약학석사학위논문

IL-1β-induced SIRT1 Upregulates Expression of Pro-inflammatory Cytokines via Deacetylation of c-Jun in Human Colon Cancer HCT-116 Cells

인체 대장암세포에서 IL-1β에 의해 유도된 SIRT1의 c-Jun 탈아세틸화를 통한 친염증성 사이토카인 생성 효과

2015년 2월

서울대학교 대학원

분자의학 및 바이오제약학과

정 재 경

Abstract

IL-1β-induced SIRT1 Upregulates Expression of Pro-inflammatory Cytokines via Deacetylation of c-Jun in Human Colon Cancer HCT-116 Cells

Jaekyung Jung

Under the supervision of Professor Young-Joon Surh

Department of Molecular Medicine and Biopharmaceutical Sciences

Seoul National University

SIRT1 is a mammalian NAD^+-dependent deacetylase, which is known to be involved in a variety of physiological events, including calorie restriction and cellular senescence. The role of SIRT1 in tumorigenesis is still controversial. However, recent studies have reported that overexpression of SIRT1 is observed in many types of cancer, suggesting its oncogenic potential. A pro-inflammatory cytokine, Interleukin-1β (IL-1β), has also been implicated as a major factor in tumor promotion and progression via production of metastatic and angiogenic small molecules. However, the mechanism by which IL-1β and SIRT1 coordinately stimulate tumorigenesis remains unclear. In the present study, IL-1β was found to upregulate SIRT1 protein expression in human colon cancer HCT-116 cells. IL-1β-induced SIRT1 upregulation led to enhanced levels of mRNA transcript of pro-inflammatory cytokines, IL-6, IL-8, and IL-1β. This phenomenon is mediated through the direct binding of SIRT1 to c-Jun, the major

component of activator protein-1 (AP-1) transcription factor complex. Knock-down of SIRT1 prevented IL-1β-induced nuclear accumulation of c-Jun, and subsequently abolished DNA binding activity of AP-1. Furthermore, nicotinamide (NAM), a SIRT1 inhibitor, abrogated colon cancer cell colony formation and migration induced by IL-1β, signifying the role of SIRT1 in colon cancer development. In addition, SIRT1 upregulated mRNA expression of IL-1R1, the specific receptor for IL-1β, indicative of a possible existence of positive feedback loop of IL-1β/SIRT1. In summary, IL-1β-induced SIRT1 upregulation stimulates production of pro-inflammatory cytokines by facilitating nuclear accumulation of c-Jun, which may lead to colon cancer promotion and progression.

Keywords: Interleukin-1β (IL-1β), Silent mating type information regulation 2 homolog 1 (SIRT1), c-Jun, Pro-inflammatory cytokines, Human colon cancer HCT-116 cells

Student Number: 2013-22737

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Introduction

Colorectal cancer is one of the most fatal malignancies in Korea (Jung et al. 2013). In United States, colon cancer is the third most prevalent cancer and also the third leading cause of cancer-associated death (Siegel et al. 2014). The colon cancer has poor prognosis, resulting in only the 60% survival rate within 5 years after diagnosis in the United States (American Cancer Society 2014).

Recently, the tumor microenvironment has been receiving increasing attention for its pivotal role in facilitating tumorigenesis (Hanahan and Weinberg 2011). In the tumor microenvironment, immune and inflammatory cells, such as macrophages and neutrophils, secrete an assorted array of bioactive molecules and growth factors. These include tumor necrosis factor-α (TNF-α), interleukins, and interferons (IFNs) that play roles in tumor progression and metastases (Kuper 2000; Wahl and Kleinman 1998). Interleukin-1β (IL-1β), one of the pro-inflammatory cytokines abundant in the tumor microenvironment, has been reported to be upregulated in human tumor specimens including melanoma, non-small cell lung cancer, and colon adenocarcinoma (Elaraj et al. 2006). A number of studies have already demonstrated the importance of IL-1β in tumor promotion (Carmi et al. 2013; Song et al. 2003). However, the exact mechanism how IL-1β promotes tumor growth is not fully revealed.

SIRT1, a mammalian NAD^+-dependent histone deacetylase, has been reported to be involved in various physiological events, including calorie restriction, cellular senescence, and aging (Rahman and Islam 2011). However, the role of SIRT1 in tumorigenesis is still under debate (Deng 2011). SIRT1 has an oncogenic function as it deacetylases p53, a well-known tumor suppressor, thereby preventing cells from undergoing DNA damage-induced apoptosis (Chen et al. 2005). On the other hand, SIRT1 has been speculated as a tumor suppressor based on the observation that SIRT1 mutant mice are prone to develop cancers (Wang et al. 2008). Moreover, some types of cancer tissues had lower SIRT1 levels than the normal ones (Wang et al. 2008). In other studies related to colorectal cancers, however, overexpression of SIRT1 is observed in colon cancer with higher tumor grades, suggesting its

oncogenic potential (Liu, Liu, and Marshall 2009; Leko et al. 2013).

Since both IL-1β and SIRT1 were overexpressed in human colon cancer cell lines, this prompted us to test the effect of IL-1β on expression of SIRT1. In the present study, I observed that IL-1β upregulated SIRT1 protein level in human colon cancer HCT-116 cells. In addition, I found that Il-1β-induced production of pro-inflammatory cytokines was abrogated by knock-down of SIRT1, suggesting the potential role of in IL-1β-induced colon carcinogensis. In the present study, I investigated the mechanism underlying IL-1β-induced SIRT1 upregulation and subsequent pro-inflammatory cytokine production in the context of its contribution to colon tumorigenesis.

Materials and Methods

Reagents

Sirtinol and nicotinamide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human IL-1β was obtained from R&D System, (Minneapolis, MN, USA). Antibodies against α-Tubulin, Lamin B1, phospho-c-Jun, total-c-Jun were provided by Santa Cruz Biotechnology (Santa Cruz, CA, USA), and antibody against IL-1β was purchased from Cell Signaling Technology (Beverly, MA, USA). Antibody against SIRT1 was obtained from Abcam (Cambridge, UK). Antibody against actin (Sigma-Aldrich) was purchased commercially. Horseradish peroxidase-conjugated secondary antibodies were obtained from Pierce Biotechnology (Rockford, IL, USA). Protein A/G-Plus Agarose was obtained from Santa Cruz Biotechnology. TRIzol® Lipofectamine® RNAiMAX, Lipofectamine® 2000, and SYBR® Green were provided by Invitrogen (Carlsbad, CA, USA). Dulbecco's modified Eagle's medium (DMEM), antibiotics, and 0.25% trypsin were products of GIBCO BRL (Grand Island, NY, USA). Fetal bovine serum was purchased from GenDEPOT (Barker, TX, USA). BCA Protein Assay Kit and Bradford Assay Reagent Kit were provided from Pierce and Bio-Rad Laboratories (Hercules, CA, USA), respectively. Protease Inhibitor cocktail tablets were from Boehringer Mannheimm (Mannheimm, Germany). Murine Leukemia Virus (MLV) reverse transcriptase and oligonucleotide probe containing AP-1 consensus sequence (5'-CGC TTG ATG AGT CAG CCG GAA-3' and 3'-GCG AAC TAC TCA GTC GGC CTT-5') was purchased from Promega (Madison, WI, USA). Illustra ProbeQuant G-50 Micro Columns were obtained by GE Healthcare (Little Chalfont, UK). SeaKem® GTG® Agarose was purchased from Lonza (Rockland, Me, USA). [γ-^{32}P]ATP T4 polynucleotide kinase was provided by Takar Bio Inc. (Shiga, Japan).

Cell Lines and Cell Culture

Human colon cancer cell line HCT-116 cells (American Type Culture Collection, Manassas, VA, USA) were maintained routinely in DMEM supplemented with 10% FBS and 100

units/ml penicillin and 100 μg/ml streptomycin at 37 ℃ in a humidified atmosphere of 5% CO2 / 95% air. The cells were plated at an appropriate density according to each experimental scale. For screening SIRT1 and IL-1β basal level, human normal colon epithelial cell line CCD 841 CoN and colon cancer cell lines HT-29, HCT-15, DLD-1, and SW480 were maintained in appropriate conditions.

siRNA Transfection

HCT-116 cells were seeded in a 60 mm dish and small interfering RNA (RNAi) transient transfection was performed with Lipofectamine® RNAiMAX (Invitrogen) according to the manufacturer's protocol. Transfected cells were then treated with IL-1β for the indicated times. 48 h after transfection, cells were harvested and processed for appropriate experiments. The target sequence for SIRT1 siRNA was sense 5'-ACU UUG CUG UAA CCC UGU A (dTdT)-3', and antisense 5'-UAC AGG GUU ACA GCA AAG U (dTdT)-3'.

