The role of JMJD4 in the regulation of cancer cell death

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The role of JMJD4 in the regulation of cancer cell death

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2년의 석사 학위 과정 동안 도움을 주셨던 많은 분들께 감사의 말씀 전합니다. 먼저 지도교수님으로써 많은 가르침과 도움을 주신 윤호근 교수님께 감사의 마음을 전합니다. 부족한 저를 믿고 기다려 주시며 좋은 결과를 낼 수 있도록 많은 기회와 관심을 주셔서 무사히 학위 과정을 마칠 수 있었습니다. 교수님의 도움으로 많이 성장할 수 있는 시간이 되었습니다. 그리고 논문을 완성하기 까지 많은 조언을 해주신 이재면 교수님, 전경희 교수님께도 감사 드립니다. 교실원으로써 다양한 것을 배울 수 있게 해주신 생화학 분자생물학 교실의 박상욱 교수님, 김건홍 교수님, 김경섭 교수님, 허만욱 교수님, 김재우 교수님께도 감사의 말씀 드립니다.

석사 과정 동안 가장 가까이서 저에게 도움을 주시고 많은 격려를 해주신 실험실 분들께도 감사한 마음을 전합니다. 대학원에 입학하고 졸업할 때까지 많은 도움을 주신 정윤오빠, 실험에 대한 조언과 관심을 주시고 제가 올바르게 연구할 수 있게 이끌어 주신 수연언니, 실험 결과가 좋지 않을 때 진심으로 위로해 주신 미정언니께 감사합니다. 그리고 좋은 본보기가 되어 지금도 미국에서 열심히 연구를 하고 계실 재성오빠와 미현언니, 그리고 모르는 것이 있으면 잘 알려주신 승현오빠에게도 감사합니다. 또한 실험실에 잘 적응하고 저의 고민을 잘 들어 주시고 같이 해결해 주려고 도와주신 수연언니, 비슷한 또래로써 즐거운 실험실 생활을 할 수 있게 해 준 현식오빠, 경은이 에게도 고마운 마음을 전합니다. 그리고 같은 동기로써 많이 의지되고 도움을 준 선호 언니와 현주언니 정말 감사합니다. 그 외에도 도움을 주신 다른 교실원들께도 감사의 마음 전합니다.

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ABSTRACT

The role of JMJD4 in the regulation of cancer cell death

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PDCD5 is known as the key regulator of apoptosis. Decreased expression of PDCD5 has been reported in various cancers. The regulation of PDCD5 stability is positively correlated with p53-dependent apoptosis and cell death. Thus, deciphering regulatory mechanism of PDCD5 function is needed for better understanding p53 signaling pathway and apoptosis.

In this study, JMJD4 was identified as a novel binding protein of PDCD5 by mass spectrometry analysis. The interaction between JMJD4 and PDCD5 was verified by co-immunoprecipitation (co-IP) and GST-pull down analysis. The level of PDCD5 protein is negatively regulated by overexpression of JMJD4, however, the levels of JMJD4 were not affected regardless of PDCD5 expression. Negative regulation of PDCD5 by JMJD4 promotes resistance to apoptosis by inhibiting p53 pathway. Colony formation assay demonstrates that JMJD4 promotes cell proliferation through reduction of PDCD5 and resistance to etoposide (ET). Therefore, this study demonstrates that JMJD4 negatively regulates cancer cell death and is provided as a potential therapeutic target for drug resistance.

Key words : JMJD4, PDCD5, cancer, p53 signaling, apoptosis

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I. INTRODUCTION

Apoptosis, a type of cell death, is the programmed cell death means it is genetically controlled[1]. In particular, apoptosis occurs in many biological processes including cell turnover, normal development, cell proliferation and cell death caused by pathologic stimuli[2]. Dysregulation of apoptosis is a hallmark of many cancers[3]. Most tumor cells are resistant to apoptosis by decreased apoptotic proteins such as p21 and BCL2 associated X, apoptosis regulator (BAX) which is controlled by p53[4]. In cancer cells, the importance of

apoptosis is emphasized more as cancer therapeutic strategy.

Programmed cell death 5 (PDCD5), also called TFAR19 (TF-1 cell apoptosis related gene-19), is a member of program cell death proteins family involved in tumor growth and cell death. The *PDCD5* gene was originally cloned from the human leukemia cell line TF-1[5]. Low expression of PDCD5 has been showed in various cancers, including breast[6], lung[7], liver[8], gastric[9], and ovarian cancer[10]. Expression of PDCD5 inhibits progression of lung carcinoma[11], and osteosarcoma cell metastasis[12]. When apoptosis is induced by various stimuli, PDCD5 stabilizes and translocates from the cytoplasm to the nucleus[13]. Overexpression of PDCD5 was shown to increase p53, p21, BAX, and caspase-3, but decrease the expression of BCL-2[14].

It has been shown that the stability of PDCD5 is regulated by several proteins. Casein kinase 2 (CK2) phosphorylates PDCD5 at serine 118 and increases the stability of PDCD5[15]. The histone acetyltransferase (HAT), Tip60 is stabilized by interacting with PDCD5 and increases expression of p53 dependent apoptotic genes by K120 acetylation of p53[16]. Serine/threonine kinase 31 (STK31) enhances stability of PDCD5 and activates p53 signaling pathway through positive regulation of PDCD5-mediated apoptosis[17]. Moreover, the serine/threonine phosphatase PPEF-1 dephosphorylated at Ser-119 of PDCD5, which led to destabilization of PDCD5[18]. YY1-associated factor 2 (YAF2) binds to PDCD5 and enhances its stability by inhibiting the ubiquitin-dependent proteasomal degradation pathway[19].

PDCD5 enhances the stability of p53 by interacting with p53 through inhibition of MDM2-induced p53 degradation[20]. Tumor suppressor p53 is an important transcriptional factor that is activated by stress signals, including DNA damage. They play a critical role in the regulation of cell cycle, apoptosis, senescence, DNA repair, and angiogenesis[21, 22]. Under normal states, p53 is degraded by MDM2 which targets p53 via ubiquitin-proteasome[23]. However, when the stress signal turns on, the p53 protein is activated and stabilized by post-translational modifications, which leads to a program that induces cell cycle arrest, cell aging or cell death[24]. Stabilization and activation of p53 regulate downstream target genes associated with cell cycle arrest and apoptosis such as *p21*, *PUMA*, and *BAX*[25, 26]. Increased expression of these apoptotic genes causes cell cycle arrest, induces apoptosis, and prevents cancer cell proliferation.

Jumonji domain-containing protein 4 (JMJD4) is located on chromosome 1q42.13 and mainly existed in the cell membrane. Proteins containing the jumonji domain are iron and 2-oxoglutarate-dependent oxygenases, which hydroxylate or demethylate substrates in an oxygen-dependent state[27]. Majority of jumonji domain proteins are involved in human physiological processes, including development, cancer, inflammation and metabolic disease[28, 29]. It has been reported that depletion of JMJD4 significantly reduces cell proliferation in mouse NIH3T3 fibroblasts[30]. Mouse *Jmjd4* gene expression is diminished upon embryonic stem cell (ESC) differentiation. Also, *Jmjd4*-null embryonic stem

cells exhibited normal colony morphology and expressed the normal pluripotent genes[31]. Biochemical catalysis of JMJD4 has been reported to be involved in translation termination by hydroxylating the translational termination factor carbon-4 lysyl eRF1 in vitro[32]. However, the biological function of JMJD4 is still unclear.

Recent studies showed that high expression of JMJD4 is associated with poor survival in colon adenocarcinoma[33]. JMJD6, an important paralog protein of JMJD4, is markedly up-regulated in various types of human cancer especially in colon cancer and represses transcriptional activity of p53 by catalyzing hydroxylation at lysine 382 of p53[34]. Elevated expression of JMJD6 has been shown in many tumor tissues, including breast[35], colon[36], lung[37, 38], and oral cancers[39]. Moreover, other isoform, JMJD5, was significantly up-regulated in colon cancer compared with normal tissues[40].

