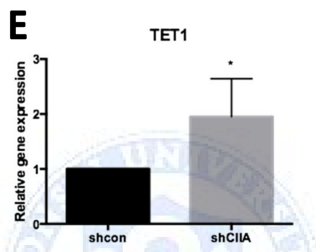
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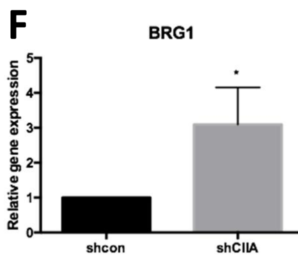
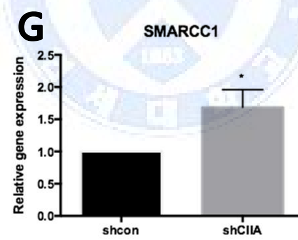

F
BRG1
Relative gene expression
5
4
3
2
1
0
*
shcon
shCIIA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Relative gene expression
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2.0
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1.0
0.5
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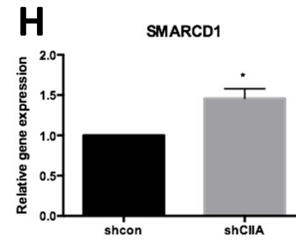
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SMARCD1
Relative gene expression
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1.5
1.0
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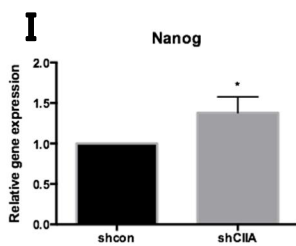
I
Nanog
Relative gene expression
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1.5
1.0
0.5
0.0
*
shcon
shCIIA

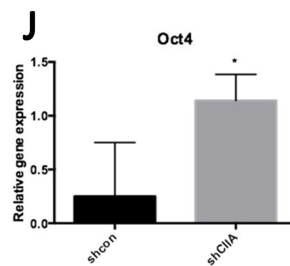

J
Oct4
Relative gene expression
1.5
1.0
0.5
0.0
*
shcon
shCIIA

Figure 3. Suppressed CIIA expression enhances the levels of epigenetic modifiers and pluripotent genes. (A-J) Extended suppression of CIIA expression induces epigenetic modifiers (Sirt1, TET1, SWI/SNF complex) and pluripotent genes (Oct4, Nanog). qRT-PCR analysis for the indicated genes at day 7 after transduction using lentiviral vector pLenti-shRNA-GFP encoding four different shRNA for CIIA in BJ fibroblasts. qRT-PCR chart values indicate expression of the specific gene normalized with β-actin. (n=3, * $P<0.05$)

following downregulation of prolonged CIIA in the stable CIIA-shRNA-expressing fibroblasts when measured with qRT-PCR (Fig. 3D-F). In previous reports, genome-wide ChIP-chip and ChIP-seq experiments revealed enrichment of BRG1 at the promoter regions of genes that were also occupied by the pluripotency regulators Oct4, Sox2 and Nanog.[27,28] BRG1 (also known as SMARCA4) is the catalytic subunit of the SWI/SNF complex, which is composed of BRG-associated factor (BAF) and polybromo BAF (PBAF). Therefore, I also performed qRT-PCR analysis targeting the remaining components of the esBAF complex,[29] an embryonic stem cell chromatin-remodeling SWI/SNF complex. The expression level of BAF60a (SMARCD1) and BAF155 (SMARCC1), essential components of the esBAF SWI/SNF complex, were also increased in CIIA-reduced cells such as BRG1 (Fig. 3G,H). More importantly, BJ fibroblasts expressing shCIIA exhibited higher expression of pluripotent genes such as Oct4 and Nanog. Together, these observations suggest that CIIA in human somatic cells is important for the maintenance of cell characteristics and that suppression of CIIA expression has a positive effect on both the early and the late stages of the reprogramming process.