SIRT1 Vector Transfection

HCT-116 cells were plated in a 60 mm dish with an appropriate number of cells, and incubated for 24 h prior to vector transfection. After incubation, SIRT1 vector transfection was performed with Lipofectamine® 2000 (Invitrogen) according to the manufacturer's protocol. 48 h after transfection, cells were harvested and processed for RT-PCR.

Western Blot Analysis

HCT-116 cells were mixed with the lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM $Na_4P_2O_7$, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 0.1 mM PMSF, and a mixture of protease inhibitor] on ice for 30 min followed by centrifugation at 4℃ for 15 min at 12,000 rpm. The supernatant was collected and stored at -80 ℃. The protein concentration was measured by using BCA Protein Assay Kit (Pierce). The lysates were solubilized with 5x loading buffer and boiled at 99℃ for 5 min. The solubilized proteins were electrophoresed in a 7-12 % sodium dodecyl sulfate

(SDS)-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes. The blots were incubated in blocking buffer (0.1 % Tween-20 in TBS containing 5 % non-fat dry milk) at room temperature for 1 h followed by incubation with appropriate primary antibodies in TBST overnight. After washing with TBST, blots were incubated with proper horseradish peroxidase-conjugated secondary antibodies in TBST with 3 % non-fat dry milk at room temperature for 1 h. The immunoblots were visualized with enhanced chemiluminescence (ECL) western blotting detection reagents.

Preparation of Cytosolic and Nuclear Proteins

Confluent cells in 100 mm dish or duplicate of 60 mm dish were scraped and centrifuged at 4 ℃ for 5min at 3000 rpm. Pellets were suspended in 50-100 μl of hypotonic buffer A [10 mM Tris-HCl (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, and 0.2 mM PMSF] with 0.1 % Nonidet P-40 for 10 min on ice. The mixture was then centrifugated at 4 ℃ for 10 min at 12000 rpm. The supernatant containing cytosolic proteins was collected. To obtain nuclear proteins, the pellets were washed with buffer A (without NP-40) and suspended in 15-50 μl of hypertonic buffer C [20 mM Tris-HCl (pH 7.9), 20 % Glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF]. After incubation on ice for 1-2 h, the samples were centrifuged and the supernatants containing nuclear proteins were harvested. The protein concentration was determined by using the BCA protein assay reagent.

Immunoprecipitation

HCT-116 cells seeded in a 100 mm dish were treated with IL-1β for 3 h after 12 h starvation. Cells were resuspended in ice-cold lysis buffer [250 mM Sucrose, 50 mM Tris-HCl (pH 8.0), 25 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 10 mM NEM, 2 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, and a mixture of protease inhibitor]. Cell suspensions were sonicated for 2 short burst of 10 sec followed by intervals for 10 sec for cooling. The protein concentration was determined by using Bradford Assay Reagent Kit (Bio-Rad). Appropriate antibodies were added to cell lysates and incubated at 4 ℃ for overnight, followed by addition of protein A/G

slurry. After centrifugation to pellet the beads, the pellets were washed and denatured at 99 ℃ for 5 min in 2x SDS-loading buffer. Supernatants were then loaded and analyzed by Western blot.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from HCT-116 cells by using TRIzol® reagent (Invitrogen) according to manufacturer's protocol. 10 μg of total RNA was reverse transcribed with MLV reverse transcriptase at 42 ℃ for 50 min and at 72 ℃ for 15 min. PCR was conducted according to the standard procedures. The primer sequences used were as follows:

Primer name	Primer sequence		Cycles	Tm
GAPDH	**Forward**	GCATGGCCTTCCGTGTCCCC	20	59℃
	Reverse	CAATGCCAGCCCCAGCGTCA		
IL-1β	**Forward**	GCATCCAGCTACGAATCTCC	36	59℃
	Reverse	CCACATTCAGCACAGGACTC		
IL-1R1	**Forward**	ACACATGGTATAGATGCAGC	30	60℃
	Reverse	TTCCAAGACCTCAGGCAAGA		
IL-6	**Forward**	GTGTGAAAGCAGCAAAGAGGC	33	55℃
	Reverse	CTGGAGGTACTCTAGGTATAC		
IL-8	**Forward**	ATGACTTCCAAGCTGGCCGTGGCT	28	61℃
	Reverse	TCTCAGCCCTCTTCAAAAACTTCT		
SIRT1	**Forward**	TCAGTGTCATGGTTCCTTTGC	28	60℃
	Reverse	AATCTGCTCCTTTGCCACTCT		

Amplified products were analyzed on 1.5-3 % agarose gel electrophoresis, stained with SYBR® Green (Invitrogen) and photographed using fluorescence in LAS-4000.

SIRT1 enzyme activity assay

SIRT1 activity was assessed by measuring fluorescence which is proportional to the deacetylation activity of the enzyme using the commercial kits (Sigma-aldrich). A blank and the assay samples with appropriate concentration were mixed with assay buffer, NAD^+, and SIRT1 substrates, followed by incubation at 37 ℃for 30 min. Two sets of standard were prepared during the incubation with or without developing solution. After adding developing solution to the samples, all the mixture were incubated at 37 ℃ for 10 min. Samples were transferred to the costar 96-well dark plate and the fluorescence was read by the plate reader.

Immunocytochemistry

HCT-116 cells were plated on four-well chamber slides or slide glasses on 12-well dishes with SIRT1 siRNA or negative control. After siRNA transfection, cells were treated with IL-1β for 3 h. Cells were rinsed with PBS, and fixed for 10 min at 4 ℃ with 5 % acetic acid in methanol. After rinsing with PBS, cells were blocked with PBST containing 5 % BSA for 1 h at room temperature, and incubated with c-Jun antibody diluted 1:200 in blocking buffer overnight at 4 ℃. Cells labeled with FITC-conjugated goat anti-rabbit IgG were examined under a fluorescent microscope.

Electrophoretic mobility shift assay

EMSA for measuring the DNA binding of AP-1 was performed using AP-1 oligonucleotide labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase (Takar Bio Inc.). Labeled oligonucleotide was purified with 1x TE buffer in micro columns. The binding reaction was carried out in 15 μl of mixture containing 3 μl incubation buffer [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4 % glycerol, and 0.1 mg/ml sonicated salmon sperm DNA], 10 μg of nuclear extracts, and 100,000 cpm of [γ-^{32}P]ATP-end labeled oligonucleotide. After 50 min incubation at room temperature, 2 μl of 0.1 % bromophenol blue was added to stop the reaction. Samples were then electrophoresed through pre-runned 6 % nondenaturing polyacrylamide gel at 170 V in a cold room. To ensure the specificity of the

binding, a competition assay was carried with the excess unlabeled oligonucleotide. The gel was dried for 1 h, and exposed to an X-ray film for 24 h at -80 ℃.

Anchorage-Independent Growth Assay

For the experiments with soft agar, stock concentration of 3.3 % agar powder was dissolved in PBS and autoclaved. After cooling down the agar solution to 55 ℃ in water bath, 60 mm dish were pre-coated with 0.5 % agarose as the bottom layer. On top of the bottom layer, the mixture of 0.3 % agarose and 25,000 cells was seeded. For one study, sirtinol (25 μM) or nicotinamide (5 mM) was treated every other day for 14 days, followed by colony staining with MTT. IL-1β (10 ng/ml) with or without nicotinamide (5 mM) was treated every other day for 7 – 10 days for another study. The number of colony formed was photographed under a microscope and counted. Minimum duplicate wells were used and three independent experiments were performed.

Cell migration assay

HCT-116 cells were plated in a culture insert on 6-well dishes and grown 90 % confluence. The silicon insert was removed, resulting in nicely defined cell patches separated by a zone of exactly the same width as the separation wall. The cells were treated with the medium containing IL-1β, nicotinamide under specific conditions. After 18 h, cells were photographed under a microscope.

Statistical Analysis

When necessary, data from three independent experiments at least was expressed as the mean ± s.e.m. The statistical analysis for single comparison was performed using the Student's *t* test. Analysis was performed using SigmaPlot (Version 10). Statistical significance was accepted at $P < 0.05$ and indicated as $P < 0.05$ with *, $P < 0.01$ with **, and $P \leq 0.001$ with ***.

