석 사 학 위 논 문

ARD1-Mediated PRMT5 Acetylation Promotes Cancer Cell Proliferation

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2022년 02월

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이 논문을 석사학위 논문으로 제출함

2022년 02월

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2022년 02월

Acknowledgment

I would like to acknowledge and give my warmest thanks to my supervisor Professor Ji Hae Seo who made this work possible. Her guidance and advice carried me through all the stages of completing my project. I would also like to thank Professor Eunyoung Ha and Professor Chul-Ho Jeong who served as the judges and gave me a lot of recomendation. I would like to express my deep gratitude to my senior, Doctor Vo Thuy Lu Tam, who taught and supported me a lot to complete my thesis. I would like to express my gratefulness to all my seniors both being here and having left and lab members who helped, encouraged, and taught me a lot during my master course.

During my master course, I have learned a lot of things. They are not only the knowledge that I have been taught from my professors and seniors but also the way how to work independently as well as how to work together as a team to make great contributions to the science.

Despite long distance, I would like to sincerely thank my family for always supporting me to successfully complete this course.

I understand that the graduation of master course is not the end but the beginning of a longer journey for which a master's course is a preparation step.

I would like to dedicate this thesis to the people whom I have always loved and respected.

2022년 02월

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1. Introduction

Arrest-defective 1 (ARD1) was first discovered in *Saccharomyces cerevisiae* (1). ARD1 is the catalytic subunit of NatA acetyltransferase (2,3) which was originally identified as an N-terminal acetyltransferase and then a lysine-acetyltransferase. ARD1 acetylates its target proteins by adding an acetyl molecule at N-termini of protein or ε-amino group of lysine residues. Recently, ARD1 was reported to acetylate the ε-amino group of lysine residues of many proteins such as β-catenin, heat shock protein 70 (Hsp70), methionine sulfoxide reductase A (MSRA), phosphoglycerate kinase 1 (PGK1), SAM domain and HD domain-containing protein 1 (SAMDH1), aurora kinase A (AuA), and Runx2 (4-11). Through lysine acetylation of these proteins, ARD1 regulates diverse cellular functions, such as cell cycle progression, cell motility, apoptosis, autophagy, and cellular stress response (5-11).

Protein arginine N-methyltransferase 5 (PRMT5) is a member of the protein arginine methyltransferase family (PRMTs), which catalyzes the transfer of one or two methyl groups to the guanidine nitrogen atoms of arginine. PRMT5 is type II PRMT which catalyzes the formation of symmetric di-methylation of arginine residues. PRMT5 is involved in a variety of biological processes, such as transcription, differentiation, and spliceosome assembly via histone and non-histone proteins methylation. Recently, several evidence suggested that PRMT5 is highly expressed in various types of cancer, including lung cancer, colorectal cancer, pancreatic cancer, hepatocellular cancer, and ovarian cancer (12-23). PRMT5 serves as an oncoprotein by regulating proliferation, invasion, and

differentiation of cancer cells (13,20,21,24,25).

In mammalian cells, PRMT5 was reported to localize in both the cytoplasm and the nucleus (26). Depending on the subcellular location, PRMT5 is supposed to have distinct cellular functions. For example, cytoplasmic PRMT5 is known to promote cancer cell growth, whereas nuclear PRMT5 suppresses the cancer cell growth (27). However, the underlying molecular mechanism for PRMT5 translocation is unknown.

Here, I found that ARD1, which is a binding partner of PRMT5, directly acetylates PRMT5 at K490 residue and that this modification is a key regulator of subcellular localization and functional activation of PRMT5. In this study, I elucidated that ARD1-mediated PRMT5 acetylation retains PRMT5 localization in the cytoplasm to promote cell proliferation by activation of NF-κB pathway.

2. Materials and Methods

2.1. Cell culture and transfection:

Human ovarian adenocarcinoma cell line OVCAR-8 (OV8) and human pancreatic adenocarcinoma cell line HPAC obtained from american type culture collection (ATCC, Manassas, VA, USA) were cultured in Roswell Park Memmorial Institute (RPMI) and Dulbecco's Modlfled Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotics, respectively.

Wild-type and mutated peGFP-c3-ARD1 were introduced to the OV8 and HPAC cells using lipofactamine 2000 (Invitrogen, Waltham, MA, USA). Wild-type and mutated PMX-IRES-PRMT5 were transduced to the cells to establish the stable cells by following the constructions for virus system transduction.

2.2. Immunoblotting:

Cells were extracted and lysed for 15 min in radioimunoprecipitation assay (RIPA) buffer (Thermo Scientific, Waltham, MA, USA) supplemented with protease and phosphatase inhibitors. The lysates were centrifuged at 13000 rpm for 15 min at 4 °C. Bicinchoninic acid (BCA) protein assay was used to determine the concentration of the lysates. Total 30 μg of protein lysate was electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) before being transferred to a membrane for further blotting with specific antibodies.

Antibodies for ARD1, PRMT5, and CDK2 were purchased from Abcam (Waltham, MA, USA). Cyclin D1, cyclin B1, cyclin E2, and acetylated-lysine antibodies were purchased from Cell signaling (Danvers, MA, USA).

2.3. Immunocytochemistry:

Cells grown on glass coverslips were fixed for 15 min with 4% paraformaldehyde (PFA) and then permeabilized for 10 min with 0.1% Triton X-100, followed by being incubated overnight at 4 °C with PRMT5 antibody. Cells were then labeled for 1 hr with an Alexa594-conjugated secondary antibody (Jackson Immuno Research Laboratories, West Grove, PA, USA), and coverslips were mounted on a drop of ProLong Diamond antifade solution containing 4',6-diamino-2-phenylindole (DAPI) (Thermo Scientific). Cells were observed under confocal microscopy (Carl Zeiss, Oberkochen, Germany).