3. Knockdown of CIIA regulates epigenetic modifiers that favor reprogramming

It was found that there is reciprocal expression of Sirt1 and CIIA in human fibroblast cells and in ESCs, i.e., CIIA is highly expressed in fibroblasts, but is quite low in ESCs. Conversely, Sirt1 is highly expressed in hESCs, whereas its expression is highly down-regulated in fibroblasts. Moreover, as shown in Figure 1, the level of CIIA is up-regulated during differentiation of hESCs. On the other hand, previous studies have shown that Sirt1 is known to be precisely down-regulated during hESC differentiation.[30] Therefore, it was hypothesized that CIIA and Sirt1 are mutually regulating. To test this hypothesis, I examined whether CIIA suppression affects Sirt1 expression and Sirt1 suppression affects CIIA expression through specific siRNA transfection in BJ fibroblasts. Analysis by qRT-PCR reveal-

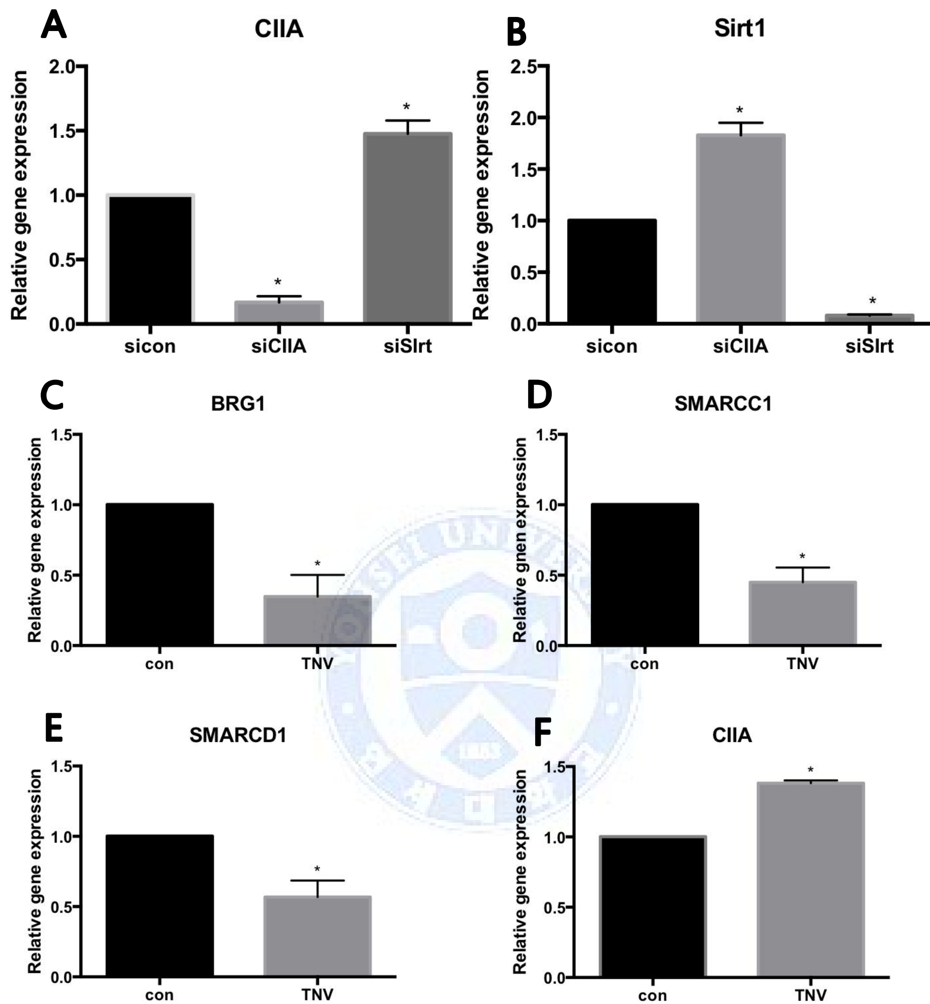


Figure 4. CIIA regulates the level of Sirt1 that modulates expression of the SWI/SNF complex. (A, B) CIIA and Sirt1 are mutually regulating. Control siRNA, CIIA siRNA, or Sirt1 siRNA was transfected twice during 7 days in BJ fibroblasts, and mRNA levels of CIIA (A) and Sirt1 (B) were quantified by qRT-PCR assays. (C-F) H9 hESCs and Sirt inhibitor (TNV)-treated H9 hESCs were analyzed for indicated gene expression using qRT-PCR analysis. TNV was treated with 1 uM concentration for 12 hr.