Results

SIRT1 and IL-1β are overexpressed in human colon cancer cell lines and SIRT1 inhibitors reduce colony formation in HCT-116 cells

The role of SIRT1 in colon carcinogenesis is debatable (Deng 2009). To confirm the basal levels of SIRT1 protein in normal and cancer cells, colon epithelial cell line CCD 841 CoN and five different human colon cancer cell lines HCT-116, HT-29, HCT-15, DLD-1 and SW480 were selected. Three of the colon cancer cell lines (HCT-116, HCT-15, and DLD-1) showed upregulated SIRT1 expression whereas HT-29 and SW480 exhibited nearly the same level of SIRT1 expression as control **(Figure 1A and B)**. To investigate the role of overexpressed SIRT1, HCT-116 cells were treated with sirtinol (25 μM) or nicotinamide (10 mM). Cells treated with these SIRT1 inhibitors showed the reduced number of colonies compared to untreated controls, suggesting the involvement of SIRT1 in colon cancer cell growth and proliferation **(Figure 1C and D)**. Furthermore, when I compared basal levels of IL-1β in the above cell lines, colon cancer cell lines exhibited upregulated expression of IL-1β level while the normal colon epithelial cell line CCD 841 CoN showed a low IL-1β level **(Figure 1E).**

IL-1β stabilizes SIRT1 protein in HCT-116 cells

IL-1β has been known to play a key role in inflammation-initiated carcinogenesis (Apte et al. 2006). Since SIRT1 showed a higher basal level in a majority of colon cancer cell lines tested than the normal colon cell line, and is proven to be oncogenic **(Figure 1A–D)**, I attempted to investigate whether IL-1β, a pro-inflammatory cytokine known for facilitating tumor promotion, could regulate SIRT1 in colon cancer cells. HCT-116 cells were incubated with serum-free media for 12 h and treated with 10 ng/ml IL-1β. Treatment of cells with IL-1β resulted in the induction of SIRT1 protein level, and the SIRT1 expression was evident at 3 h **(Figure 2B)**. Notably, IL-1β treatment did not influence the SIRT1 mRNA level **(Figure 2A)**, suggesting that IL-1β regulates stabilization of SIRT1 protein, not its transcription. To explore

the subcellular localization of SIRT1, cytosolic and nuclear extracts were separated. I analyzed that when cells were stimulated with IL-1β, SIRT1 resides only in the cytosol **(Figure 2C)**. IL-1β also stimulated deacetylase activity of SIRT1 **(Figure 2D)**.

IL-1β-induced SIRT1 regulates pro-inflammatory cytokine transcription

IL-1β has been known to stimulate synthesis of other pro-inflammatory cytokines, including IL-6 and IL-8 (Kim et al. 2010; Cahill and Rogers 2008). Moreover, it has already been reported that in a certain kind of inflammation-related diseases, such as rheumatoid arthritis synovium, SIRT1 can induce the production or secretion of pro-inflammatory cytokines (Niederer et al. 2010). IL-1β was treated to HCT-116 cells for various time periods. As illustrated in figure 3A, mRNA levels of IL-6 and IL-8, two representative cytokines related to inflammations were highly elevated by IL-1β treatment. Notably, the mRNA expression of IL-1β itself was also upregulated **(Figure 3A)**. To confirm the role of IL-1β-induced SIRT1 in induction of IL-6, IL-8, and IL-1β transcription, SIRT1 siRNA was utilized. Level of IL-6, IL-8, and IL-1β transcription, whose transcription was upregulated by IL-1β, was reduced when SIRT1 was knocked down. In addition, the IL-1β level was decreased when SIRT1 was silenced, suggesting the existence of a probable positive feedback loop between SIRT1 and IL-1β in HCT-116 cells. Among the three cytokines, the transcription of IL-6 was completely abolished in SIRT1 siRNA-treated group **(Figure 3B)**.

Besides in inflammation-related conditions, SIRT1 may function as an endogenous regulator of the cytokines in unstimulated cells. When HCT-116 cells were transfected with SIRT1 siRNA or an overexpression vector, the mRNA expression of IL-6, IL-8, and IL-1β was highly upregulated **(Figure 3C)** or suppressed **(Figure 3D)**, respectively.

SIRT1 inhibition attenuates nuclear accumulation and DNA binding of c-Jun by blocking SIRT1 deacetylase activity

For SIRT1 to regulate the expression of pro-inflammatory cytokines, it needs a transcription factor that binds to their promoter of the genes. It is reported that AP-1 binds to pro-

inflammatory genes and induces the transcription (Thomas and Schroder 2013). However, the role of SIRT1 in AP-1 regulation is controversial. To verify the relationship between SIRT1 and AP-1 in human colon cancer cells, the levels of c-Jun and c-Fos, the major components of AP-1, were first checked in IL-1β-treated HCT-116 cells. As level of SIRT1 was increased, levels of phospho- and total c-Jun were also upregulated by IL-1β **(Figure 4A)**. However, I failed to detect protein level of c-Fos. Since AP-1 is a transcription factor, nuclear levels of phospho- and total c-Jun was also examined. As illustrated in **Figure 4B**, SIRT1 knock down by siRNA transfection abrogated the accumulation of both phospho- and total c-Jun in the nuclear fraction of the cells stimulated with IL-1β. Immunocytochemical analysis also reveals that SIRT1 knock down reduces the level of c-Jun nuclear accumulation, proposing the involvement of SIRT1 in c-Jun expression **(Figure 4C)**.

Since SIRT1 is a histone/protein deacetylase, I determined whether SIRT1-mediated regulation of AP-1 is attributable to its deacetylase activity. To act as a deacetylase, SIRT1 should bind the lysine residue of the substrate and remove the acetyl group (Liu, Liu, and Marshall 2009). Direct binding of SIRT1 to c-Jun was already demonstrated in some studies, but only in mouse epithelial and macrophage models (Dey et al. 2008; Gao and Ye 2008). In this study, an immunoprecipitation assay was initially conducted to examine whether the binding of SIRT1 to c-Jun increases after IL-1β induction. In an IL-1β treated group, SIRT1 and c-Jun showed higher physical interaction **(Figure 4D)**. To further check if the binding is mediated by deacetylase activity of SIRT1, the level of nuclear c-Jun was measured after SIRT1 activity was inhibited. When treated with nicotinamide, a SIRT1 activity inhibitor, the nuclear levels of phospho-c-Jun and total c-Jun were decreased **(Figure 4E)**. The DNA binding activity of AP-1 in SIRT1 silenced group was significantly diminished than that in the control group **(figure 4F)**.

IL- 1β and SIRT1 induce colon cancer colony formation and cell migration

To check if IL-1β-induced SIRT1 contributes to colon cancer cell growth, the effect of SIRT1 inhibition on colon cancer cell colony formation was investigated. As shown in **Figure 5A**,

HCT-116 cell colony formation induced by IL-1β was suppressed when treated with nicotinamide, a SIRT1 inhibitor. Next, the effect of IL-1β and SIRT1 on cell migration was investigated by a wound healing assay. HCT-116 cells treated with IL-1β migrated to the center of the wound. However, when treated with nicotinamide, the migration induced by IL-1β was markedly inhibited **(Figure 5B)**.

SIRT1 increases expression of IL-1R1 transcription

According to the previous study, IL-1β is a positive regulator of SIRT1, and conversely, endogenous SIRT1 promotes IL-1β transcription via deacetylation of c-Jun. It is known that IL-1β can influence the cells that possess IL-1 receptor type 1 (IL-1R1), a specific receptor of IL-1β. The mRNA level of IL-1R1 in HCT-116 cells transfected with SIRT1 siRNA or overexpression vector was measured. Interestingly, IL-1R1 also exhibited the same pattern as the other pro-inflammatory cytokines did; the mRNA level of IL-1R1 was diminished when SIRT1 was silenced and highly induced when SIRT1 was overexpressed **(Figure 6A and B)**.