2.4. Quantitative real time-polymerase chain reaction (qRT-PCR):

The TRizol reagent (Thermo Scientific) was used to extract total RNA of cells according to the manufacturer's instructions. The purity and concentration of RNA were measured using NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). qRT-PCR analysis was conducted using a LightCycler 96 instrument (Roche Life Science, MA, Germany) with the SYBR green mix (Thermo Scientific). Samples were normalized against β-actin. All

reactions were performed three times.

2.5. Cell cycle analysis:

Cells were fixed by 70% ethanol in phosphate buffered saline (PBS) overnight at 4 °C, washed three times by ice-cold PBS and then treated with 200 μg/mL of ribonuclease A and propidium iodide for 20 min. Data was acquired using a flow cytometry BD FACS Canto II system (BD, Franklin, NJ, USA).

2.6. Colony formation assay:

Cells were seeded at 5000 cells/well into 6-well plate. Cells were grown at 37 °C, 5% CO_2 for 2-3 wk and then fixed by 4% PFA, stained with 0.5% Crystal Violet. Colonies were counted and images of the stained colonies were taken with microscope.

2.7. Cell proliferation assay:

Cells were plated at 1000 cells/well into 96-well plate in 100 μL media and each well was pulsed by addition of 10 μL cell counting kit 8 reagent (CCK8, Dojindo Molecular Technologies, Kumamoto, Japan). Absorbance was measured by reading at a wavelength of 450 nm after 1 hr.

2.8. In vitro acetylation assay:

BL21 cells were grown to an optical density of 0.6–0.8 after being transformed using plasmids pGEX4T1-ARD1 or pGEX4T3-PRMT5. After inducing glutathione s-transferase (GST)-tagged PRMT5 or ARD1 with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), cells were cultured for 8-12 hr at 25 °C. Proteins were extracted from cells using a lysis solution comprising 50 mM Tris-HCl (pH 8.0), 250 mM NaCl, 2.5 mM ethylenediamine tet raacetic acid (EDTA), and 1% Triton X-100. Glutathione sepharose 4B (GE Healthcare, Chicago, IL, USA) was used to purify GST-tagged proteins, which were then eluted using an elution solution comprising 50 mM Tris-HCl (pH 8.0) and 10 mM reduced glutathione (GSH). One μg of purified recombinant was incubated in the reaction mixture containing 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM diothiothreitol (DTT), 10% glycerol and 10 mM acetyl-CoA at 37 °C. Reaction products were separated by SDS-PAGE and analyzed by western blotting using an anti-Lys-Ac antibody. Input proteins were visually quantified by incubating with ARD1 and PRMT5 antibodies.

2.9. Statistical analysis:

All statistical analyses were performed using Excel Microsoft. All experiments were performed at least three independent experiments and all data represented as mean ± SD. Statistical analyses were conducted using the *t*-test and the significance was determined when $p < 0.05$.

Table 1. Details of Primer Pair Sequences

Name	Sequences of primers	℃	bp
cyclin D1	Forward: 5'-GATCAAGTGTGACCCGGACT- 3' Reverse: 5'-CTCAGATGTCCACGTCCC- 3'	60	182
β-actin	Forward: 5'-AGCCATGTACGTAGCCATCC- 3' Reverse: 5'-TTTCCCTCTCAGCTGTGGTG- 3'	60	233

bp: base pair.

3. Results

3.1. ARD1 regulates methyltransferase activity and localization of PRMT5:

To determine whether the presence of ARD1 is essential for PRMT5 activity and localization, I used siRNA to temporarily knock down the expression of ARD1 and PRMT5. H4R3Me2 is the symmetrically di-methylated form of histone H4 which is methylated by PRMT5 (26,28). The level of H4R3me2 protein significantly decreased in ARD1 deficient cells while the expression of histone H4 did not change (Figure 1A&B). In addition, I observed that PRMT5 was translocated into nucleus when ARD1 was depleted (Figure 2A). In the presence of ARD1, most of cells exhibited cytosolic localization of PRMT5. In contrast, PRMT5 was localized in nucleus in more than 90% population of cells when ARD1 was deficient (Figure 2B). These results suggest that ARD1 regulates the subcellular localization and enzyme activity of PRMT5.

3.2. ARD1 acetylates K490 residue of PRMT5:

Next, I examined whether PRMT5 serves as a direct substrate of ARD1-mediated acetylation. In vitro acetylation assay was performed with GST-PRMT5 and GST-ARD1 recombinant proteins. As shown in Figure 3A, PRMT5 acetylation was detected in the presence of ARD1.

Based on the domain architecture of human PRMT5 (29), I

constructed deletion mutants of TIM, Rossman, and β-barrel domain and subjected them into an in vitro acetylation assay (Figure 3B). Among deletion mutants, only β-barrel domain recombinant was acetylated by ARD1 (Figure 3C). Then, to identify the specific target site of acetylation, I prepared the control (without ARD1) and acetylated (with ARD1) β-barrel domain recombinants, which were digested into peptides and then analyzed by micro-liquid chromatography-tandem mass spectrometry (LC-MS/MS). The peptides containing K490 residues were identified as acetylated peptides (Figure 3D).

3.3. Acetylation of PRMT5 regulates its localization and activity:

To determine whether PRMT5 acetylation regulates cellular localization and methyltransferase activity of PRMT5, I established the stable cell lines overexpressing PRMT5 wild-type (WT), K490R mutant (deacetylation mimic mutant), and K490Q mutant (acetylation mimic mutant). While WT and K490Q mutant PRMT5 mostly localized in cytosol, about 50% of K490R mutant PRMT5 showed nuclear localization (Figure 4A&B). The protein level of H4R3me2 was also increased by the overexpression of PRMT5 WT, but not K490R mutant (Figure 4C).

These results suggested that acetylation is required for cytosolic localization and enzyme activity of PRMT5. To confirm the importance of acetylation in cytosolic localization of PRMT5, I treated histone deacetylase inhibitors, trichostatin A (TSA) and valproic acid (VPA), in ARD1-deficient cells. As expected, TSA and

VPA restored the cytosolic localization of PRMT5 in ARD1-depleted cells (Figure 4D&E), indicating that acetylation/deacetylation state decides the cellular localization of PRMT5.