In this study, JMJD4 was identified as a new interacting protein of PDCD5 by mass spectrometer analysis. GST-pull down assay showed that JMJD4 directly interacts with PDCD5. Overexpression of JMJD4 decreased PDCD5 at the protein level, which leads to inhibition of p53 signaling pathway. Finally, JMJD4 promoted cell proliferation and resistant to chemoresistance.

II. MATERIALS AND METHODS

1. Cell culture and reagents

Human colorectal carcinoma cell line (HCT116), human lung adenocarcinoma cell line (A549) and human embryonic kidney cell (HEK293FT) from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 units/ml penicillin and 0.1 mg/ml streptomycin (Hyclone, Logan, UT, USA) at 37ºC under 5% CO_2. Etoposide was purchased from Sigma-Aldrich (St. Louis, MO, USA). Etoposide was prepared in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA). Transient transfection was performed using TransIT 2020 (Mirus, Madison, WI, USA) and Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA).

2. Plasmid and cloning

Wild-type, full length JMJD4 was generated by PCR and cloned into plasmid vectors pCDNA 3.1-Myc-his A (Invitrogen, Carlsbad, CA, USA) or pSG5- Flag plasmid vector. Wild type, full length growth factor independent 1 transcriptional repressor (GFI1), Pim-2 proto-oncogene, serine/threonine kinase (PIM2) was generated by PCR and cloned into plasmid vectors pSG5-Flag plasmid vector. Wild type, full length PDCD5 was generated by PCR and

cloned into plasmid vectors pSG5-HA plasmid vector or pSG5-Flag plasmid vector. All plasmid constructs were verified by DNA sequencing.

3. Mass spectrometry

The eluted immune complexes were precipitated with 20% trichloroacetic acid and the pellets were washed four times with cold acetone. The precipitated proteins were resuspended in 100 mM ammonium bicarbonate (pH 8.0) with 10% acetonitrile and incubated with sequencing-grade trypsin (Promega, San Luis Obispo, CA, USA) at a concentration of 12.5 ng/ml at 37°C for 4 hours. Trypsin reactions were quenched by addition of 5% formic acid, and peptides were desalted using the C18 Stage Tip method. For each liquid chromatography–MS/MS analysis, 4 μl of sample was loaded onto an EASY-Spray C18 column (Thermo Scientific, Waltham, MA, USA) and eluted using a 90 minutes 8–26% acetonitrile gradient. Mass spectra were acquired with an LTQ Orbitrap XL linear ion trap mass spectrometer (Thermo Scientific, Waltham, MA, USA) using a data-dependent Top-10 method. Each sample was shot twice in succession, followed by a wash with 70% acetonitrile and 30% isopropanol. MS/MS data was analysed using the Coon OMSSA Proteomics Software Suite[41]. Z-score is representative of the identification of candidate interacting proteins. Total Spectral Count (TSC) is for each identified protein from each immunoprecipitation–MS/MS experiment.

4. Site-directed mutagenesis

The various deletion constructs were created by High-Fidelity DNA Polymerases & Master Mixes (Thermo scientific, Waltham, Massachusetts, USA), DNA templates, 10 pM primer were mixed for PCR reaction. PCR cycling conditions used in site directed mutagenesis were 30 cycles of amplification of following reaction: after initial denaturation at 94 ºC for 4 minutes, denaturation at 94 ºC for 30 seconds, annealing 55 ºC for 30 seconds, and extension at 72 ºC for 45 seconds. And final extension was performed at 72 ºC for 5 minutes. Amplified mixtures were treated with *Dpn* I (Aglient Technologies, Santa clara, CA, USA) at 37 ºC for 1 hour 30 minutes and PCR products were used to transform competent *E.coli* (Real Biotech Corporation, Banqiao, Taiwan). All the constructs were confirmed by DNA sequencing.

5. GST fusion proteins purification and in vitro translation

GST or GST fusion protein expression was purified from *E. coli* BL21 (DE3) (Real Biotech Corporation, Banqiao, Taiwan) transformed with GST or GST fusion proteins expression plasmid. The *E. coli* were induced with 0.025 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA) for 72 hours at 16°C. The cells were lysed by sonication in MBP buffer containing 20 mM Tris-HCL (pH 7.4), 150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 1 mM DTT. The GST-tagged recombinant proteins were purified using Glutathione-agarose 4 bead (Peptron, Daejeon, Korea). The purified proteins

were resolved with SDS-PAGE to quantitate and assess purity. The same amount of aliquot of the protein-agarose bead complex was used in GST-fusion protein pull down assays.

All *in vitro* translations were performed by TNT T7 quick coupled transcription/translation kit according to the manufacturer's protocol (Promega, San Luis Obispo, CA, USA). Plasmid DNA template, TNT T7 quick master mixture and [^{35}S]-methionine (1,175.0 Ci/mol, PerkinElmer Life Science Inc., Waltham, MA, USA) were mixed and incubated at 30℃ for 90 minutes.

6. GST-pull down assay

The purified GST fusion proteins (5μg) were incubated with Glutathione-agarose 4 bead (Peptron, Daejeon, Korea) for 16 hours at 4°C and washed three times with 1 ml MBP buffer. After that, 10 μl of the *in vitro* translated products were added and incubated in HEMG buffer at 4°C for 16 hours. The reaction mixtures were centrifuged at 2,000 rpm for 3 minutes at 4°C. The supernatants were removed and the pellets were washed five times with cold MBP buffer. The bound proteins were eluted by heating at 100°C for 5 minutes with 5x sample buffer and separated by a SDS-PAGE. The SDS-PAGE gel was dried and exposed to X-ray film using image-intensifying screen (Kodak, Rochester, NY, USA).

7. Immunoprecipitation

Cells were lysed in lysis buffer. After centrifugation, 500 μg of the clarified cell lysate was pre-cleared with G plus/protein A-agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) by incubating for 1 hour. The supernatant was collected and 1 μg of antibody was added. After overnight incubation at 4℃, 20 μl of 50% slurry of G plus/protein A-agarose was added and the mixture was incubated for 1 hour. The agarose bead was centrifuged, washed four times with ice-cold lysis buffer, and suspended in electrophoresis sample buffer, and boiled for 5 minutes. The immunoprecipitated protein was further analyzed by Western blotting.

8. Western blot analysis

Cells were lysed in lysis buffer (20 mM Tris-Cl, 150 mM NaCl, 1% Triton X-100, 1.5% $MgCl_2$, 1 mM EDTA, 1 mM Na_2VO_4, 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor cocktail, pH 7.5). Lysates were briefly vortexed and sonicated and cleared by centrifugation at 13,000 rpm for 20 minutes at 4℃. The supernatants were collected and transferred to fresh tubes. Protein concentrations were determined by 660 nm protein assay reagent (Thermo Scientific, Waltham, MA, USA). Equal amount of protein extracts was subjected to electrophoresis on SDS-polyacrylamide gels and then transferred to Nitrocellulose transfer membranes (Whatman, Dassel, Germany). The membranes were blocked in PBS containing 0.1% (v/v) Tween 20

(Sigma-Aldrich, St. Louis, MO, USA) and 5% (w/v) nonfat Difco™ skim milk (BD Biosciences, Sparks, MD, USA) and 3% BSA (Affymetrix, Santa clara OH, USA) and probed with primary antibodies. The following antibodies were used: anti-JMJD4 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA)), anti-PDCD5 (Proteintech, Chicago, IL, USA), anti-HA, anti-p53 (DO-1), anti-Myc, anti-BAX (AbCam, Cambridge, MA, USA), anti-PUMA (Abcam, Cambridge, MA, USA), anti-Flag, anti-β-actin (Sigma-Aldrich, St. Louis, MO, USA). The signals were developed by substrate (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol.

