약학석사학위논문

ARD1 regulates cell cycle via Aurora kinase A

ARD1의 Aurora kinase A를 통한 세포주기 조절에 관한 연구

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ABSTRACT

ARD1 regulates cell cycle via Aurora kinase A

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ARD1 (N-acetyltransferase arrest defective 1) is first identified to have N-α-acetylation activity in yeast and classified as a subunit of NatA acetyltransferase. It is also reported to catalyze both N-α-acetylation and N-ε-acetylation in mammalian cells. ARD1 overexpression has been found in many cancers such as breast cancer, prostate cancer, lung cancer, cervical cancer. It has been considered as an critical molecule in cell proliferation. The autoacetylation activity of ARD1 has been discovered as a key regulator of cell cycle. However, the biological mechanism of ARD1 in cell cycle hasn't been well-defined yet. Whereas Aurora kinase A (AURKA) is a member of Aurora kinase protein family, Aurora kinase A is known as

serine/threonine-protein kinase 6 and involved in centrosome maturation, separation as well as mitotic spindle assembly. Therefore, Aurora kinase A has a very important role in regulation of cell cycle. Recent studies revealed that Aurora kinase A overexpression is correlated with aneuploidy, supernumerary centrosomes, defective mitotic spindle and has been seen in some cancers, including breast, prostate, ovarian, cervical, and colorectal cancer. We found that ARD1 localizes to the centrosome during mitotic phases. Immunoprecipitation assays indicated that Aurora kinase A is an ARD1 binding partner. Furthermore, Aurora kinase A was seen to be acetylated by *in vitro* acetylation assay. In addition, cell proliferation was seen in overexpressed ARD1 WT but not its mutants. Taken together, these results suggest that the acetylation activity of ARD1 is related to cell cycle regulation possibly via Aurora kinase A.

Key words: ARD1, Aurora kinase A, acetyltransferase, acetylation, cell cycle, centrosome

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I. INTRODUCTION

1. ARD1

Arrest defective protein 1 (ARD1) was first identified in yeast to have effects on mitotic cell cycle which is involved in mating, stationary phase and sporulation (Whiteway & Szostak, 1985). ARD1 is known as a catalytic subunit of N-acetyltransferase (Nat) (Park & W.Szostak, 1992; Polevoda & Sherman, 2003; Whiteway & Szostak, 1985).

Various homologues of ARD1 have been identified as mARD198, mARD225, and mARD235 in mouse cells and hARD225 and hARD235 in mammalian cells (Chun et al., 2007; Kim et al., 2006). These variants share a conserved N-acetyltransferase domain, a putative NLS (Nuclear Localization Signal) (KRSHRR), and acetyl-CoA binding domain [(Q/R)XXGX(G/A)], but contain different sequences and lengths in their C-terminal region (Thomas Arnesen et al., 2005; Kim et al., 2006).

Recent studies indicate that ARD1 is expressed in both nucleus and cytoplasm (Thomas Arnesen et al., 2005; Asaumi et al., 2005). However, predominant expression of ARD1 in cytoplasm has been reported (Ren et al., 2008). It is claimed that ARD1 functions may be related to its localization in cells (Kuo & Hung, 2010).

N-α-acetylation is a catalytic process in which an acetyl group is transferred from acetyl CoA to the N-terminal of nascent peptide (Driessen, Jong, Tesser, & Bloemendal*, 1985; Polevoda & Sherman, 2003), while N-ε-acetylation is the acetylation of ε-lysine groups on internal sites of protein (Marmorstein, 2003). ARD1 is the only known protein representing

both N-α-acetylation and N-ε-acetylation activity (Thomas Arnesen et al., 2005). ARD1 was reported to acetylate hypoxia-inducible factor 1 α (HIF-1 α) at K532 leading to the instability and degradation of HIF-1α via ubiquitin-proteasome system (Jeong et al., 2002). ARD1 was also shown to have acetylation activity on β-catenin inducing the binding of TCF4 to cyclinD1 promoter and promoting lung cancer cell proliferation (Lim, Park, & Chun, 2006). In addition, myosine light chain kinase was demonstrated as a substrate of ARD1 and inhibited tumor cell migration and invasion after being acetylated (Shin, Chun, Lee, Shin, & Park, 2009). Recently, in the report of Seo et al., the autoacetylation of ARD1 has been discovered as an crucial process in cancer cell development. In this report, lysine residue 136 (K136) is important for autoacetylation of ARD1 and arginine residue 82 (R82) and tyrosine residue 122 (Y122) are necessary for ARD1 catalytic activity (Seo et al., 2010).