3.4. ARD1-mediated PRMT5 acetylation promotes cell proliferation:

To figure out the relevance of ARD1-mediated PRMT5 acetylation and cancer progression, I examined the growth rate of cancer cells overexpressing WT and K490R mutant PRMT5. OV8 cells overexpressing PRMT5 WT grew two-fold faster than cells overexpressing K490R mutant (Figure 5A). The colony formation assay also showed the similar pattern. PRMT5 WT significatly increased the number of colonies, compared to K490R mutant (Figure 5B). Interestingly, when ARD1 was depleted, the colony number did not show any difference between PRMT5 WT- and K490R mutant- overexpressing cells (Figure 5C). These results suggest that ARD1-mediated PRMT5 acetylation is essential for cancer cell proliferation.

3.5. ARD1-mediated PRMT5 acetylation regulates cell cycle:

To examine further mechanism by which PRMT5 WT enhances the cell proliferation, I analyzed the cell cycle progression of PRMT5 WT- and K490R mutant-overexpressing cells. Interestingly, the amount of PRMT5 WT-overexpressing cells at G0/G1 was

significantly higher than that of K490R mutant-overexpressing cells (Figure 6A&B). Furthermore, I also checked the expression of cell cycle marker proteins: cyclin B1 for M phase marker, cyclin E2/CDK2 complex for S phase marker, and cyclin D1 for G1 phase marker. The western blot data showed that PRMT5 WT increased the expression of cyclin D1 compared to K490R mutant (Figure 6C). These results suggest that PRMT5 acetylation enhances the cell proliferation through the regulation of cell cycle progression.

3.6. PRMT5 promotes cell growth by activating NF-κB pathway:

Previous studies showed that NF-κB is activated by PRMT5-mediated methylation of p65 on R30 (30,31). Based on this, I examined the activation of NF-κB pathway. Western blotting assay revealed that phosphorylation of NF-κB was increased by the overexpression of PRMT5 WT, but not K490R mutant (Figure 7A&B). To confirm the involvement of NF-κB in PRMT5 acetylation-mediated cell proliferation, NF-κB inhibitor, caffeic acid phenethyl ester (CAPE), was treated to cells overexpressing PRMT5 WT and K490R mutant. Under CAPE treatment condition, either PRMT5 WT or PRMT5 K490R did not show any effect on cellular growth (Figure 7C). The results indicat that PRMT5 acetylation is required for NF-κB activity, leading to cell proliferation.

3.7. Cytosolic localization of PRMT5 is necessary for cancer cell proliferation:

A nuclear localization signal (NLS) is an amino acid sequence that 'tags' a protein for nuclear transport into the cell nucleus. To confirm again the necessary of cytosolic PRMT5 in cancer cell proliferation, I constructed PRMT5 with NLS to induce nuclear translocation of PRMT5 (Figure 8), then examined the cell proliferation. Compared to PRMT5 WT, NLS inserted mutant inhibited the proliferation and colony formation of cancer cells (Figure 9A&B). The expression of cyclin D1 showed the similar pattern with cellular growth (Figure 9C). Western blotting data also revealed that cyclin D1 and phosphorylation of NF-κB were increased by PRMT5 WT, but not NLS inserted mutant (Figure 9D). These results indicated that cytosolic localization of PRMT5 is essential for activating NF-κB pathway and cancer cell proliferation.

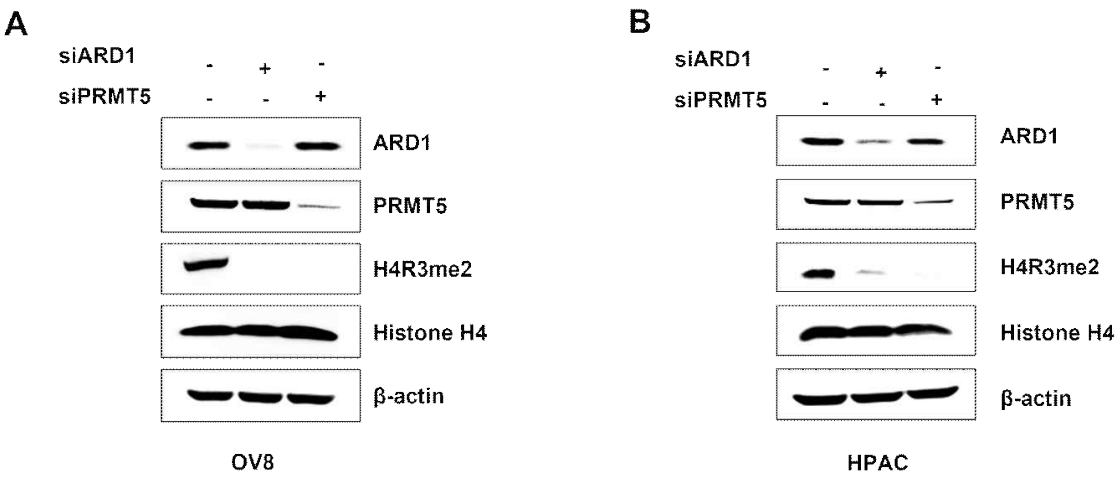

Figure 1. ARD1 regulates the methyltransferase activity of PRMT5. (A&B) OV8 (A) and HPAC (B) cells were transfected with ARD1 and PRMT5 siRNA. Methylation of histone H4 was analysed by western blotting.