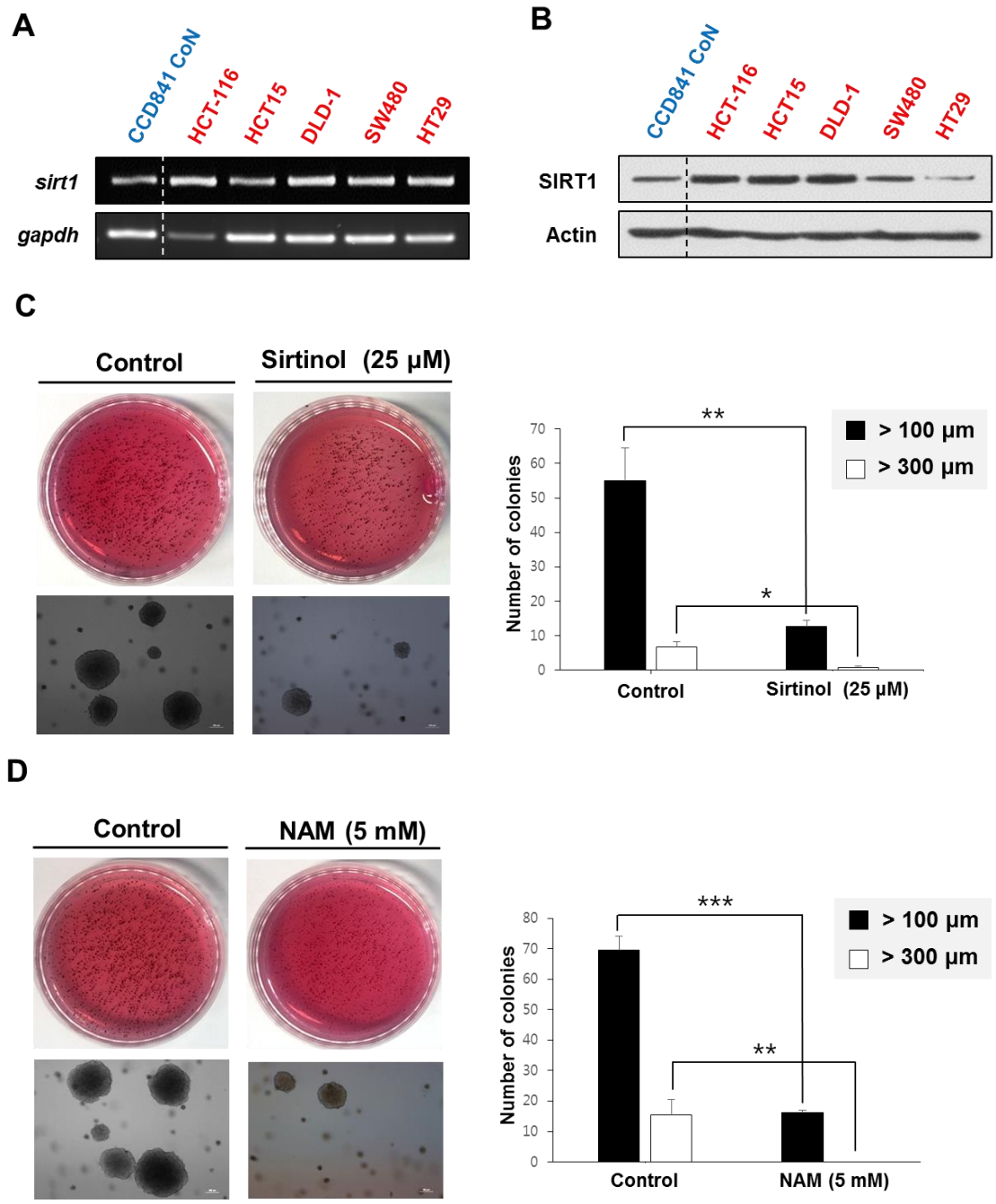

Figure 1. Overexpression of SIRT1 and IL-1β in human colon cancer cell lines and the role of SIRT1 in colony formation of HCT-116 cells. (A and B) mRNA and Protein lysates of human normal colon epithelial cell line CCD 841 CoN and colon cancer cell lines HCT-116, HT-29, HCT-15, DLD-1, and SW480 were assessed by RT-PCR (A) and western blot analysis (B), respectively. *GAPDH* or Actin was included to ensure equal cDNA or protein was loaded. (C and D) HCT-116 cells were seeded at 2.5 x 10^5 cells per well in

complete medium in a 60 mm dish. After 48 hours of incubation, cells were treated with sirtinol (25 μM) (C) or nicotinamide (5 mM) (D) for 14 days. The number of colonies larger than 100 μm or 300 μm was counted under a microscope and quantification of the colony number in three independent experiments is shown. Data represent mean ± s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

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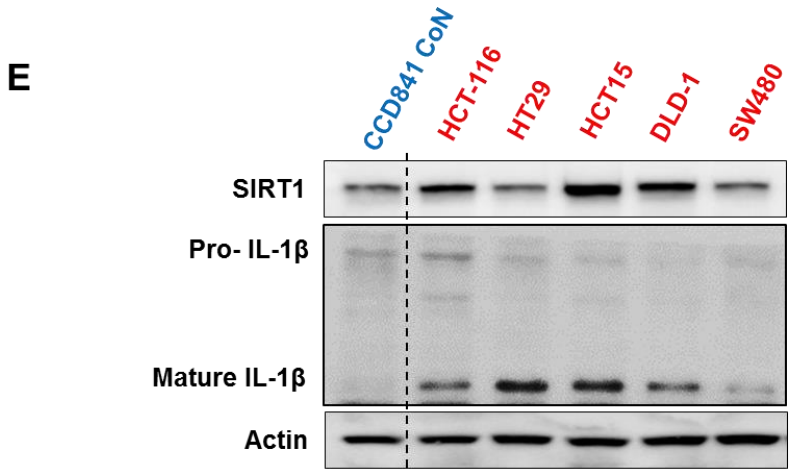

Figure 1. Overexpression of SIRT1 and IL-1β in human colon cancer cell lines and the role of SIRT1 in colony formation of HCT-116 cells. (E) Protein lysates of human normal colon epithelial cell line CCD 841 CoN and colon cancer cell lines HCT-116, HT-29, HCT-15, DLD-1, and SW480 were assessed by western blot analysis.