9. RNA isolation and Reverse Transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was extracted using Trizol reagent following the manufacturer's protocol (Takara Bio Inc, Otsu, Shiga, Japan). The 500 ul of Trizol was added to cells culture dishes (6 well) and cells were collected to tube. The 100 ul of chloroform was added to samples and the samples were vortexed. The samples were incubated for 3 minutes at room temperature and centrifuged at 12,000 rpm for 15 minutes at 4℃. The 250ul of supernatant was collected and transferred to fresh tube. The 250 ul of isopropanol was added to samples and samples were centrifuged at 12,000 rpm for 10 minutes at 4℃. The supernatant was removed and the 500 ul of 75% ethanol was added to samples. The sample was centrifuged at 13,000 rpm for 5 minutes at 4℃. The supernatant was

removed and pellet was dried at room temperature. The 30 ul of DEPC was added to dried pellet. The concentration of RNA was measured by Nanodrop1000 (Thermo scientific, Waltham, MA, USA). After RNA isolation, the 1ug of total RNA was mixed with 10 pM oligo dT and 2.5 mM dNTP (Takara Bio Inc, Otsu, Shiga, Japan). The samples were heated for 5 minutes at 65℃. Then, 5X buffer, MMLV-Reverse transcriptase (Takara Bio Inc, Otsu, Shiga, Japan) and distilled water were added to samples. The samples were incubated to anneal at 40℃ for 1 hour and to transcript at 65℃ for 10 minutes in PCR cycler (BioRad, Hercules, CA, USA). RT-qPCR was performed using the ABI PRISM 7000 Sequence Detection System instrumentation and software (Applied Biosystems, Carlsbad, CA, USA) according to the manufacture's protocol with minor modification. Briefly, the appropriate amount of the reverse transcription reaction mixture was amplified with specific primers (Table 1) using SYBR green PCR master mix (Applied Biosystems, Carlsbad, CA, USA). Expression levels of genes were determined by generating a five point serial standard curve. The concentration of cDNA was normalized by GAPDH. All reactions were performed in triplicate. Relative expression levels and SD values were calculated using the comparative method.

Table 1. Primers of RT-qPCR

GAPDH	F-5'-CCCATGTTCGTCATGGGTGT-3'
	R-5'-TGGTCATGAGTCCTTCCACGATA-3'
JMJD4	F-5'-AACCCCAAAGAGCACATGAC-3'
	R-5'-AGCCAGTCGGACGAGAAGTA-3'
PDCD5	F-5'-AAAGCACAGGGAAGCAGAAA-3'
	R-5'-TTGTCCATATCTTGCCATCTG-3'
BAX	F-5'-TCTACTTTGCCAGCAAACTGGTGC-3'
	R-5'-TGTCCAGCCCATGATGGTTCTGAT-3'

10. Colony forming assay

After transfection of plasmid or siRNA, 2 x 10^2 or 1 x 10^3 cells were seeded in 6 well plate (Cornng incorporated, Corning, NY, USA). The next day DMSO or 0.5 uM of etoposide is added. Cells were incubated 37℃ under 5% CO_2 for 10–14 days. After washing Colony with PBS, it was stained with 0.5% crystal violet. The number of colonies was counted and calculated as plate efficiency.

11. Statistical analysis

Statistical significance was examined using Student's *t*-tests. The two-sample *t* test was used for two-group comparisons. Values were reported as means ± standard deviations (SD). *P* values < 0.05 were considered significant.

III. RESULTS

1. JMJD4 directly interacts with PDCD5

Previous study showed that liquid chromatography–mass spectrometry (MS)/MS analysis was applied to identify binding partners of PDCD5[54] (Table 2). PIM2 and GFI1 were identified by performing Yeast-two hybrid screening[17].

In this study, to verify the interaction between PDCD5 and candidate proteins, Flag-tagged JMJD4, PIM2, GFI1 and HA-tagged PDCD5 were co-transfected into 293FT cells and immunoprecipitation analysis was performed with anti-HA antibody. As a result, JMJD4, PIM2, and GFI1 bound to PDCD5 (Figure 1A). Next, GST-pull down assay was performed to identify a protein directly binds to PDCD5 by using GST-PDCD5 fusion protein and *in vitro* translated proteins. This results showed that PDCD5 directly interacted with JMJD4 but not with PIM2 and GFI1 (Figure 1B). These results demonstrate that JMJD4 directly interacts with PDCD5.

Table 2. List of PDCD5 interacting proteins

Identified gene list	z-score
WD repeat domain 74 (WDR74)	13.27
Programmed cell death 5 (PDCD5)	12.98
Histone deacetylase 3 (HDAC3)	12.38
Jumonji domain containing 4 (JMJD4)	12.38
Transducin-like enhancer of split 3 (TLE3)	12.38
UBX domain protein 7 (UBXN7)	12.38
DDB1 associated factor 7 (DCAF7)	3.2
Replication factor C (activator 1) 2 (RFC2)	1.59
WD repeat domain 77 (WDR77)	1.13
TATA box binding protein (TBP)-associated factor, RNA polymerase I, C (TAF1C)	0.24
TAF15 RNA polymerase II, TATA box binding protein (TBP)-associated factor (TAF15)	0.08
Eukaryotic translation initiation factor 3, subunit I (EIF3I)	-0.02
Fused in sarcoma (FUS)	-0.14
RNA binding motif protein 14 (RBM14)	-0.18
Ewing sarcoma breakpoint region 1 (EWSR1)	-0.18
Histone cluster 1, H2ab (HIST1H2AB)	-0.22
Y box binding protein 1 (YBX1)	-0.3
TIA1 cytotoxic granule-associated RNA binding protein-like 1 (TIAL1)	-0.48
Eukaryotic translation elongation factor 1 alpha 1 (EEF1A1)	-0.72
Nucleolin (NCL)	-0.73
Nucleophosmin (NPM1)	-0.84

*Z-score is representative of the identification of candidate interacting proteins.

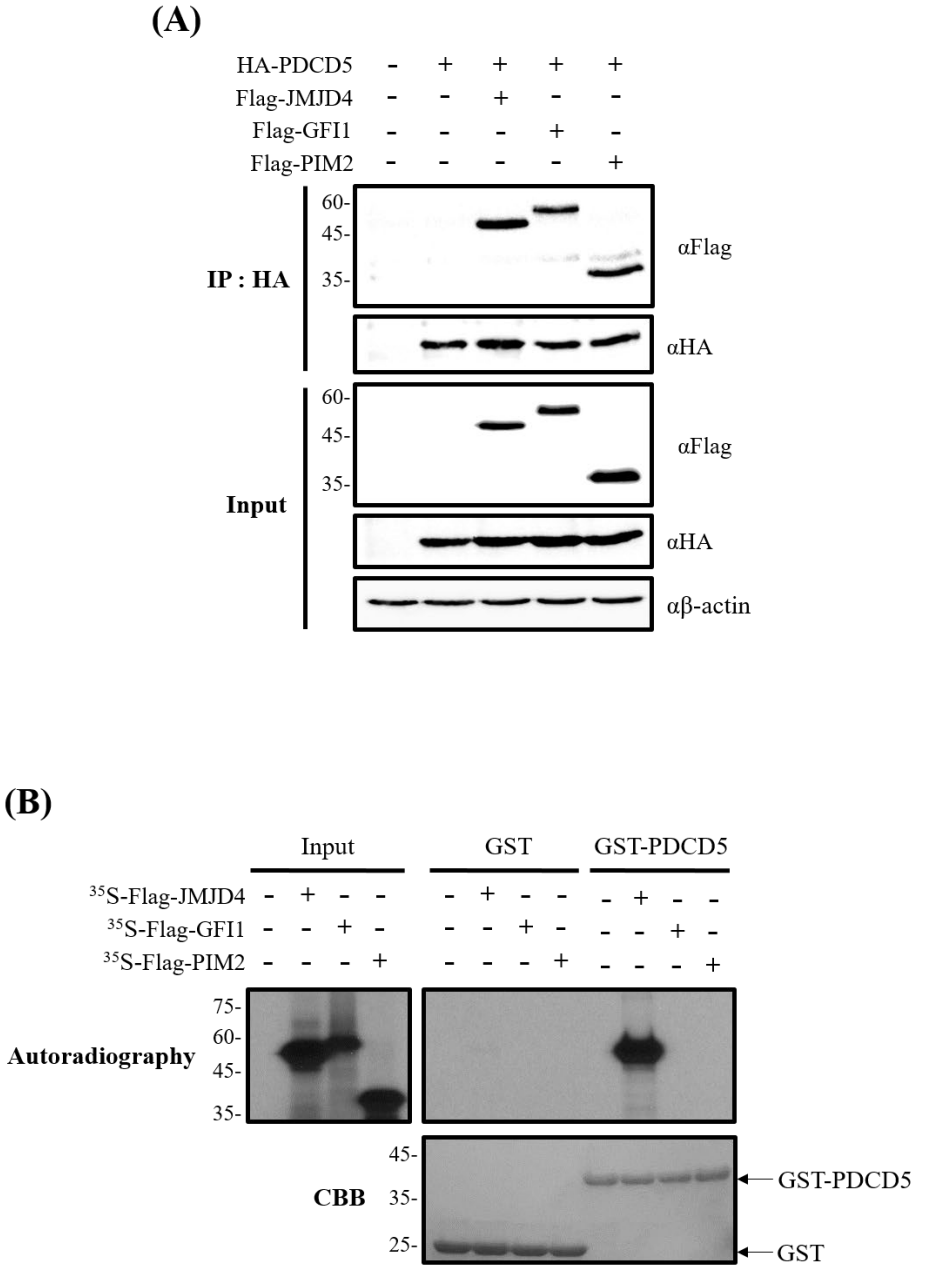
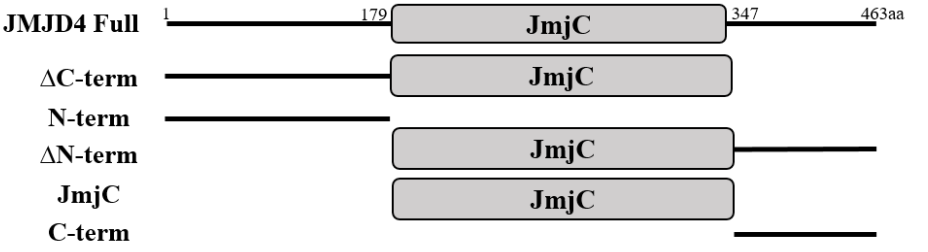