-ed that Sirt1 expression was upregulated in CIIA siRNA expressing BJ fibroblasts, whereas CIIA expression was unregulated in Sirt1 siRNA expressing BJ fibroblasts (Fig. 4A, B). Sirt1 is a member of the sirtuin family of NAD+-dependent protein deacetylases. It is also known that this enzyme can directly interact and deacetylate a number of transcription factors and coregulators, leading to the positive and negative regulation of target gene expression. Furthermore, previous studies have shown that Sirt1 regulates chromatin-remodeling complexes such as the Clock-Bmal133[31] and SWI-SNF complexes.[32] Therefore, I also confirmed that Sirt1, resulting from inhibited CIIA expression, regulates the SWI-SNF complex during cellular reprogramming. When the Sirt1 inhibitor Tenovin-6 (TNV) was treated in H9 hESCs to examine whether SWI-SNF complex subunits are linked with Sirt1 activation, all of the subunits were decreased by TNV (Fig. 4C-E). I also confirmed the enhanced level of CIIA by TNV (Fig. 4F). Thus, these data suggest that CIIA regulates the level of Sirt1 that modulates the expression of the SWI/SNF complex.

4. Down-regulation of CIIA opens epigenetic marks of key reprogramming factor

Cellular reprogramming requires erasing the somatic repressive chromatin and establishing a permissive chromatin state involving opening histones and changing the DNA structure.[33] Thus, I analyzed the effect of suppressed CIIA expression on the transition of chromatin state because CIIA regulates the epigenetic modifiers, as shown in Figure 4. Initially, to examine whether suppressed CIIA enhances the open chromatin state, I assessed immunocytochemistry to detect trimethylation of histone3 at lysine 4 (H3K4me3). This epigenetic modification marks transcriptionally active genes.[29,34] Immunocytochemistry showed that suppressed CIIA in shCIIA-expressing BJ fibroblasts increased H3K4me3 (Fig. 5A), whereas overexpressed CIIA in Dox-inducible GFP-CIIA-expressing BJ fibroblasts decreased (Fig. 5B). To further test that suppression of CIIA was required for elevating the open chromatin state to induce the pluripotent state, chromatin immu-