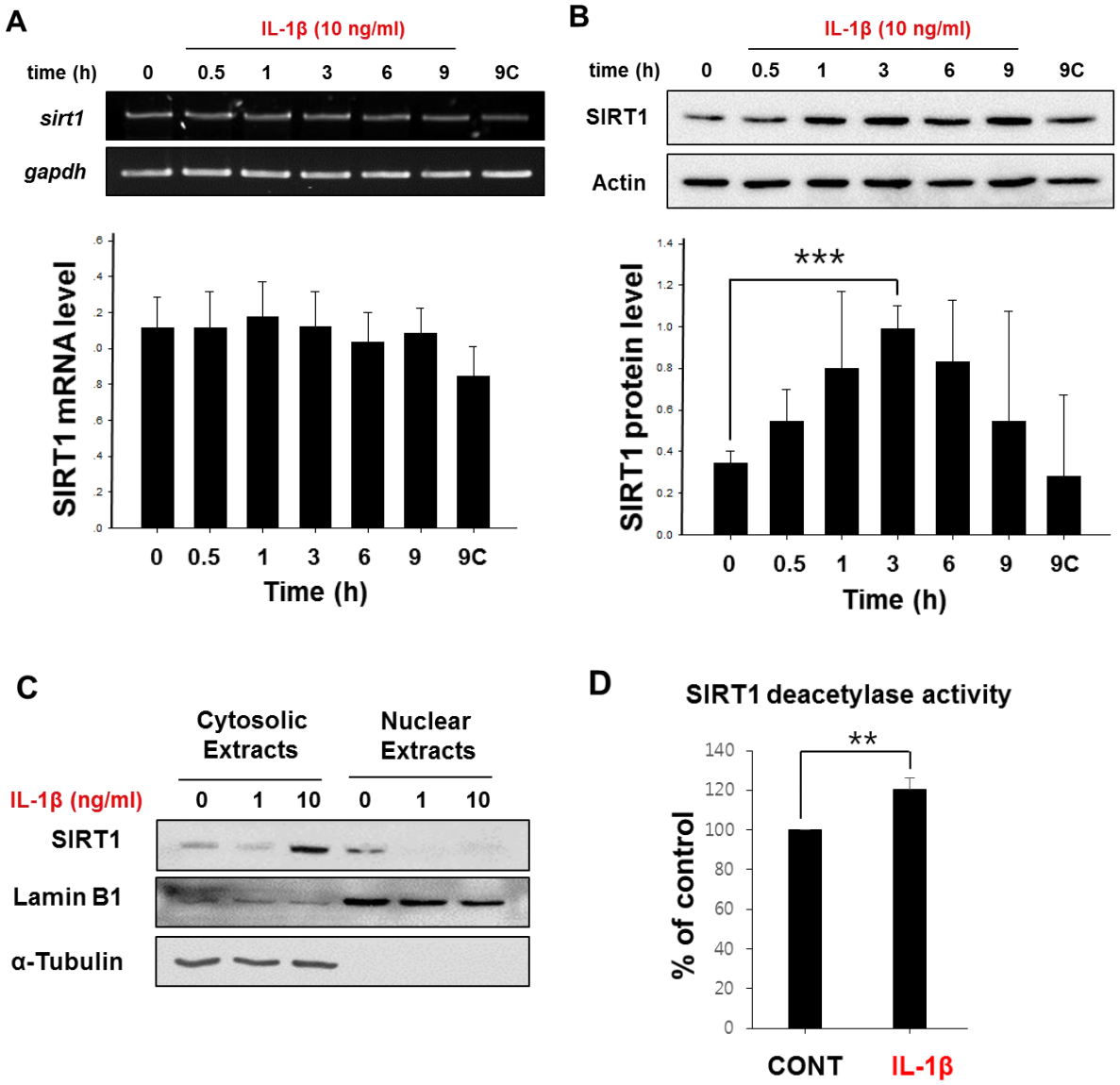

Figure 2. Role of IL-1β in the expression of SIRT1 in the human colon cancer HCT-116 cells. (A and B) After 12 hours of starvation, HCT-116 cells were treated with IL-1β (10 ng/ml) or 0.1% BSA in PBS as control for indicated time periods. The mRNA and protein levels of SIRT1 were measured in whole cell lysates by RT-PCR (A) and Western Blot analysis (B), respectively. Quantification graphs of SIRT1 mRNA and protein expression level are shown. (C) HCT-116 cells were treated with IL-1β for 3h with indicated doses and cytosolic extracts were separated from nuclear extracts. Lamin B1 and α-Tubulin were included as loading controls for cytosolic and nuclear fractions, respectively. (D) SIRT1 deacetylase activity was measured after IL-1β treatment for 3 h. Data represent mean ± s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

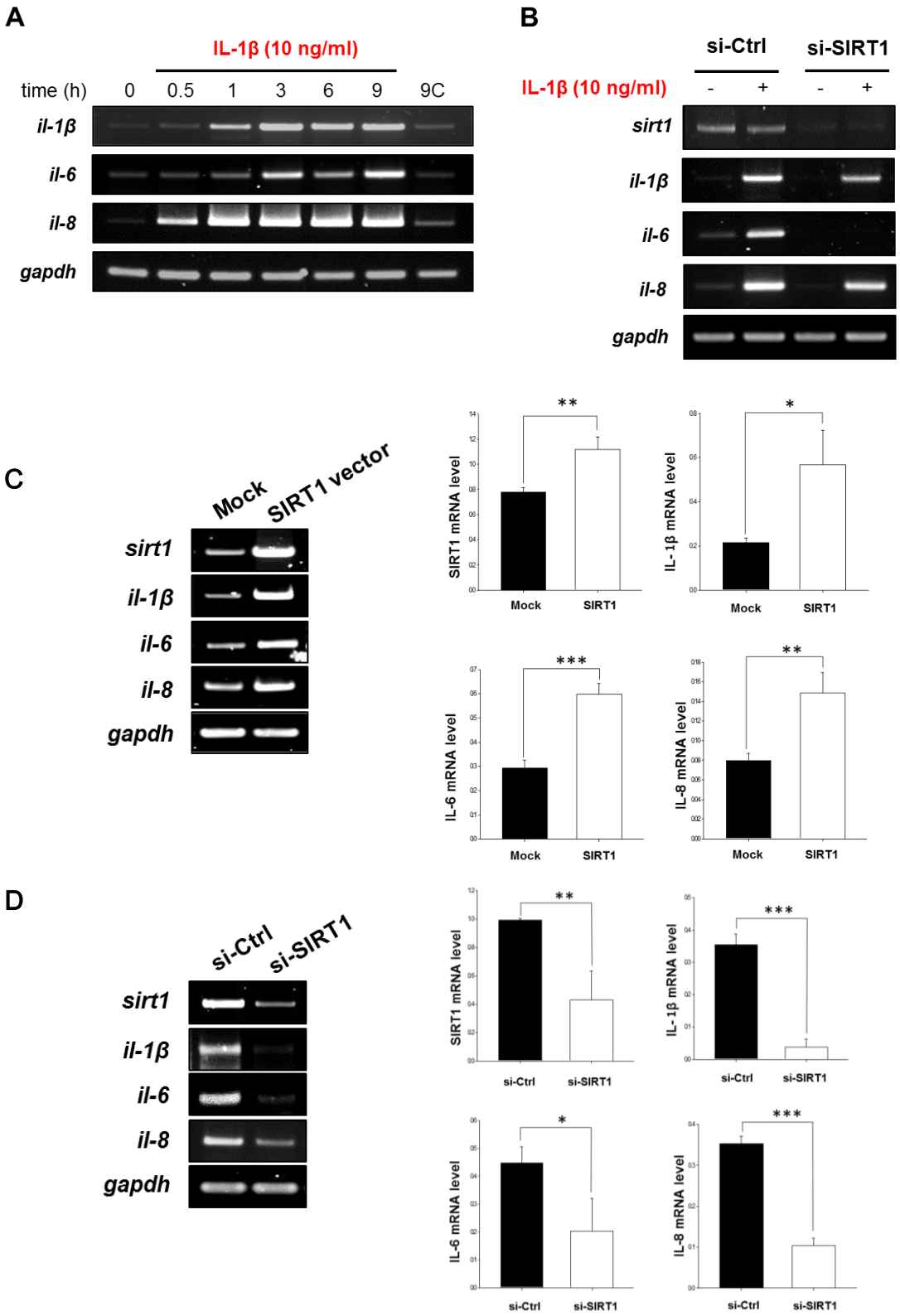

Figure 3. Role of SIRT1 in IL-1β-induced expression of pro-inflammatory cytokines. (A) Total RNA extracted from HCT-116 cells treated with IL-1β (10 ng/ml) was

subjected to RT-PCR with IL-1β, IL-6, and IL-8 sequence-specific primers. (B) HCT-116 cells were transfected with SIRT1 siRNA for 48 h, followed by IL-1β (10 ng/ml) treatment for 3 h. mRNA levels of SIRT1, IL-1β, IL-6, and IL-8 were determined by RT-PCR. (C and D) HCT-116 cells were transfected either with SIRT1 overexpression vector (C) or siRNA (D) for 48 h. Total RNA was then extracted and analyzed by RT-PCR to evaluate the mRNA level of IL-1β, IL-6, and IL-8. *SIRT1* was used as a positive control. Quantification graphs of SIRT1, IL-1β, IL-6, and IL-8 mRNA are shown. Data represent mean ± s.e.m * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$