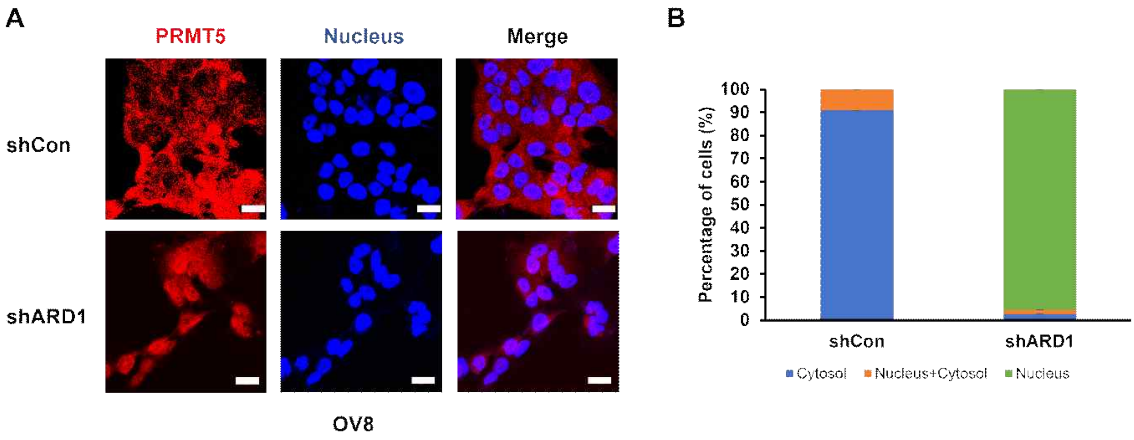

Figure 2. ARD1 determines the cellular localization of PRMT5. (A) OV8 cells were transfected with ARD1 siRNA. Representative confocal microscopic images show the localization of florescent-tagged PRMT5 (red) and the merge with DAPI staining (blue) of nuclei. Bar, 20 μm. (B) Quantification of florescent-tagged PRMT5. Error bars represent ± SD.

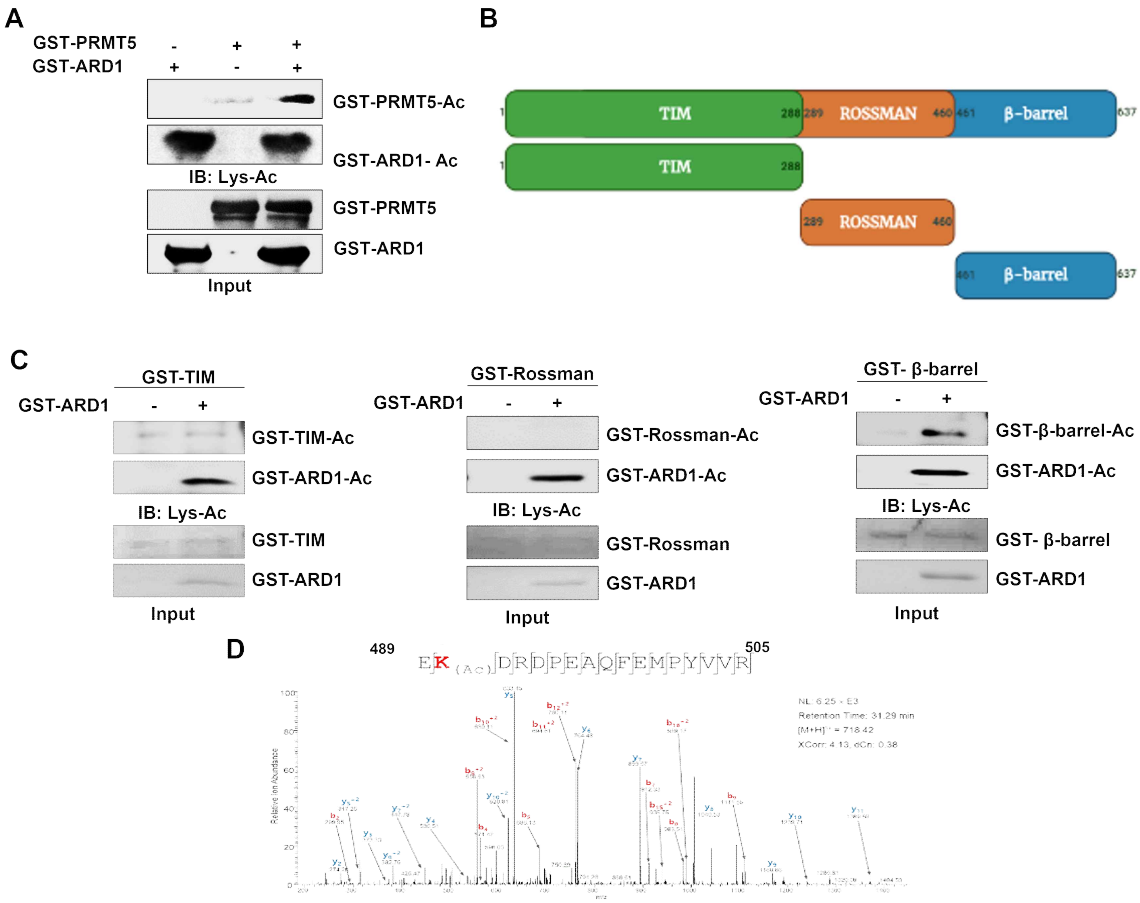

Figure 3. ARD1 acetylates K490 residue of PRMT5. (A) GST-ARD1 and GST-PRMT5 were subjected to an in vitro acetylation assay. Acetylation levels of recombinants were detected by western blotting using anti-Lys-Ac antibody. (B) Structure of three domains PRMT5. (C) Deletion mutants of PRMT5 and ARD1 recombinants were subjected to an in vitro acetylation assay, then acetylation level were analysed by western blotting. (D) The acetylation site in GST-β barrel was identified by LC-MS/MS.