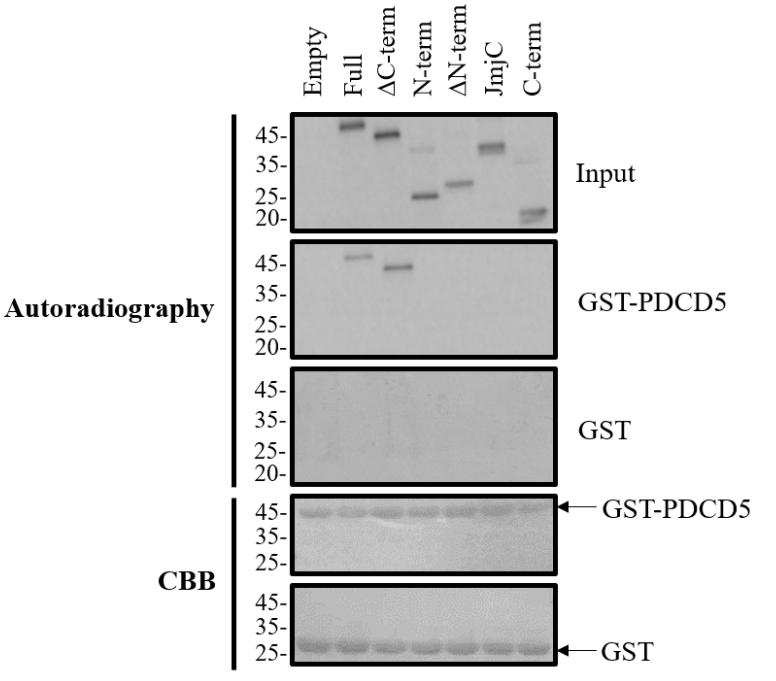
Figure 1. JMJD4 directly binds to PDCD5. (A) Immunoprecipitation analysis. HEK293FT cells were transfected with Flag-tagged candidate genes and HA-tagged PDCD5 plasmids. Whole cell lysates were immunoprecipitated with anti-HA antibody, and then immunoblotted with anti-Flag antibody. β-actin was used as a loading control. (B) GST-pull down analysis. GST-PDCD5 fusion protein was extracted in *E.coli* and ^{35}S labeled JMJD4, PIM2, GFI1 were synthesized by in vitro translation. ^{35}S labeled proteins were incubated with GST-PDCD5 proteins at 4℃ for overnight in MBP buffer. Sample were eluted and analyzed by SDS-PAGE and autoradiography.

2. JMJD4 directly interacts with PDCD5 through amino acids 180 to 221 of JMJD4

To elucidate the structure and function of PDCD5 and JMJD4 with respect to their interacting domains, the various fragments of the JMJD4 protein were *in vitro* translated, and their interaction with PDCD5 was assessed by GST-pull down analysis. As shown in Figure 2A, PDCD5 failed to interact to all constructs except the c-terminus-deleted JMJD4 (ΔC-term). To find more accurate binding region, *in vitro* binding assay was carried out with deletion constructs of JMJD4. As a result, the deletion construct of JMJD4 (Δ180-221) failed to interacts with GST-PDCD5 protein (Figure 2B). Taken together, amino acids 180 to 221 of JMJD4 directly interact with PDCD5.

(A)





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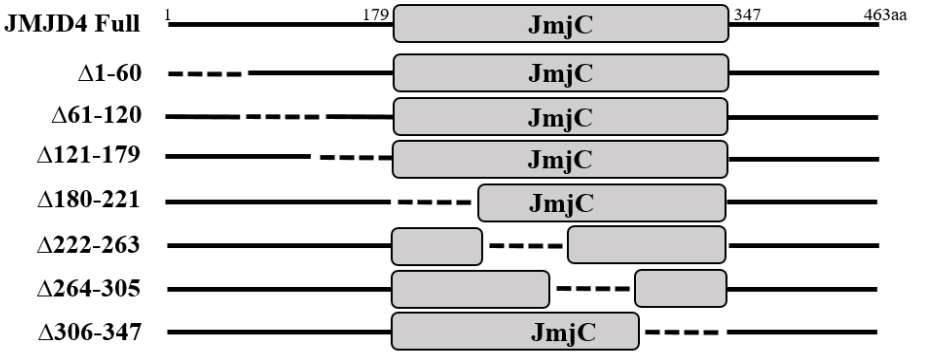


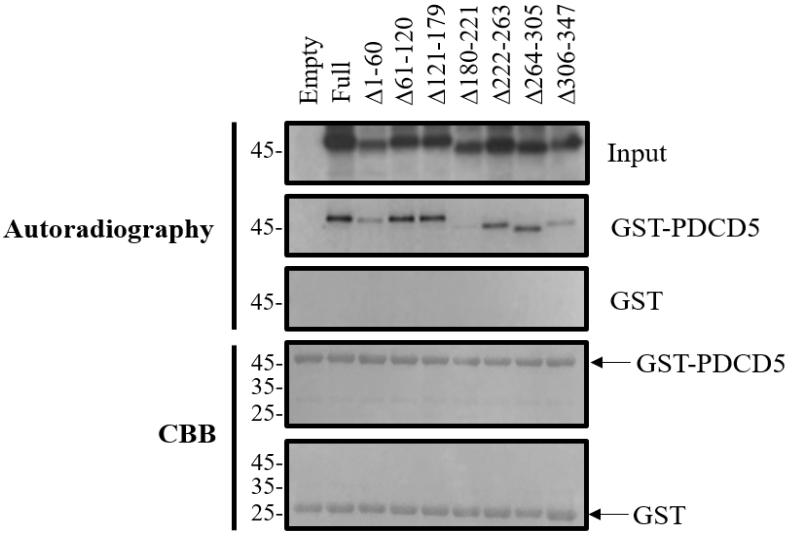


Figure 2. Amino acids 180 to 221 of JMJD4 interact with PDCD5. (A-B) GST-pull down analysis. The upper panels, a schematic diagram shows the structure of JMJD4 and the deletion constructs used for the mapping experiments. *In vitro* pull down assay was carried out using GST-PDCD5 fusion protein and *in vitro* translated ^{35}S-labeled constructs of JMJD4. Binding reactions were performed at 4℃ for overnight in MBP buffer. Bound proteins were eluted and analyzed by SDS-PAGE and autoradiography.