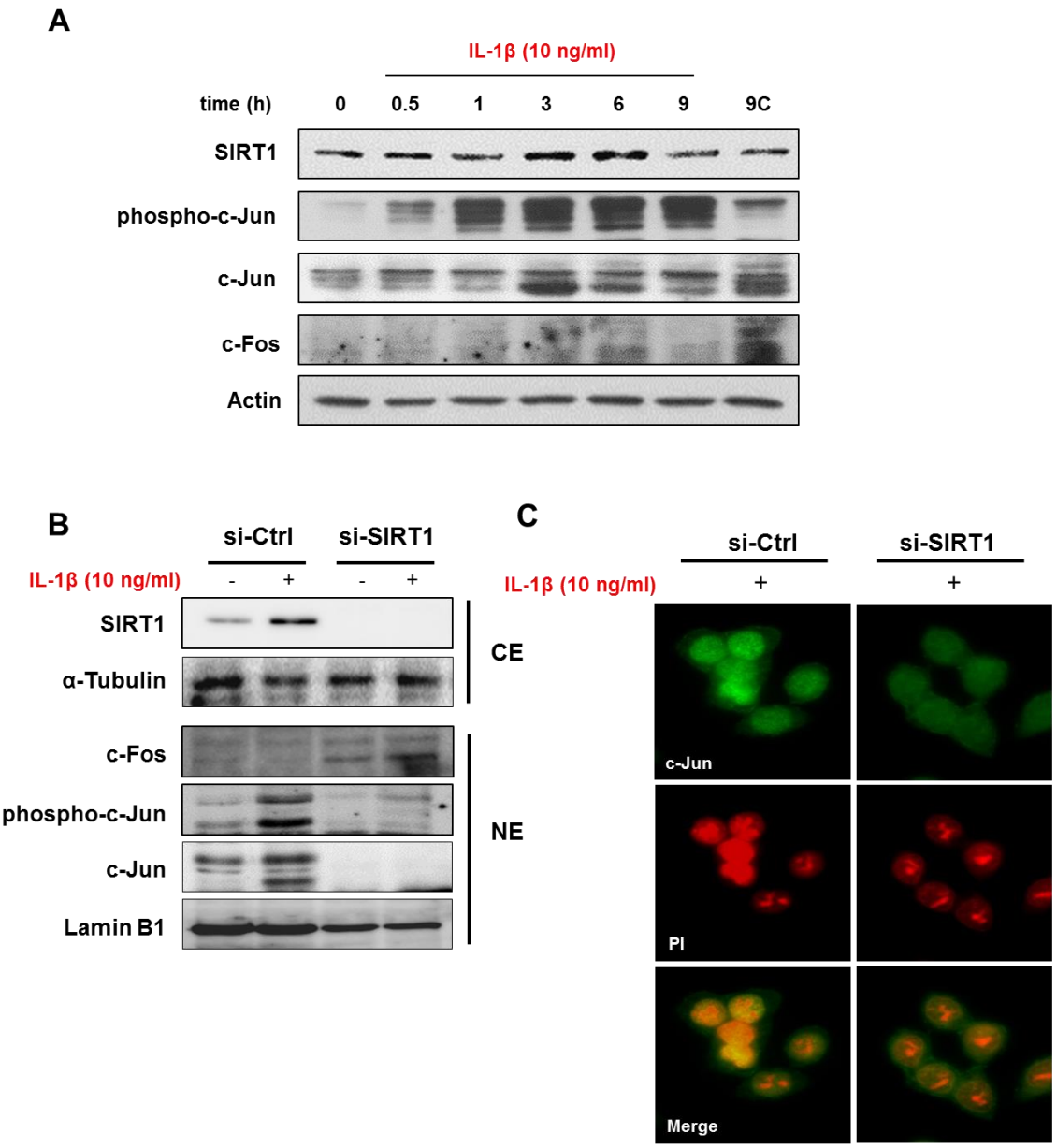

Figure 4. Effects of SIRT1 on nuclear accumulation and DNA binding activity of c-Jun. (A) Whole lysates from HCT-116 cells treated with IL-1β (10 ng/ml) at various time periods were subjected to Western blot analysis. (B) HCT-116 cells were transfected with SIRT1 siRNA for 48 h, followed by IL-1β (10 ng/ml) treatment for 3 h. Cytosolic and nuclear extracts were obtained and verified by Western blot analysis (C) HCT-116 cells were co-treated with SIRT1 siRNA and IL-1β. Decreased expression of c-Jun nuclear level was determined by immunocytochemistry.

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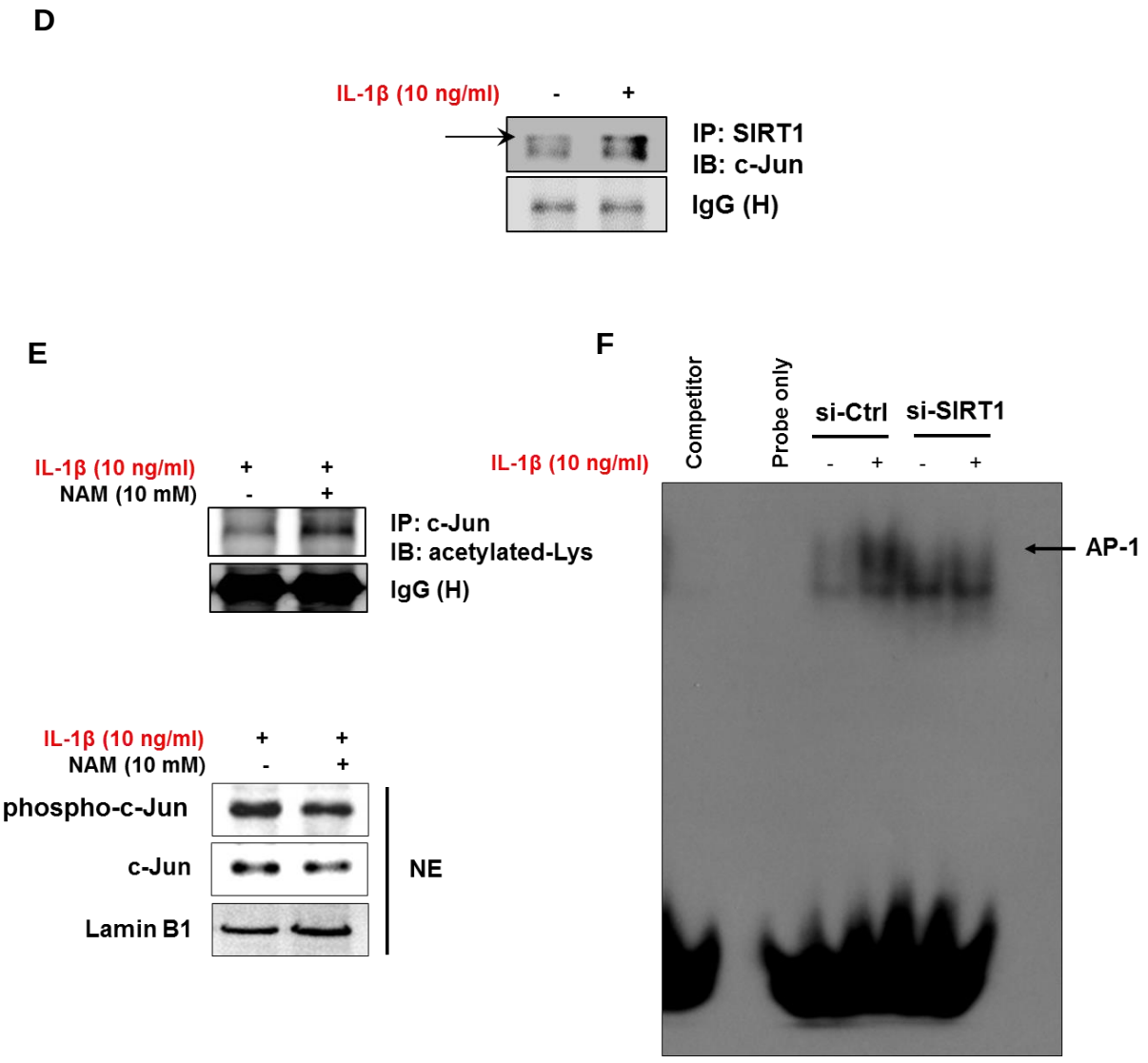

Figure 4. Effects of SIRT1 on nuclear accumulation and DNA binding activity of c-Jun. (D) HCT-116 cells were treated with IL-1β (10 ng/ml) for 3 h. nuclear extracts was immunoprecipitated with c-Jun antibody and analyzed by Western blot using SIRT1 antibody. IgG was included as loading control. (E) HCT-116 cells were pre-treated with nicotinamide (10 mM), a SIRT1 activity inhibitor, 1 h prior to IL-1β (10 ng/ml) treatment. c-Jun was immunoprecipitated with the c-Jun antibody and analyzed by Western blot with the acetyl-lysine antibody. IgG was included as loading control. After the same treatment in HCT-116 cells, nuclear extracts were obtained and verified by Western blot analysis. (F) Nuclear

extracts were incubated with the [γ-^{32}P]-labeled oligonucleotides containing AP-1 consensus sequence. Protein-DNA complexes were separated from the free probe by electrophoretic mobility shift assay.