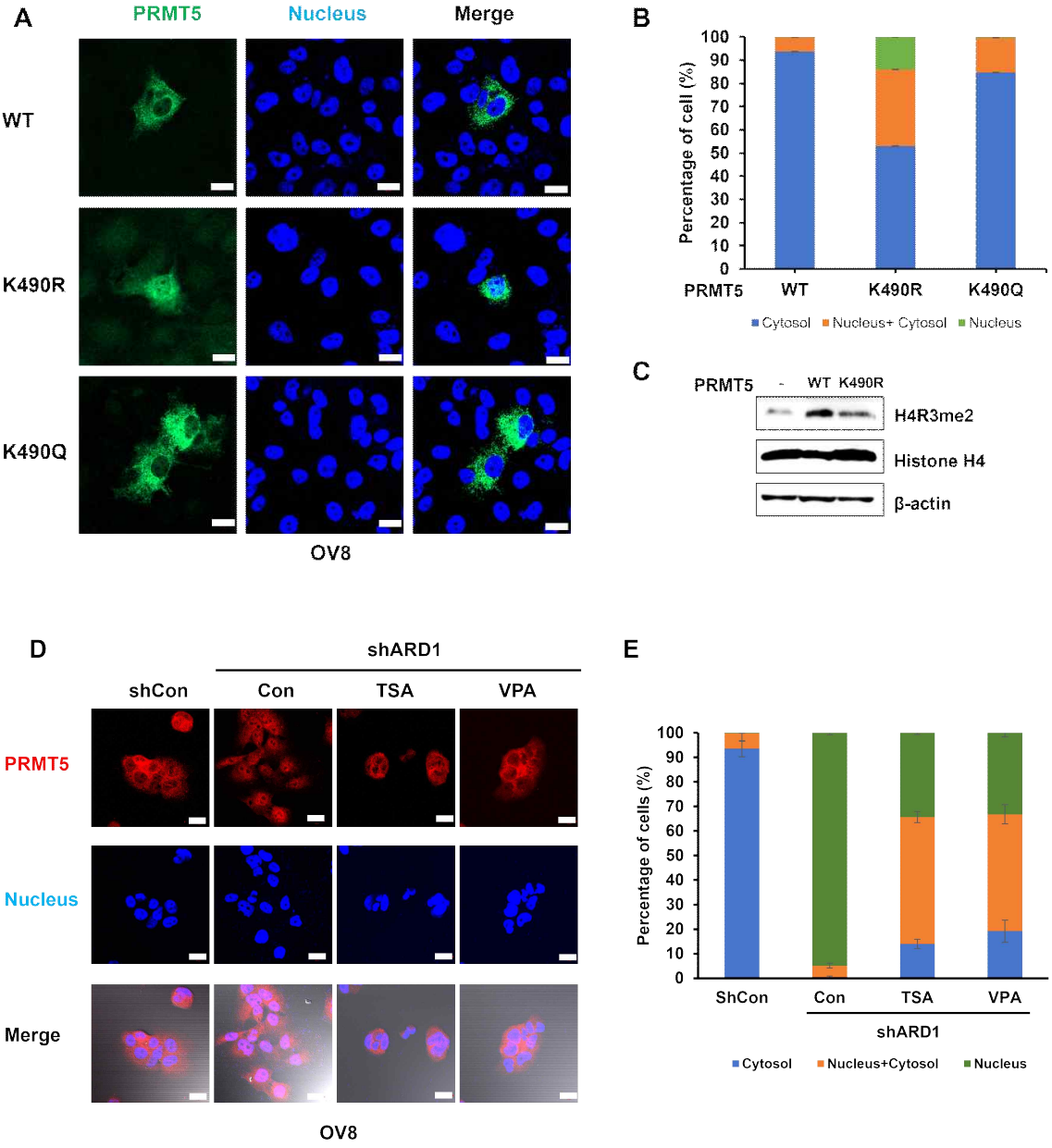

Figure 4. Acetylation is essential for cytosolic localization and methyltransferase activity of PRMT5. (A) OV8 cells were transfected with Flag-PRMT5 WT, K490R, and K490Q mutant plasmids. Representative confocal microscopic images show the localization of florescent-tagged PRMT5 (green) and the merge with DAPI staining (blue) of nuclei. Bar, 20 μm. (B) Quantification of florescent-tagged PRMT5. Error bars represent ± SD. (C) OV8 cells were transfected with Flag-mock, Flag-PRMT5 WT, and Flag-PRMT5 K490R mutant plasmids. H4R3me2 levels were analysed by western blotting. (D) ARD1 depleted cells were treated with TSA and VPA. Florescent-tagged PRMT5 (red) was captured by confocal microscopy. (E) Quantification of florescent-tagged PRMT5. Error bars represent ± SD.