3. JMJD4 decreases the level of PDCD5 protein

To determine whether JMJD4 regulates the levels of PDCD5 protein and mRNA, JMJD4 was transfected in HCT116 and A549 cells, and then PDCD5 level was observed by western blot analysis and RT-qPCR. The results showed that overexpression of JMJD4 reduces protein expression of PDCD5 by western blot analysis (Figure 3A). On the other hand, when the mRNA expression of PDCD5 was not changed by JMJD4 (Figure 3B). Furthermore, to determine whether JMJD4 is regulated by PDCD5, protein and mRNA expression levels of JMJD4 were assessed by western blot analysis and RT-qPCR when PDCD5 was overexpressed. The results showed that the both protein and mRNA level of JMJD4 were not changed by overexpressed PDCD5 (Figure 3C and D).

Next, to confirm the negative regulation of PDCD5 by JMJD4, knocking-down effect of JMJD4 on PDCD5 was investigated in HCT116 and A549 cells. JMJD4 knockdown was verified using #1 to #5 siRNAs by immunoblot assay and RT-qPCR. Since the both protein and mRNA levels of JMJD4 were efficiently reduced by JMJD4 siRNA #4, siJMJD4 #4 was selected for following experiments (Figure 4A and 4B). The results showed that PDCD5 was increased at protein level by knocking-down of JMJD4 (Figure 4C). As shown in Figure 3B, JMJD4 knockdown had no effect on the mRNA expression of PDCD5 (Figure 4D). On the other hand, knocking-down of PDCD5 leads to reduction of p53 but not JMJD4 in both HCT116 and A549 cells (Figure 4E). Consistently, the mRNA level of JMJD4 was not changed by knockdown of

PDCD5 (Figure 4F). These results suggest JMJD4 negatively regulates PDCD5 at the protein level.

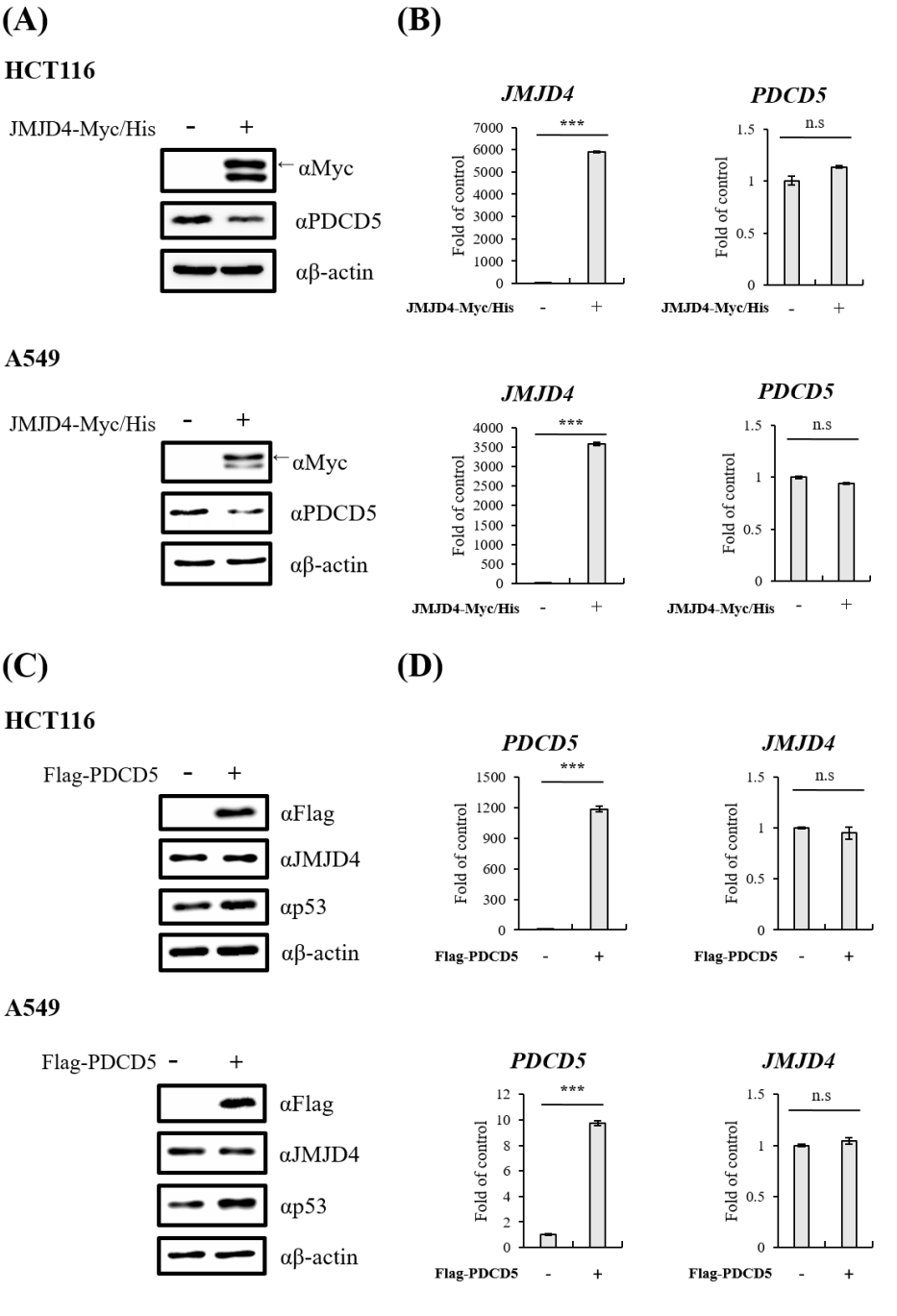
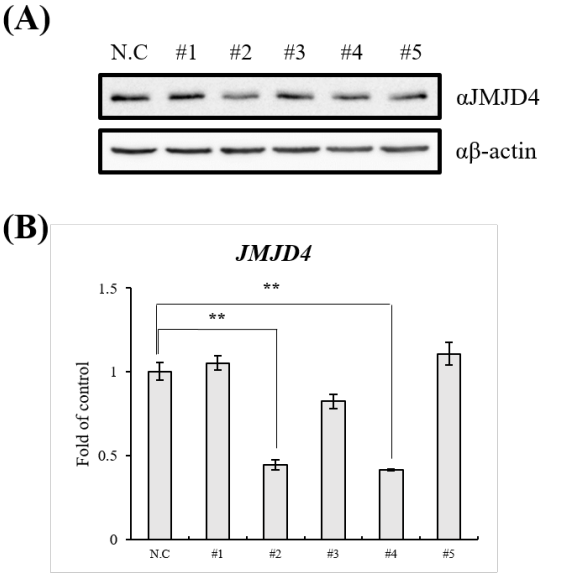
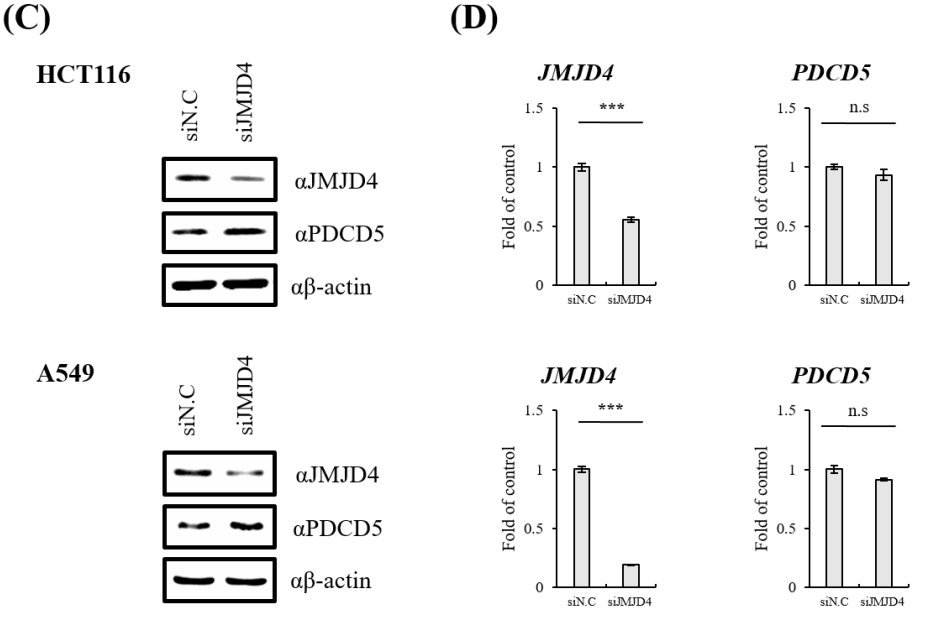