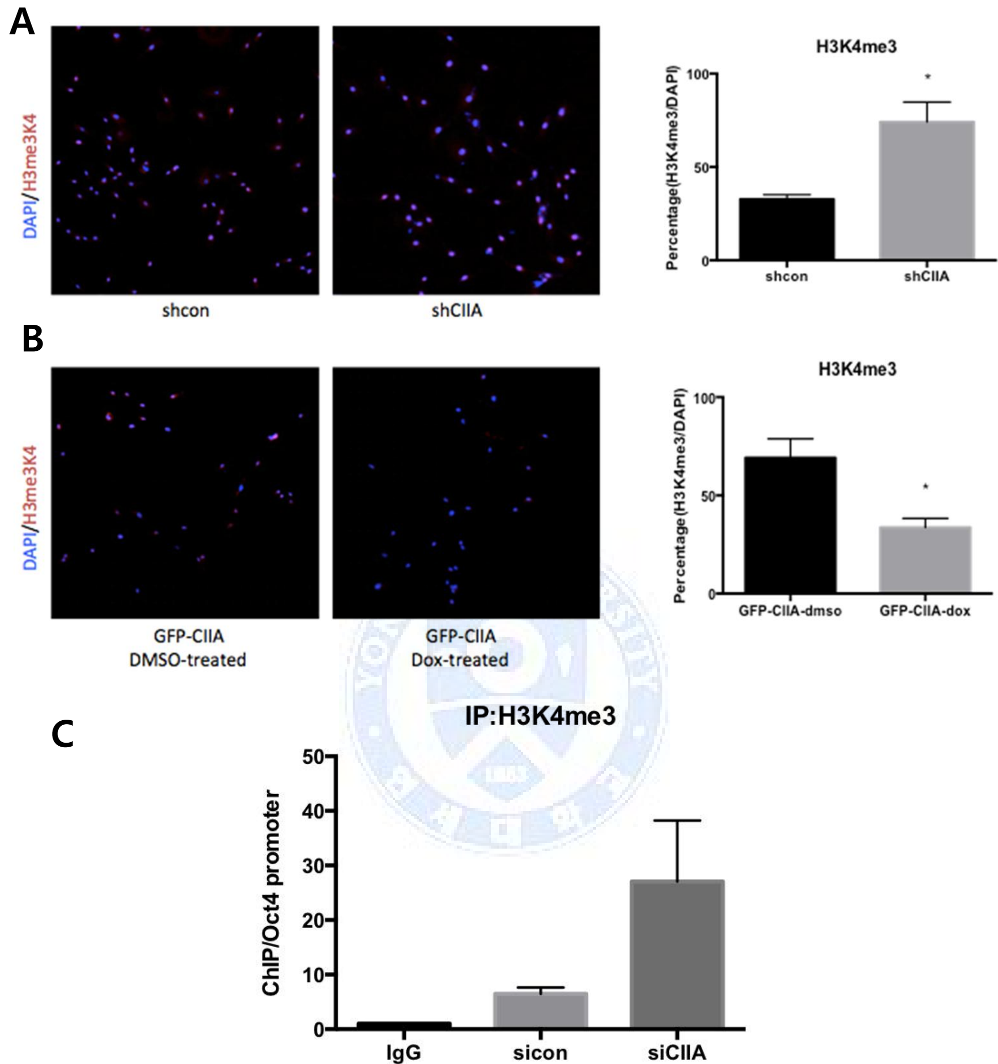
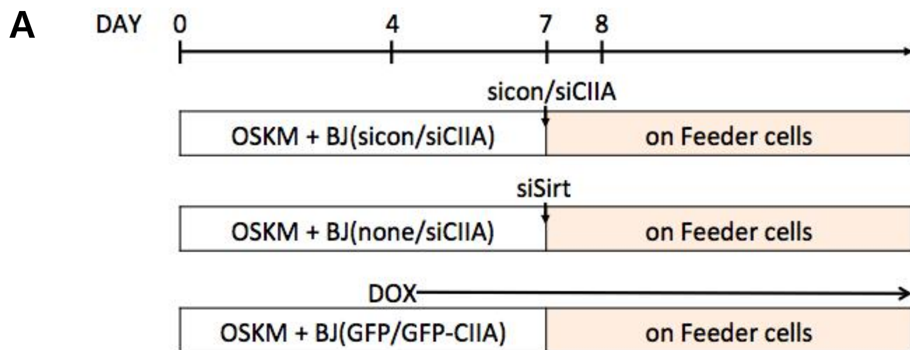


Figure 5. Knocking down of CIIA elevates permissive chromatin state on Oct4 promoter region. (A) shcon or shCIIA expressing BJ fibroblasts were analyzed with immunochemistry analysis with specific antibody against H3me3K4. Graph showing the percentage of H3K4me3^{+} cells in BJ fibroblasts (right). (B) Chromatin immunoprecipitation (ChIP) assay in BJ fibroblasts transfected with control siRNA or CIIA siRNA. ChIP assay were performed using specific antibody against H3me3K4. Specific region of Oct4 promoter was amplified by qRT-PCR.

-noprecipitation followed by PCR analysis (ChIP-PCR) was performed to detect H3K4me3 on an Oct4 promoter region. CIIA-downregulated 293T cells showed elevated levels of H3K4me3 on an Oct4 promoter region (Fig. 5C). These results suggest the downregulation of CIIA enhanced H3K4me3 expression in the Oct4 promoter region, preparing the permissive chromatin state for successful reprogramming.