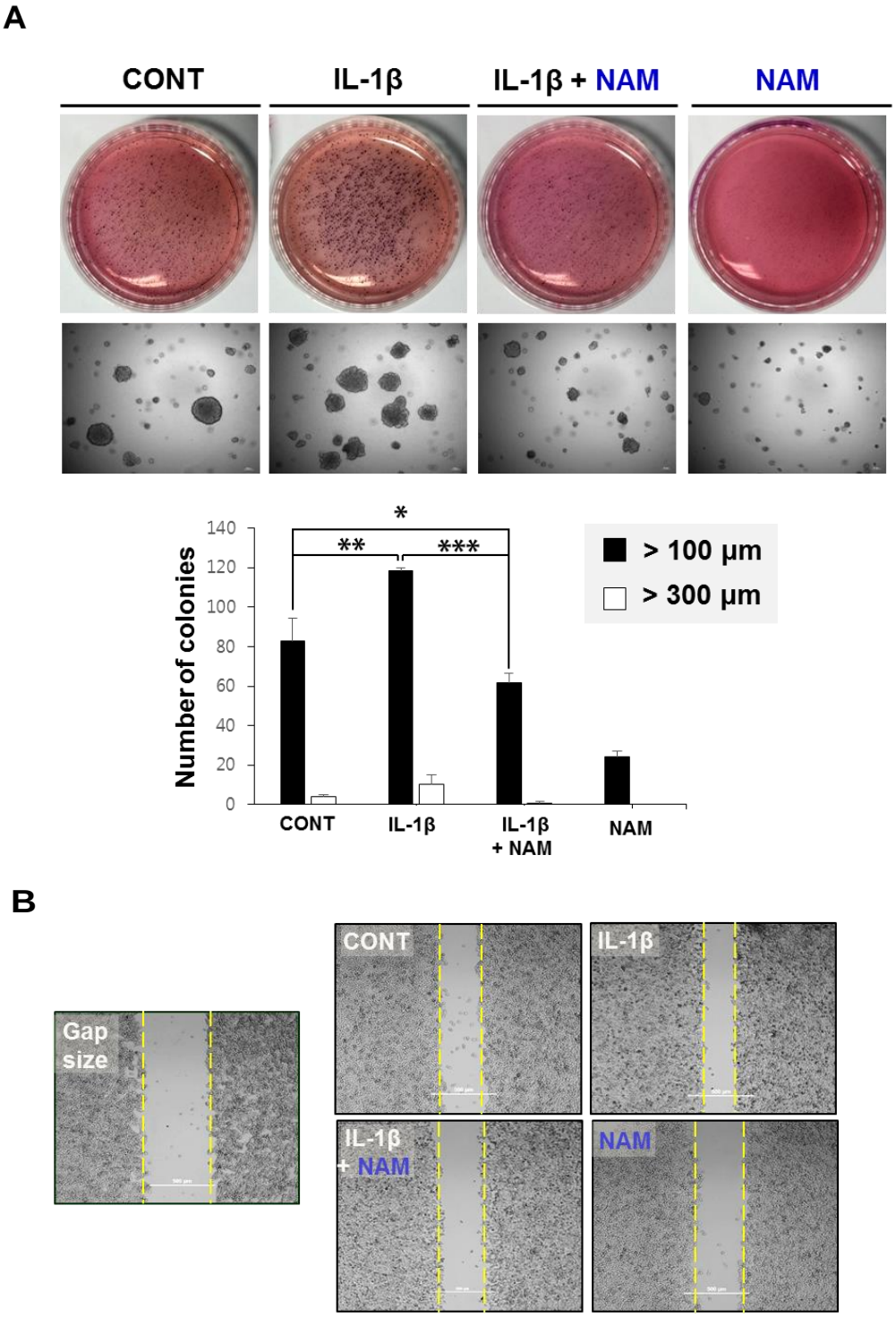

Figure 5. The inhibitory effect of nicotinamide on IL-1β-induced colony formation and cell migration. (A) HCT-116 cells were seeded at 2.5 x 10^5 cells per well in complete medium in a 60 mm dish. After 48 h of incubation, cells were treated with IL-1β in the presence or absence of nicotinamide (10 mM) for 7 days. The number of colonies

bigger than 100 μm or 300 μm was counted under a microscope and quantification graph of the colony number in three independent experiments is shown. Data represent mean ± s.e.m. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (B) HCT-116 cells were exposed to IL-1β alone or in combination with nicotinamide (10 mM) for 18 h and the cell migration was assessed by measuring the width of un-migrated space under a microscope.

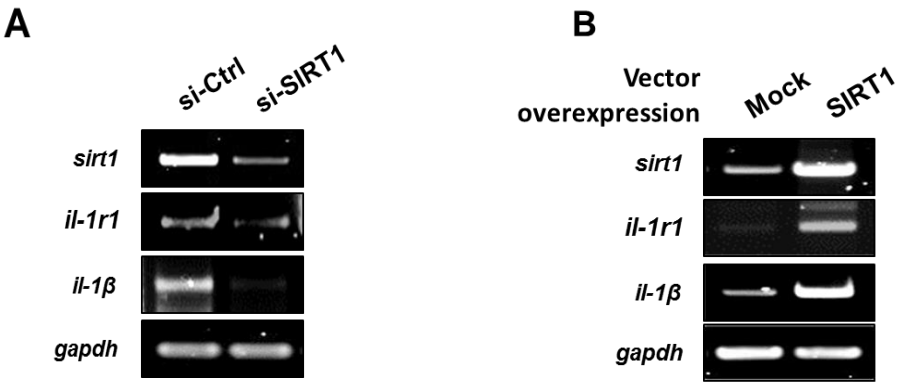

Figure 6. Effect of SIRT1 on expression of IL-1R1, a specific receptor of IL-1β. (A and B) Total RNA extraction from HCT-116 cells transfected with either SIRT1 siRNA (A) or overexpression vector (B) were subjected to RT-PCR with IL-1β and IL-1R1 sequence-specific primers.

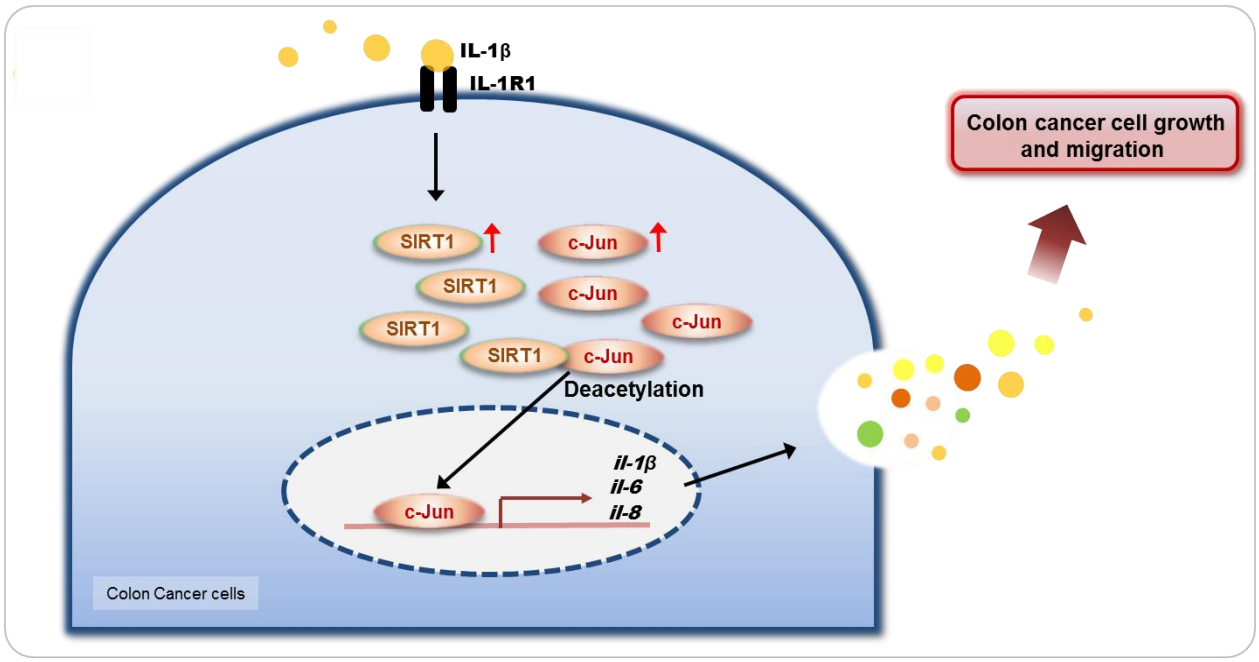

Figure 7. A proposed mechanism underlying the possible role of IL-1β-induced SIRT1 in transcription of pro-inflammatory cytokines in human colon cancer HCT-116 cells. The study proposes a mechanism responsible for induction of pro-inflammatory cytokines by IL-1β-induced SIRT1 via deacetylation of c-Jun in HCT-116 cells. The crucial role of SIRT1 as a mediator of IL-1β-induced production of pro-inflammatory cytokines is suggested.

Discussion

Although the function of SIRT1 in tumor is still under debate, I noticed overexpression of SIRT1 in colon cancer cell lines, such as HCT-116, HT-29, HCT-15, DLD-1, and SW480. Other colon cancer cell lines such as SW620, RKO and LoVo also expressed high levels of SIRT1 mRNA or protein, supporting its oncogenic function in colon cancer (Ford, Jiang, and Milner 2005). Consistent with the overexpressed SIRT1 level in colon cancer cell lines, the reduced colony formation in HCT-116 cells after treatment of SIRT1 activity inhibitors further supports that SIRT1 indeed serves as an oncoprotein. Recently, several studies have proposed that the function of SIRT1 may be determined by its subcellular localization (Byles et al. 2010; Song and Surh 2012). Thus, SIRT1 is predominantly localized in the nucleus of normal cells while it is mainly localized in the cytosol of cancer cells. Based on these findings, subcellular localization of SIRT1 in HCT-116 cells was checked. Notably, I also confirmed that SIRT1 is localized in the cytosol, which might function as a tumor promoter.