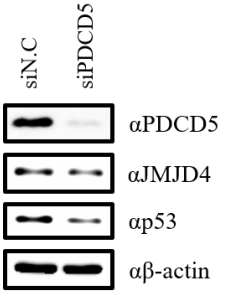
Figure 3. Overexpression of JMJD4 decreases the level of PDCD5 protein. (A) Western blot analysis. HCT116 and A549 cells were transfected with JMJD4-Myc/His. After transfection for 48 hours, harvested cells were immunoblotted with indicated antibodies. (B) RT-qPCR. RNA was extracted from transfected cells. After RNA isolation, cDNA was synthesized and mRNA expression level was measured by RT-qPCR. The level of *GAPDH* mRNA was normalized by RT-qPCR using the same RNA samples. Error bars, SD (n=3) ***p<0.001. (C) Western blot analysis. HCT116 and A549 cells were transfected with Flag-PDCD5. After transfection for 48 hours, cells were harvested, lysed, and immunoblotted with specific antibodies. (D) RT-qPCR. RNA was extracted from transfected cells. After RNA isolation, cDNA was synthesized and used for RT-qPCR. As a control, the level of *GAPDH* mRNA was normalized by RT-qPCR using the same samples. Error bars, SD (n=3) ***p<0.001.



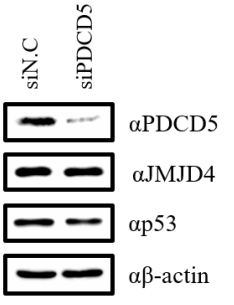


(E)

HCT116



A549



(F)

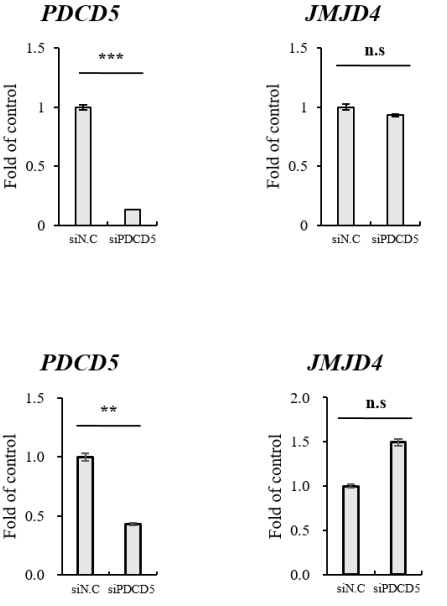


Figure 4. Knocking-down of JMJD4 increases the level of PDCD5 protein. (A) Western blot analysis. HCT116 and A549 cells were transfected with the indicated sets of 50 nM siRNA. After transfection for 48 hours, harvested cells were lysed and immunoblotted with indicated antibodies. (B) RT-qPCR. RNA was isolated from harvested cells, cDNA was synthesized and used for RT-qPCR. As a control, the level of *GAPDH* mRNA was normalized by RT-qPCR using the same RNA samples. (C) Western blot analysis. HCT116 and A549 cells were transfected with 300 nM JMJD4 siRNA #4 and incubated for 48 hours, and then cells were harvested and whole cells lysates were immunoblotted with indicated antibodies. (D) RT-qPCR. RNA was extracted from transfected cells. After RNA isolation, cDNA was synthesized and mRNA expression level was measured by RT-qPCR. As a control, the level of *GAPDH* mRNA was normalized by RT-qPCR using the same RNA samples. Error bars, SD (n=3) $^{**}p<0.01$, $^{***}p<0.001$. (E) Westernblot analysis. HCT116 and A549 cells were transfected with the PDCD5 siRNA #4. After transfection for 48 hours, cells were harvested, lysed and immunoblotted with specific antibodies. (F) RT-qPCR. RT-qPCR was done in PDCD5 siRNA #4 transfected cells. RNA was isolated from transfected cells and then cDNA was synthesized. As a control, the level of *GAPDH* mRNA was normalized using the same samples. Error bars, SD (n=3) $^{**}p<0.01$, $^{***}p<0.001$.

4. JMJD4 inhibits p53 signaling pathway via negative regulation of PDCD5

Since JMJD4 decreases the level of PDCD5 protein, it was hypothesized that JMJD4 negatively regulates p53 signaling by reduction of PDCD5 protein. To verify this, HCT116 cells were transfected with Myc/His-tagged JMJD4 plasmid and treated with etoposide (ET), a topoisomerase inhibitor. As a result, overexpression of JMJD4 reduced p53, and p53-target proteins as well as PDCD5 (Figure 5A). Reverse transcription polymerase chain reaction (RT-PCR) analysis also showed that the mRNA expression of *PUMA, BAX* was decreased by overexpression of JMJD4 (Figure 5B).

Next, the knocking-down effect of JMJD4 on p53 signaling was assessed upon genotoxic stress response. JMJD4 knockdown further increased the levels of PDCD5, p53, PUMA, and BAX in a response to genotoxic stress (Figure 6A). Similarly, mRNA expressions of *PUMA* and *BAX* were further increased by knocking-down of JMJD4 (Figure 6B). Collectively, JMJD4 inhibited p53 signaling pathway via negative regulation of PDCD5.

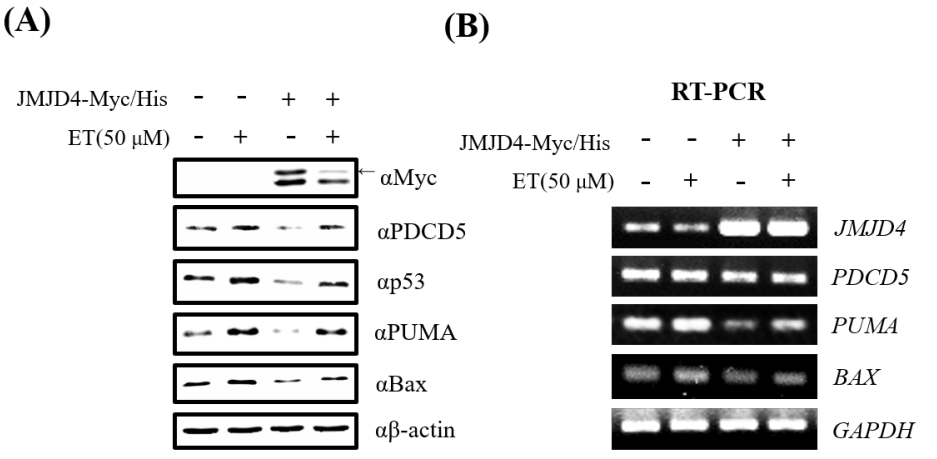


Figure 5. Overexpression of JMJD4 decreases p53 signaling via negative regulation of PDCD5. (A) Western blot analysis. JMJD4-Myc/His transfected HCT116 cells were treated with 50 μM of ET for 12 hours, and then immunoblot analysis was done with indicated antibodies. (B) RT-PCR. JMJD4-Myc/His transfected HCT116 were treated 50 μM of ET for 48 hours, total mRNA was prepared from each samples and used for RT-PCR to measure *PDCD5, PUMA, BAX* gene expression. As a control, the level of *GAPDH* mRNA was showed by RT-PCR using the same RNA samples.