5. Inhibition of CIIA in human fibroblasts promotes reprogramming efficiency

Since CIIA counteracts the reprogramming and suppression of CIIA is required for the maintenance of self-renewal and pluripotency in hESCs, I investigated whether knockdown of CIIA can enhance OSKM-induced reprogramming. To test this hypothesis, a combination of CIIA siRNA and OSKM episomal vectors was transfected in BJ fibroblasts. When CIIA expression was inhibited by siRNA, it exhibited a more efficient and faster reprogramming process than OKSM alone (Fig. 6B, C). Furthermore, prolonged suppression of CIIA by repeated transfection of CIIA siRNA showed more increased AP+ colonies than single-instance transfection (Fig. 6B, C). On the other hand, to examine whether forced expression of CIIA with OSKM expression affects reprogramming, I established cells expressing GFP or GFP-CIIA by doxycycline (Dox) treatment, transfected OSKM in these cells, and treated them with Dox or DMSO. In contrast with the CIIA knockdown condition, AP+ colonies were very sparse in the CIIA overexpressed condition (Fig. 6B, C). These data showed that suppression of CIIA enhanced the efficiency of reprogramming, demonstrating that CIIA plays an important role in cellular reprogramming. Next, I also identified the axis of CIIA-Sirt1 in cellular reprogramming. When Sirt1, the counter-regulator of CIIA, was downregulated by siRNA transfection, the efficiency of reprogramming was greatly diminished, implying their reciprocal regulation (Fig. 6B, C).


A
DAY 0 4 7 8
sicon/siCIIA
OSKM + BJ(sicon/siCIIA)
on Feeder cells
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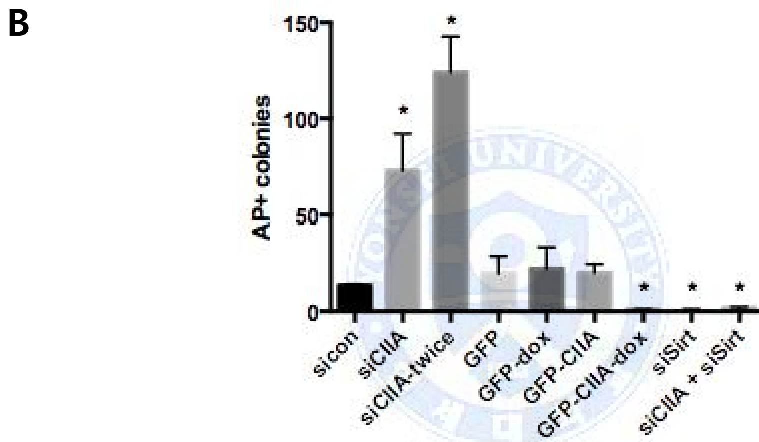
OSKM + BJ(none/siCIIA)
on Feeder cells
DOX
OSKM + BJ(GFP/GFP-CIIA)
on Feeder cells


B
AP+ colonies
150
100
50
0
sicon
siCIIA
siCIIA-twice
GFP
GFP-dox
GFP-CIIA
GFP-CIIA-dox
siSirt
siCIIA + siSirt