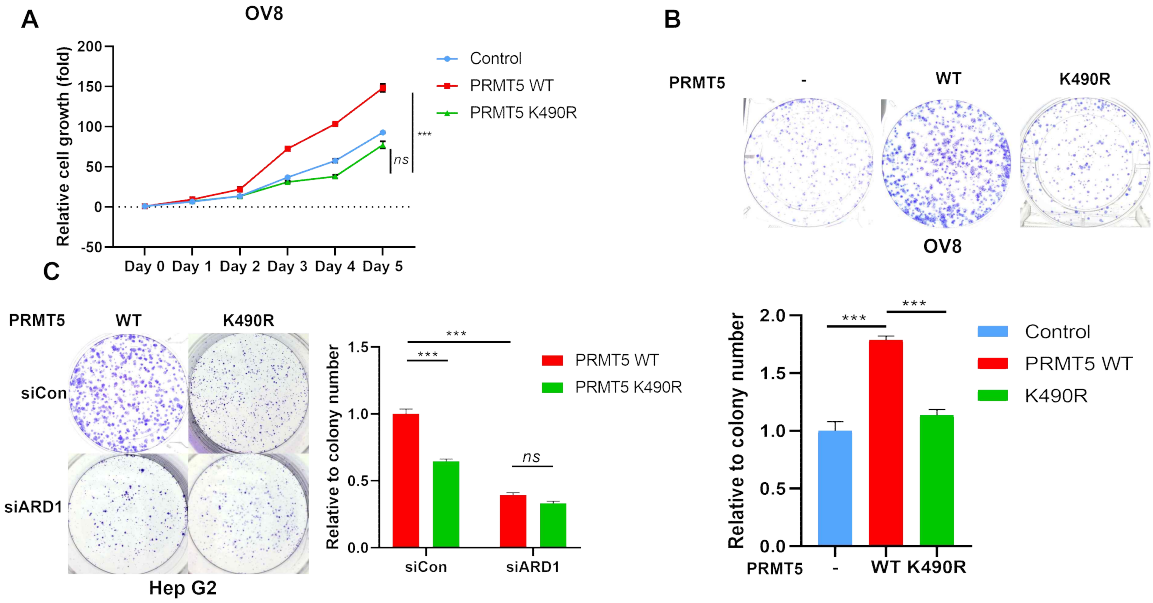

Figure 5. ARD1-mediated PRMT5 acetylation is essential for cancer cell growth. (A&B) OV8 cells were transfected with Flag-mock, Flag-PRMT5 WT, and Flag-PRMT5 K490R mutant plasmids. Cell proliferation was examined by CCK8 assay (A) and colony formation assay (B). (C) ARD1-depleted HepG2 cells were co-transfected with Flag-PRMT5 WT and Flag-PRMT5 K490R mutant plasmids then cell proliferation was examined by colony formation assay. All experiments were repeated three times. Error bars represent ± SD. ***: $p < 0.001$, ns: non-significant.

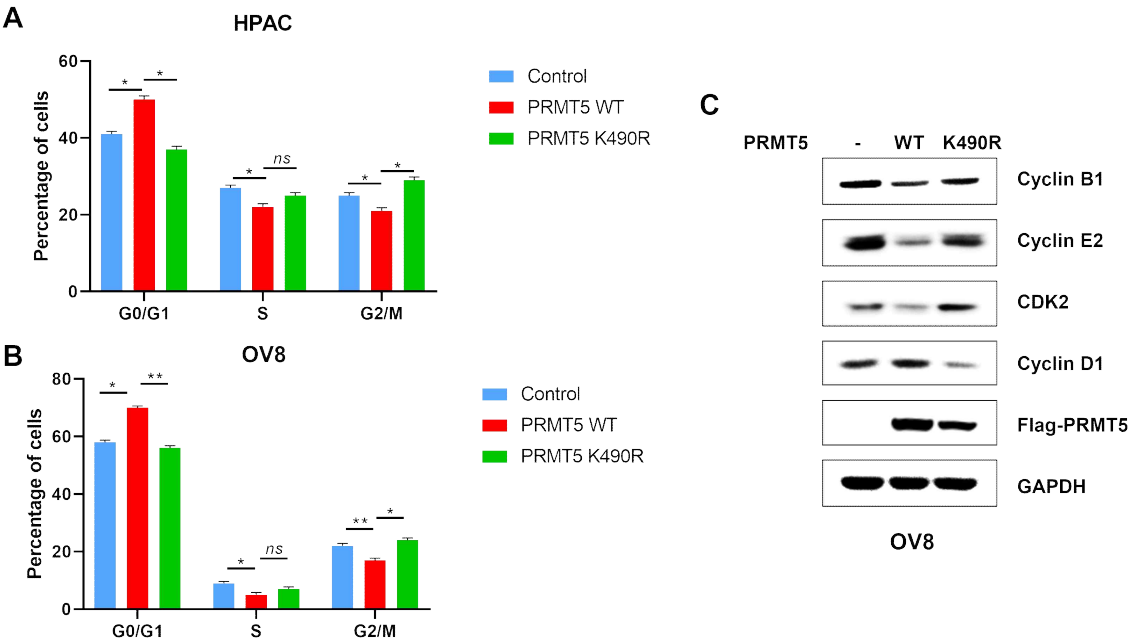

Figure 6. Acetylated PRMT5 increases the expression of cyclin D1 and the cell population at G0/G1 phase. (A&B) OV8 and HPAC cells were transfected with Flag-mock, Flag-PRMT5 WT, and Flag-PRMT5 K490R mutant plasmids. Cells were subjected into cell cycle analysis. (C) The level of cell cycle marker proteins were analysed by western blotting. All experiments were repeated three times. Error bars represent ± SD. *: $p < 0.05$, **: $p < 0.01$, ns: non-significant.