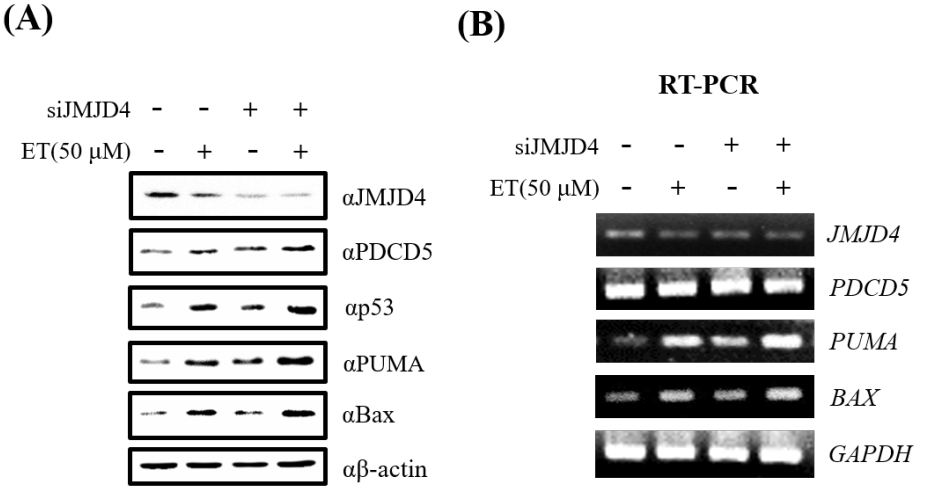


Figure 6. Knocking-down of JMJD4 increases p53 signaling via negative regulation of PDCD5. (A) Western blot analysis. JMJD4 siRNA #4 transfected HCT116 cells were treated with 50 μM of ET for 12 hours, and then immunoblot analysis was done with indicated antibodies. (B) RT-PCR. siRNA-mediated knockdown HCT116 were treated 50 μM of ET for 48 hours, total mRNA was prepared from each samples and used for RT-PCR to measure *PDCD5* and p53 dependent apoptotic gene expression. As a control, the mRNA level of *GAPDH* was showed by RT-PCR using the same RNA samples.

5. JMJD4 promotes cell proliferation via suppression of PDCD5

The previous results showed that overexpression of JMJD4 decreased PDCD5 at the protein level, which leads to inhibition of the p53 pathway. Finally, colony formation assay was performed to investigate whether JMJD4 affects cancer cell proliferation through negative regulation of PDCD5 and p53. As a results, overexpression of JMJD4 increased colony formation of HCT116 cells compared with the control HCT116 (Figure 7A). On the other hands, knocking-down of JMJD4 reduced colony formation compared to the control (Figure 7B). ET treatment reduced the cell proliferation, however, overexpression of JMJD4 overcame the ET-induced cell death (Figure 8A). Consistently, JMJD4 knockdown cell further enhanced the effect of ET on cell death (Figure 8B). Thus, these results suggested that JMJD4 increases chemoresistance by promoting proliferation of cancer cell.

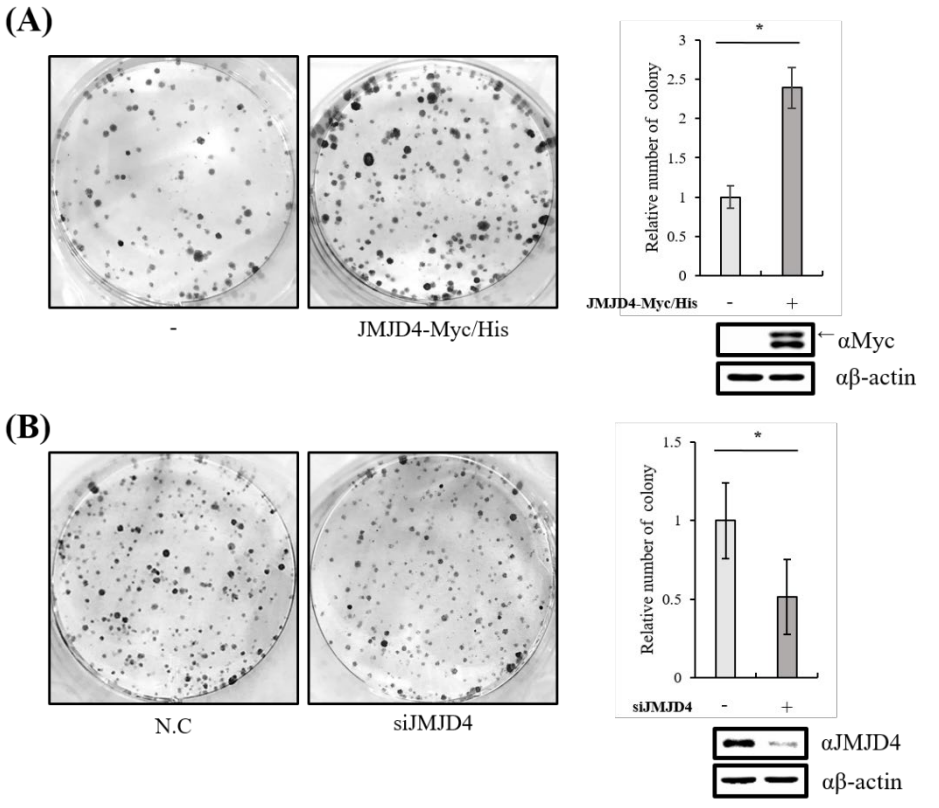


Figure 7. JMJD4 promotes colony formation. (A-B) Colony formation assay. HCT116 cells were transfected with JMJD4 expressing plasmid (A) or JMJD4 siRNA #4 (B). $2x10^2$ transfected cells were seeded in 6 well plate and incubated 10 days. Colonies were stained with 0.5% crystal violet and counted. Error bars, SD (n=3) *p<0.05.

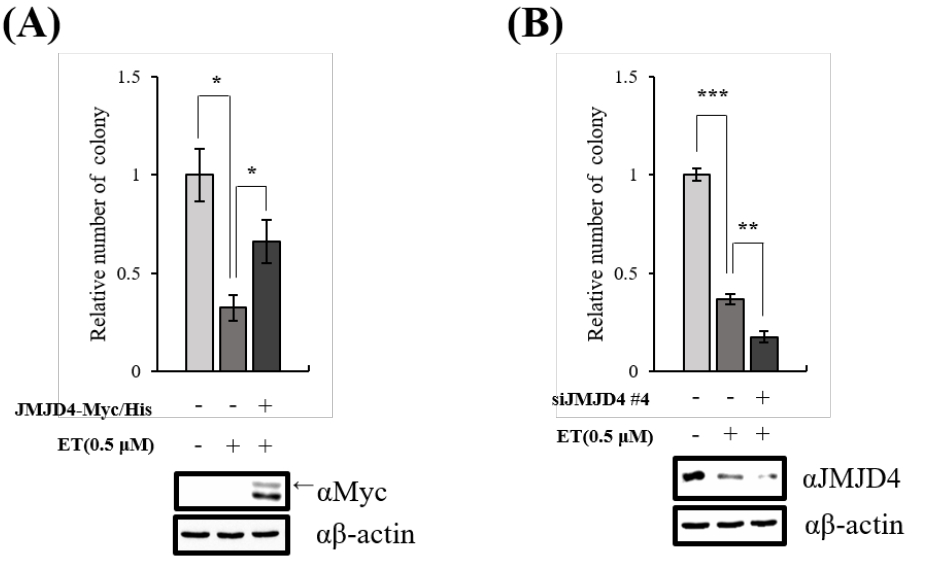


Figure 8. JMJD4 increases chemoresistance. (A-B) Colony formation assay. HCT116 cells were transfected with JMJD4 expressing plasmid (A) or JMJD4 siRNA #4 (B). $1x10^3$ transfected cells were seeded in 6 well plate. Next day, cells were treated with 0.5 μM of ET or DMSO (Control) and incubated for 14 days. Colonies were stained with 0.5% crystal violet and calculated with plate efficiency. Error bars, SD (n=3) *p<0.05, **p<0.01, ***p<0.001.