Until now, not a single paper has explored cytokine-induced SIRT1 overexpression in a cancer model. The present study focuses on the possible induction of SIRT1 by IL-1β. Interestingly, IL-1β induced upregulation of SIRT1 protein expression is not related to its mRNA expression. According to a preliminary study from this laboratory, c-Jun N-terminal kinase (JNK) is a possible mediator of IL-1β-dependent SIRT1 stability. Ford and Nasrin have proposed that JNK1/2 increases SIRT1 stability and enzymatic activity by phosphorylating SIRT1 at its specific residues (Ford et al. 2008; Nasrin et al. 2009). Since JNK has been reported as one of the major downstream kinase of IL-1β (Wuyts et al. 2003), SIRT1 stabilization induced by the JNK pathway is highly plausible. However, further investigation upon IL-1β-induced SIRT1 mediated by JNK should be required.

This study is considered novel as it provides an insight into mechanism underlying regulation of pro-inflammatory cytokines, such as IL-6, IL-8, and IL-1β by SIRT1 in cancer. Overproduction of these cytokines is believed to increase colon cancer cell growth and migration. I speculate that, AP-1, a transcription factor composed primarily of c-Jun and c-Fos

as a positive mediator in this process. However, the relationship between SIRT1 and AP-1 has not yet been clarified. Ren et al. have reported that SIRT1 positively regulates c-Jun subunit of AP-1 transcription factor, resulting in induction of HBV transcription and replication in human hepatoma cells (Ren et al. 2014). On the other hand, SIRT1 suppresses AP-1 transcriptional activity via deacetylase activity in macrophages (Zhang et al. 2010). By demonstrating that overexpressed SIRT1 induced by IL-1β directly binds to c-Jun, and increases nuclear accumulation and DNA binding activity of the protein, it was confirmed that IL-1β-induced SIRT1 overexpression positively regulates AP-1 in this colon cancer model. Moreover, as c-Jun is highly influenced by knockdown of SIRT1, I speculated that SIRT1 activity plays a significant role in this pathway. In contrast to our findings, several studies support that SIRT1 deacetylation results in suppressed activity of c-Jun (Zhang et al. 2010; Zhang et al. 2009). Since it appears that the role of SIRT1 varies depending on the cell types (Rahman and Islam 2011), the inconsistency between these studies may be due to different cell types and experimental conditions.

While the present study suggests a possible positive regulation of c-Jun by SIRT1 daecetylation, it is still unclear how the deacetylation induces c-Jun nuclear accumulation. Here I suggest three possible underlying mechanisms how SIRT1 deacetylation increases c-Jun nuclear accumulation. First, SIRT1 may deacetylate and prevent nuclear export of c-Jun. It is reported that SIRT1-mediated deacetylation impedes nuclear export of FoxO1 and SOX2 in hepatocytes and bone marrow-derived mesenchymal stem cells, respectively (Frescas, Valenti, and Accili 2005; Yoon et al. 2014) Similar to the aforementioned proteins, SIRT1 may possibly extend c-Jun nuclear residency. Next, SIRT1 deacetylation may contribute to stabilization of c-Jun. For instance, it is known that matrix metalloproteinase-2 (MMP2), which is a member of zinc-dependent endopeptidases that degrade extracellular matrix to promote cancer cell invasion, is deacetylated and stabilized by SIRT1 in prostate cancer cell lines (Lovaas et al. 2013). Moreover, DNA methyltransferase 1 (DNMT1), which is the crucial enzyme that maintains DNA methylation, is also stabilized by deacetylation mediated by other class of HDAC, HDAC1 and thereby protecting DNMT1 from degradation in

various cancer cells (Du et al. 2010). In a similar manner, SIRT1 may deacetylate and stabilize c-Jun, thereby preventing it from undergoing degradation. Lastly, SIRT1 may protect c-Jun from its inhibitor by deacetylating the c-Jun region targeted by a specific repressor. Vries et al. have proposed that a specific kind of repressor, E1A, requires acetylation in c-Jun basic region in order to repress its transcriptional activity (Vries et al. 2001). Based on these findings, SIRT1 may positively regulate c-Jun by removing the acetyl group from the site of c-Jun that is targeted by repressors. Further investigation on the effect of SIRT1 deacetylation on c-Jun should be conducted to address a specific function of SIRT1 on c-Jun nuclear accumulation.

Taken all together, the above findings suggest that IL-1β-upregulated SIRT1 might stimulate production of pro-inflammatory cytokines, such as IL-6, IL-8 and IL-1β, via c-Jun nuclear accumulation in human colon cancer HCT-116 cells. IL-1β and SIRT1 would be consistently overexpressed in this scheme, and this positive feedback mechanism might further support the development of colon cancer. According to the results from this study and those done by others, I propose that SIRT1 may function as a crucial mediator in colon cancer promotion and progression.

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국 문 초 록

SIRT1은 히스톤 (histone) 및 단백질 탈아세틸효소 (deacetylase)로, 세포 주기, 칼로리 제한 등의 다양한 생체 내 신호 전달에 관여한다. 이러한 SIRT1의 발암과정에서의 역할은 분분하다. 하지만 최근 학계에 발표된 연구 결과에 따르면, SIRT1은 다양한 암세포 및 암조직에서 높은 수준으로 발현되어 있으며, 이것이 발암과정을 가속화할 것이라고 보여지고 있다.

인체 대장암세포에 과발현되어 있는 IL-1β는 친염증성 사이토카인으로써, 전이나 신혈관생성 인자들의 분비를 통해 종양의 촉진과 진행에 중요한 역할을 한다고 알려져 있다. 이렇듯 IL-1β 와 SIRT1 모두 발암과정에 관여한다고 알려져 있지만, 두 단백질이 협력하였을 때 어떠한 기전을 통해 발암과정에 기여하는지는 증명된 바가 없다. 따라서, 본 연구에서는 두 단백질의 발암과정 촉진 효과를 탐구하고 그 기전을 규명하고자 하였다.

인체 대장암 상피 세포 (HCT-116)에 IL-1β를 처리하였을 경우, SIRT1의 단백질 발현이 증가되어 있음을 확인하였다. 또한, 대장암 조직의 여러 소분자들이 암세포의 성장을 촉진시킨다는 보고에 기반하여, 본 연구에서는 IL-1β에 의해 과발현된 SIRT1이 다른 친염증성 사이토카인의 생성을 촉진시킬 수 있는지 규명하고자 하였다. 과발현된 SIRT1은 친염증성 사이토카인으로 알려져 있는 IL-6, IL-8과 IL-1β의 mRNA 전사를 증가시켰다. 이러한 과정에서, SIRT1이 친염증성 사이토카인 전사조절인자 AP-1의 구성요소인 c-Jun 을 조절함을 확인하였고, 이 것이 SIRT1과 c-Jun간의 직접적인 결합에 의한 것임을 Immunoprecipitation assay를 통해 알 수 있었다. siRNA를 이용하여 SIRT1 유전자를 knockdown시킨 경우, IL-1β 처리에 의해 유도된 c-Jun의 핵 내 이동 및 DNA 결합능력이 현저히 억제되었다. 이러한 현상은 SIRT1 활성 억제제인 nicotinamide (NAM)에 의해서도 제한되었고, 이는 곧 SIRT1이 c-Jun을 조절함에 있어서 탈아세틸화 활성 (deacetylase activity)이 중요한

역할을 할 것임을 시사한다. Anchorage independent growth assay 및 wound healing assay에서 IL-1β에 의해 유도된 인체대장암 세포의 증식 및 이동성이 nicotinamide에 의해 억제되는 현상 역시 SIRT1의 탈아세틸화 활성이 중요함을 뒷받침한다. SIRT1은 IL-6와 IL-8, IL-1β 뿐만 아니라, IL-1β의 수용체인 IL-1 Receptor 1의 mRNA 전사를 증가시킴을 확인하였고, 이에 따른 IL-1β 와 SIRT1 간의 positive feedback loop이 형성되어 암화과정에 기여하는 것으로 사료된다.

결론적으로, 종양미세환경에 과발현되어 있는 IL-1β는 다른 친염증성 사이토카인의 분비를 증가시켜 암화를 촉진시킬 수 있는데, SIRT1은 이 기전의 중요 매개체로 작용하여 이 유전자들의 전사인자인 c-Jun의 핵 내 이동을 조절함으로써 발암 기전을 제어할 수 있다. 따라서 SIRT1의 활성을 조절함으로써 종양미세환경에서 분비되는 다양한 물질들에 의한 발암 과정 제어의 가능성을 제시하는 바이다.