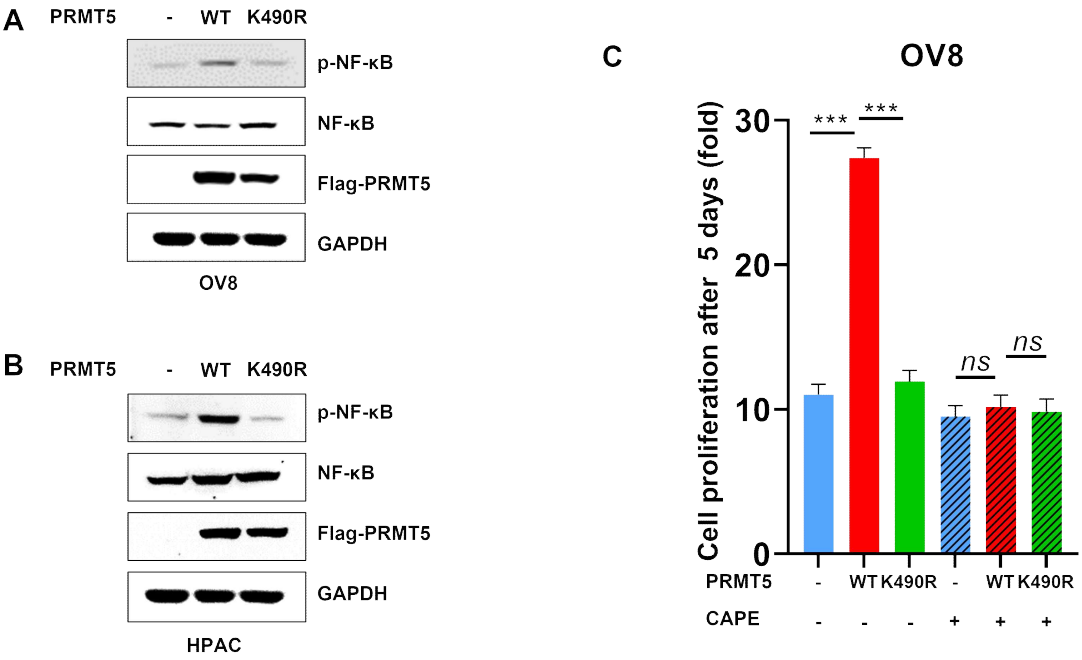

Figure 7. Acetylated PRMT5 is required for NF-κB activity, leading to cell proliferation. (A&B) OV8 cells were transfected with Flag-mock, Flag-PRMT5 WT, and Flag-PRMT5 K490R mutant plasmids. Cell lysates were immunoblotted for p-NF-κB. (C) Cells were treated with 10 μM CAPE, NF-κB inhibitor. Cell proliferation was analysed by CCK8 assay. All experiments were repeated three times. Error bars represent ± SD. ***: $p < 0.001$, ns: non-significant.

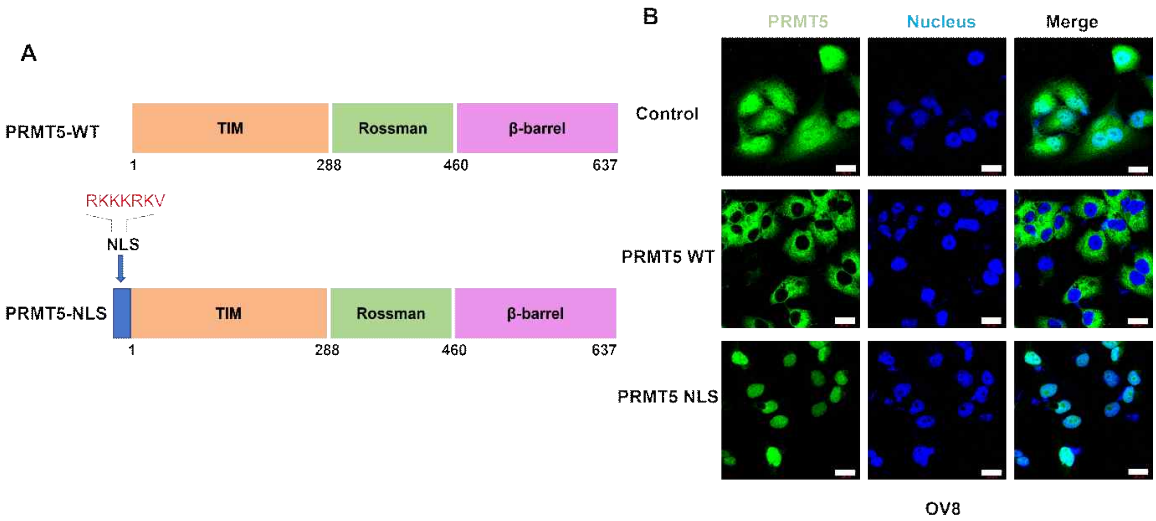

Figure 8. Construction of nuclear localization signal (NLS) PRMT5. (A) Construction of PRMT5 NLS inserted mutant. (B) GFP-mock, GFP-PRMT5 WT, and GFP-PRMT5 NLS plasmids were transfected to OV8 cells. Representative confocal microscopic images show the localization of GFP-PRMT5 (green) and the merge with DAPI staining (blue) of nuclei. Bar, 20 μm.

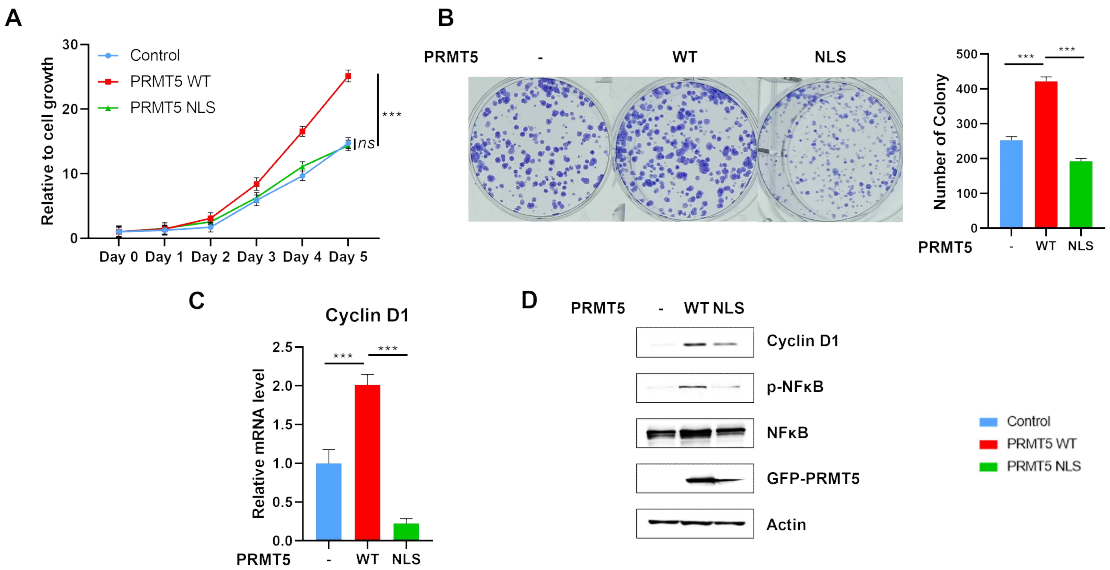

Figure 9. Cytosolic localization of PRMT5 is necessary for cancer cell proliferation. (A&B) OV8 cells were transfected with GFP-mock, GFP-PRMT5 WT, and GFP-PRMT5 NLS plasmids. Cell proliferation was analysed by CCK8 (A) and colony formation assay (B). (C) Cyclin D1 expression level was determined by qRT-PCR. (D) Cell lysates were immunoblotted for cyclin D1 and p-NF-κB. All experiments were repeated three times. Error bars represent ± SD. ***: $p < 0.001$, ns: non-significant.