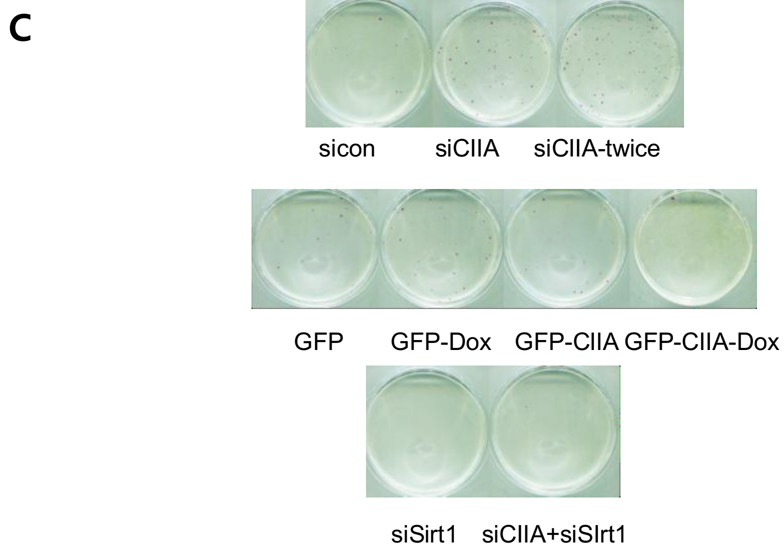

C
sicon
siCIIA
siCIIA-twice
GFP
GFP-Dox
GFP-CIIA
GFP-CIIA-Dox
siSirt1
siCIIA+siSIrt1

Figure 6. Axis of CIIA-Sirt1 is crucial in the reprogramming process. (A) Schematic picture of reprogramming methods using GFP-CIIA, CIIA siRNA, and Sirt1 siRNA. (B) Quantification of AP^+ colonies in episomal plasmid (OSKML)-transfected BJ fibroblasts (at Day 22). BJ fibroblasts were co-transfected with the indicated combination. (C) Representative AP-stained plates during reprogramming.

IV. DISCUSSION

The present study has identified that CIIA constitutes a cellular and molecular barrier to reprogramming, with different expressions of CIIA between fibroblasts and pluripotent stem cells. First, short-term repression of CIIA negatively regulates EMT, which is known to be a major driving force in tumor formation and in embryonic development.[35] Furthermore, the prolonged suppression of endogenous CIIA induces pluripotent genes and epigenetic modifiers that are abundant in PSCs, but are minimally expressed in fibroblasts. Finally, knocking down CIIA in combination with OSKM in fibroblasts could establish more ESC-like colonies than with just OSKM alone.

As previous investigators have reported, CIIA identical to VPS28 playing an endocytotic role,[36] functions as an apoptotic protein.[21] In addition, CIIA physically associates with SOS1 and promotes the SOS1-mediated activation of Rac1.[25] It also suppresses the SOS1-mediated activation of Ras[37] that provides instructions for cell growth and development. This study demonstrated a new role of CIIA in cellular reprogramming. Thus, this study also newly revealed that the knockdown of CIIA in fibroblasts exhibits more efficient reprogramming, forming more iPSC colonies than OSKM alone.

Another interesting link here is that CIIA modulates Sirt1 expression, and this link controls a significant aspect of reprogramming. It has been observed that Sirt1 accelerates iPSC reprogramming via the miR-34a and p53 pathways,[38] consistent with prior evidence that Sirtuin mediates p53 in cancer.[39] Furthermore, Sirt1 modifies histones and DNA in epigenetic regulation, as well as interactions with a broad range of transcription factors.[40] Sirt1 has been shown to regulate chromatin-remodeling complexes such as the Clock-Bmal1[31] and SWI-SNF complexes.[32] This study also demonstrated that Sirt1, resulting from inhibited CIIA expression, regulates the SWI/SNF complex during cellular reprogramming. BRG1

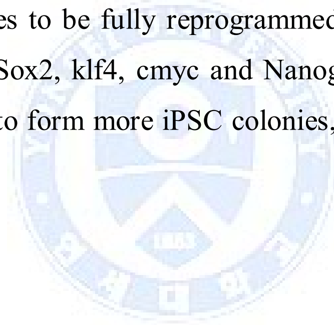
of *bona fide* subunits in the SWI/SNF complex, including BAF complex components, has been observed to regulate self-renewal and pluripotency.[41] Prior studies also show that the BAF complex can replace cmyc,[41] one of the reprogramming factors, and open chromatin structures on binding sites of key pluripotent factors. Therefore, these results suggest that enhanced Sirt1, regulating epigenetic modification, potentially promotes the SWI/SNF complex, and that this chromatin-remodeling complex controls the H3K4me3 level on the Oct4 promoter region.