IV. DISCUSSION

Jumonji domain proteins are involved in numerous biological processes, including mammalian development and the epigenetic regulation of cancer by demethylation[42, 43] or hydroxylation [44]. The biochemical catalysis of JMJD4 has been reported that translational termination is enhanced by hydroxylating eRF1 carbon-4 lysyl *in vitro*[32]. Increased expression of JMJD4 and JMJD6 has been reported in various cancers[33-39]. JMJD6 which shares 34% sequence similarity with JMJD4 plays a critical role as a key regulator in transcriptional pause release[45], histone modification[46], and RNA splicing[47, 48]. However, there are no reports that JMJD4 protein is involved in the regulation of methylation of proteins including histones. Further study is needed to unravel the biological function of JMJD4.

In normal condition, apoptosis occurs during maturation and aging to maintain cell populations in tissues. However, imbalance of apoptotic genes is showed in various cancer cells. In the case of cancer cells, apoptotic genes are abnormally regulated, causing mutations or reduced expression[49]. PDCD5 is known as a protein that increases apoptosis by various stimuli[50, 51]. Expression of PDCD5 is lowered in various cancers, which is associated with tumor formation[52]. In HepG2 cells, stable transfection of the PDCD5 gene can inhibit the proliferation, invasion, cause cell cycle arrest in the G_2/M phase and enhance sensitivity to cisplatin[53]. This suggests that PDCD5 plays an important

role in inhibiting tumor development. PDCD5 has been reported to promote apoptosis by regulation of p53. PDCD5 increases stability of p53 by inhibiting Mdm2-mediated degradation[20]. In this study, JMJD4 was identified as a novel interaction protein for PDCD5 by mass spectrometry and validated by co-immunoprecipitation. GST-pull down assay showed that JMJD4 directly interacts with PDCD5 through amino acids 180 to 221 of JMJD4. The level of PDCD5 protein was altered by overexpressing or knocking-down JMJD4. In contrast, levels of protein and gene expression of JMJD4 were not changed regardless of PDCD5 expression. These results demonstrated that JMJD4 decreases the level of PDCD5 protein via direct interaction.

In a previous study, PDCD5 has been reported to interact with other proteins. In genetic toxic stress, PDCD5 enhances p53 activation via PDCD5-dependent HDAC3 dissociation[54]. DNAJB1 inhibits p53 activation by promoting the ubiquitination and degradation of PDCD5[55]. OTU deubiquitinase5 (OTUD5) interacts with PDCD5 and increases the PDCD5 stabilization by mediating deubiquitination of PDCD5 at lysine-97/98[56]. YY1-associated factor 2 (YAF2) binds to and increases PDCD5 stability by inhibiting the ubiquitin-dependent proteasomal degradation pathway[19]. According to these results, the stability of PDCD5 is increased by inhibition of ubiquitination. Therefore, it is necessary to investigate whether the JMJD4-mediated reduction of PDCD5 stability is correlated with ubiquitination of PDCD5.

PDCD5 is a positive regulator of p53 and is known to regulate apoptosis through the p53-mediated pathway[20]. The tumor suppressor p53 regulates transcription of downstream target genes involved in cell cycle arrest, apoptosis, DNA repair, senescence[57]. Numerous human cancers have mutations in the p53 gene[58]. Mutant p53 shows enhanced tumor progression, metastasis, and drug resistance[59]. Therefore, regulation of p53 and related target genes is essential for apoptosis. The p53 protein can activate various cellular processes, including cell cycle arrest, senescence, DNA damage repair, and apoptotic cell death. One of the p53 functions is as a transcription factor that regulates the expression of various responsive genes. For instance, p21 which is the cyclin-dependent kinase inhibitor is central role of p53-mediated cell cycle arrest[60]. The p53 activation promotes induction of BH3-only proteins (BIM, PUMA, NOXA) and then inhibits of BCL2 family members, increases apoptosis effectors such as BAX, BAK[61]. Thus, p53 dependent apoptotic markers were analyzed with overexpression or knocking-down of JMJD4. As a result, overexpression of JMJD4 decreased PDCD5 and p53, thereby reducing levels of pro-apoptotic signaling proteins. Similarly, knocking-down of JMJD4 promoted the activation of the p53 signaling pathway as well as increase of PDCD5 level. These results suggest that JMJD4 inhibits p53 signaling via negative regulation of PDCD5. Finally, overexpression of JMJD4 promoted colony formation and knocking-down of JMJD4 inhibits colony formation, suggesting that JMJD4 increases cell proliferation. Importantly, overexpression of JMJD4 reversed the

genotoxic effect of ET on cancer cell growth. In contrast, the knocking-down of JMJD4 further enhanced the genotoxic effect of ET. This suggests that JMJD4 increases chemoresistance by negative regulation of PDCD5-p53 pathway.

This study showed that JMJD4 inhibits p53 pathway and apoptosis through negative regulation of PDCD5 stability. Consequently, JMJD4-mediated negative regulation of PDCD5 promotes cancer cell proliferation, which consequently leads to chemoresistance. Majority of JMJD proteins play a role as demethylase or hydroxylase. Recently, it has been reported that JMJD6 mediates phosphorylation of Y39 at histone H2A.X by having tyrosine kinase activity[62]. Further study is necessary to examine whether JMJD4 negatively regulates PDCD5 in a demethylation or hydroxylation-dependent way. Based on our study, elevated expression of JMJD4 confers chemoresistance to cancer cell. Thus, JMJD4 may be a promising therapeutic target to overcome chemoresistance of cancer cell.

V. CONCLUSION

In this study, JMJD4 was identified as a novel binding protein of PDCD5 by mass spectrometry analysis. JMJD4 directly interacts with PDCD5 through amino acids 180 to 221 of JMJD4. JMJD4 negatively regulates PDCD5 at the protein level, which leads to inhibition of p53 signaling pathway. JMJD4 promoted cell proliferation and diminished genotoxic stress response. This study demonstrates the crucial role of JMJD4 on chemoresistance of cancer cell and potential therapeutic target for drug resistance.

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ABSTRACT (IN KOREAN)

암세포 사멸 시 JMJD4의 생물학적인 기능 연구

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장 수 빈

PDCD5는 세포사멸의 핵심 조절자로써 알려져 있다. 여러 암에서 PDCD5의 발현이 감소된 것이 보고 되어 있고, PDCD5 안정성의 조절은 p53에 의존하는 세포사멸과 관련 있다. PDCD5 기능의 조절 기전을 밝히는 것은 p53 신호 전달 경로와 세포사멸을 잘 이해하는 데 필요하다.

본 연구에서는, 질량분석법를 통해 PDCD5의 새로운 결합 단백질로 JMJD4를 발견하였고, JMJD4와 PDCD5 사이의 상호작용을 확인하였다. PDCD5 단백질 발현 수준은 JMJD4의 과발현에 의해 저해되었다. 그러나, JMJD4의 발현은 PDCD5의 발현과 관계 없이 영향을 받지 않았다. JMJD4에 의한 PDCD5의 조절을 통해 p53 신호 전달 경로를 막음으로써 세포사멸에 대한 저항성을 촉진시켰다. 또한, 콜로니 형성 분석으로 JMJD4가 PDCD5의 감소를 통해 세포 증식을 촉진시키고 etoposide에 저항성을 보였다. 그러므로, 본 연구는 암세포에서 JMJD4가 세포사멸을 저해시키고 약에 대한 저항성으로 잠재적인 치료 목표물이 될 수 있음을 설명하였다.

핵심되는 말: JMJD4, PDCD5, 암, p53 신호 전달, 세포사멸