4. Discussion

ARD1 plays a role as an oncoprotein in human cancer. ARD1 regulates many important molecular mechanisms for carcinogenesis. In deed, increased ARD1 expression in cancer tissues is correlated with worse outcomes of cancer patients. ARD1 regulates a series of biophysical process to support for cancer development by protein acetylation (32). In this study, it was investigated the relevance of ARD1 and PRMT5. ARD1 is important for methyltransferase activity and cellular localization of PRMT5. When ARD1 is knockdowned, the methylation of H4R3 is downregulated and PRMT5 is translocated into the nucleus. PRMT5 is acetylated by ARD1 at lysine residue at position 490. This modification regulates enzymatic activity and subcellular localization of PRMT5. Moreover, it was observed an enhancement of cancer cell growth by overexpression of PRMT5 WT, but not K490R mutant, indicating an important role of PRMT5 acetylation in cell proliferation. Mechanistic studies revealed that PRMT5 acetylation activates NF-κB pathway to promote cancer cell growth.

Recently, several evidence showed that PRMT5 is highly expressed in diverse tumor. PRMT5 is required for cell proliferation and plays a role in the regulation of cell cycle progression (34). Previous study showed that PRMT5 controls cell cycle transition from G1 to S phase in CD4 Th1 cells (35). This study provided more evidence to show that PRMT5 promotes cancer cell proliferation via cell cycle regulation after being acetylated by ARD1. To investigate the effect of PRMT5 acetylation on cell proliferation, I used PRMT5 mutant, K490R, which mimics

deacetylation state of PRMT5. Whereas PRMT5 WT enhanced the cell population at G0/G1 phase, PRMT5 K490R reduced that population. In addition, phosphorylation of NF-κB was increased by overexpression of PRMT5 WT, but not PRMT5 K490R mutant. It was elucidated that PRMT5 acetylation regulates cell cycle by NF-κB activation.

In conclusion, this study reveals the molecular mechanism by which ARD1 and PRMT5 promote the cancer cell proliferation. In particular, this study shows the importance of cytosolic localization of PRMT5 in promoting cancer cell growth. Therefore, ARD1-mediated PRMT5 acetylation might be a good target for anticancer treatment to prevent cancer development. Future studies toward elucidating the potential therapeutic benefit of inhibiting both ARD1 and PRMT5 would be of great importance in this respect.

5. Summary

ARD1 and PRMT5 have been reported as oncogene. They play important roles in cancer development. In this study, I identified an interaction between PRMT5 and ARD1. It was found that ARD1 directly acetylates PRMT5 on lysine 490 residue to regulate PRMT5 localization and methyltransferase activity. Acetylated PRMT5 is localized in the cytoplasm, triggering NF-κB activation. PRMT5 stimulates cell cycle progression via NF-κB activation. Taken together, this study suggests that ARD1-mediated PRMT5 acetylation promotes cancer cell proliferation.

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ARD1-mediated PRMT5 acetylation promotes cell proliferation

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Protein Arginine Methyltransferase 5 (PRMT5) is known as an oncoprotein and contributes to the development of many types of cancer. In previous studies, PRMT5 was discovered to localize in cytosol in several types of cancer cells but localize in nucleus in almost of normal tissues. However, the molecular mechanism that regulates PRMT5 localization and its function remains unclear. In this study, I found that PRMT5 localization is regulated by ARD1-mediated acetylation. I observed that wild-type PRMT5 localizes in cytosol, while non-acetylated form (K490R) of PRMT5 translocates into nucleus. In addition, I found that cytosolic localization of PRMT5 is essential for cancer cell proliferation. Wild-type PRMT5, but not PRMT5 K490R, enhances the cancer cell growth. Mechanistic study revealed that cytosolic localization of PRMT5 is required for NF-κB pathway activation, leading to the expression of NF-kB target genes required for cell cycle progression. Taken together, ARD1-mediated PRMT5 acetylation is essential for its cytosolic localization, then promoting cell proliferation by activating NF-κB activity.

ARD1에 의한 PRMT5 아세틸화가 암세포 증식에 미치는 영향 연구

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(초록)

단백질 아르기닌 메틸전달효소 5(PRMT5)는 종양유발단백질로 다양한 종류의 암 발생에 기여한다고 알려져 있다. 이전의 연구에서 PRMT5는 여러 종류의 암세포에서 세포질에 위치하는 반면, 대부분의 정상 조직에서는 핵에 위치하는 것으로 밝혀졌다. 그러나 PRMT5의 위치와 기능을 조절하는 분자 메커니즘에 대해서는 알려진 바가 없다. 본 연구에서는 PRMT5의 위치가 ARD1에 의한 아세틸화에 의해 조절된다는 것을 발견했다. PRMT5는 세포질에서 국소적으로 발현되지만, 490번 라이신 잔기가 변이되어 탈아세틸화 된 PRMT5 K490R은 핵으로 전위되었다. 또한 정상 PRMT5는 암세포 성장을 촉진하는 반면, PRMT5 K490R은 암세포 증식을 촉진하지 못하였다. 기전 연구를 통해 PRMT5의 세포질 위치는 NF-κB 경로의 활성화에 필요하며, 이는 세포 주기 진행에 필요한 NF-kB 표적 유전자의 발현으로 이어짐을 규명하였다. 따라서 본 논문에서는 ARD1에 의한 PRMT5 아세틸화가 세포질의 NF-κB 활성을 증가시켜 암세포의 증식을 촉진함을 규명하였다.