The reprogramming of human fibroblasts using small molecules such as siRNA, miRNA, and chemicals may be especially challenging for the formation of safe human iPSCs.[42-45] However, the replacement of Oct4 by small molecules has been one major issue to overcome. This research has shown that the knockdown of CIIA increased Oct4 expression, but it was not sufficient to fully turn on the endogenous Oct4 expression. Therefore, this study cannot preclude the possibility that siRNA of CIIA may replace Oct4, one of the key Yamanaka factors.

These data showed that CIIA is a hurdle to be surmounted during cellular reprogramming. It is important to note, however, that the suppression of CIIA would stimulate Oct4 through the modulation of epigenetic modifiers. Further observations are needed to substitute small molecules for reprogramming factors, but these findings suggest the possibility of forming safer and more efficient iPSCs. In addition, our understanding of the mechanism underlying cellular reprogramming through this study suggests strategies to improve modeling and curing disease, and advances our insight into the regulation of pluripotency.

V. CONCLUSION

To summarize, this study has presented the finding that CIIA is closely involved in cell fate change, negatively regulating the transcriptional network and chromatin configuration. Decreased CIIA gene expression not only blocks EMT in the initial reprogramming stage, but also remodels histone modification by enhancing the active H3K4me3 levels on the Oct4 promoter region. In this process, a chromatin remodeling complex, SWI/SNF, is promoted by up-regulated Sirt1 expression, and then activates the H3K4me3 structure, opening the Oct4 promoter region. This transcriptional activity of Oct4, one of the key stemness factors, could allow more iPSC candidates to be fully reprogrammed while turning on the other pluripotent genes such as Sox2, klf4, cmyc and Nanog. In conclusion, suppressed CIIA expression is crucial to form more iPSC colonies, conquering reprogramming barriers.



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ABSTRACT (in korean)

세포리프로그래밍에서 CIIA 유전자의 역할

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역분화 기술은 배아를 사용하지 않고 환자로부터 채취한 체세포에서 전분화능을 지닌 유도만능줄기세포를 생성하는 것으로 재생의학에서 매우 각광받고 있는 분야이다. 그러나 임상 적용을 위한 안정성 확보와 현저히 낮은 역분화 효율 문제는 여전히 개선이 필요한 부분이다. 본 연구에서는 CIIA 유전자의 발현이 세포리프로그래밍시 낮은 역분화 효율을 일으키는 걸림돌 중 하나임을 밝혔다. 먼저, siRNA, shRNA 기술을 이용하여 CIIA의 발현량을 낮추었을 때 상피중간엽세포이행관련 유전자 발현이 줄어드는, 즉 세포리프로그래밍 초기에 관찰할 수 있는 특징이 보일 뿐 아니라 후생학적 변이와 관련된 유전자의 발현 변화 또한 관찰 할 수 있었다. 이는 CIIA를 단독으로 감소시키는 것만으로도 세포의 특성을 변화시킬 수 있는 것을 의미한다. 그리고 분자적 수준에서의 실험들을 통해 CIIA가 Sirt1(히스톤변형인자)발현을 조절함으로써 세포리프로그래밍을 저해함을 밝혔다. 마지막으로 CIIA발현을 감소시킨 체세포에서 OSKM(역분화줄기세포 생성 유전자)의 도입은 OSKM만 도입했을 때에 비해 더

많은 배아줄기세포유사 역분화줄기세포를 생성함을 관찰할 수 있었다. 이러한 연구 결과를 통해 세포리프로그래밍에서 역할을 하는 CIIA 유전자의 새로운 기능을 알 수 있었다. 또한 이를 통해 리프로그래밍에서 일어나는 현상을 더 이해함으로써 역분화줄기세포 생성효율 문제점을 해결할 만한 새로운 방법을 제시했다.

핵심되는 말: 역분화 줄기세포, CIIA유전자, 상피중간엽세포이행(EMT), 후생학적변이, 세포리프로그래밍