Although ARD1 functions in mammalian cells have not been well-defined, ARD1 overexpression has been found in many cancer tissues including hepatocellular carcinoma, breast cancer, lung cancer, and colorectal cancer (Midorikawa Y, 2002; Min Yu, 2009; Ren et al., 2008; Yu et al., 2009). ARD1 was also overexpressed in the urinary bladder cancer and cervical cancer (Min Yu, 2009). There are several reports demonstrating the contribution of ARD1 to the cell proliferation, cell differentiation and apoptosis and showing the knockdown of ARD1 causing cell proliferation inhibition and G1 arrest (T Arnesen et al., 2006; Lim, Chun, & Park, 2008; Lim et al., 2006; Sugiura, Adams, & Corriveau, 2003). However, the mechanism regulation of ARD1 has not been unclear. In this study, we tried to find out the role of ARD1 in cell cycle regulation.

2. Aurora kinase A

Aurora kinase A is a member of Aurora kinase family including Aurora kinase A, Aurora kinase B, and Aurora kinase C (Nigg, 2001). It was first described in yeast in a screen for mitotic mutants (Chan & Botstein, 1993). The particular attention has been paid on Aurora kinase A among those three mammalian Aurora kinases since it has a vital role in centrosome functions composed of centrosome maturation and separation, assembly of mitotic spindle, trigger of mitotic entry, alignment of chromosomes in the metaphase, and cytokinesis. Aurora kinase A localizes at centrosomes from centrosome duplication until mitotic exit (Nikonova, Astsaturov, Serebriiskii, Dunbrack, & Golemis, 2013) (Fig. 1).

Aurora kinase A regulates centrosome functions by phosphorylating and recruiting microtubule-associated proteins kinesin Eg5 (Giet, Uzbekov, Cubizolles, Le Guellec, & Prigent, 1999), TACC (Transforming Acidic Coiled Coil protein number)(Giet et al., 2002).

Highly expression of Aurora kinase A has been observed in many types of cancer and tumor tissues such as colorectal, breast, gastric, cancer (Bischoff et al., 1998; Sakakura et al., 2001; Subrata Sen, 1997; Zhou et al., 1998). Especially, Aurora kinase A was found to bind to BRCA1 (Breast Cancer-Associated gene product) and phosphorylate this protein inducing mitosis entry (Ouchi et al., 2004).

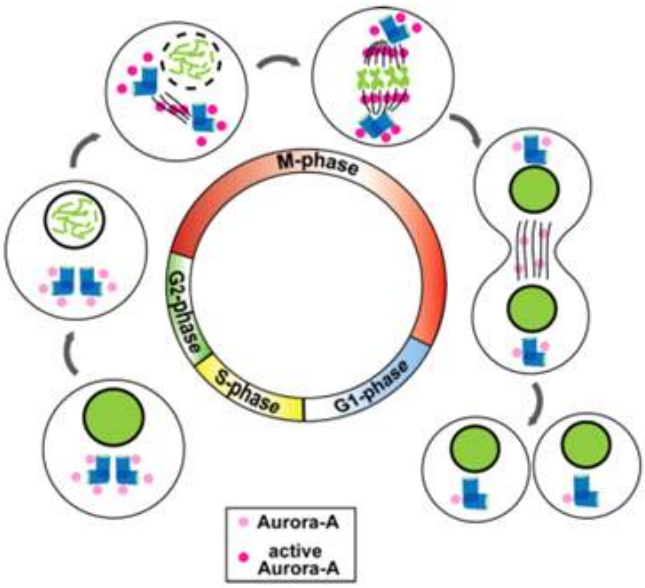

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Figure 1. Aurora kinase A in the cell cycle.

Aurora-A begins to accumulate significantly at centrosomes in S phase, and is activated at the boundary between G2 and M phase. Active Aurora-A propagates along the mitotic spindle to the midzone, with most of the protein inactivated and degraded before cytokinesis, with only low levels detectable in early G1 cells.

II. MATERIALS AND METHODS

1. Antibodies

Antibodies anti-Aurora A, anti-GFP were purchased from AbCam. Antibodies anti-actin, anti-Flag M2 were purchased from Sigma-Aldrich. And antibodies anti-Cyclin B1, anti-ARD1 were obtained from Santa Cruz Biotechnology. We used antibodies specific for acetyl lysine (Cell signaling).

2. Plasmids

hARD1 expression vectors were constructed by PCR and subcloned into pCMV-tag2b and pEGFP-C3. Oligonucleotide primers were designed to amplified ARD1 gene and Aurora kinase A gene (AURKA). Each primer covers additional sequences of restriction enzyme. ARD1 PCR product was digested and cloned into pCMV-tagb2 and pEGFP-C3. Aurora A PCR product was digested and cloned into pGEX-4T3.

3. Cell culture, transfection and stable cell lines

HEK 293T (Human embryonic kidney cells), Hela (human cervix carcinoma) were grown in DMEM medium containing 10% FBS (fetal bovine serum) with penicillin and streptomycin in a 5% CO2 humidified atmosphere at 37oC. Cells were transfected using Polyethylenimine (PEI) reagent following manufacturer's recommendation. For Hela and Raw264.7 stably overexpressing GFP-ARD1, Hela cells and Raw264.7 cells were transfected with pEFGP-C3-ARD1 using Lipofectamine 2000 (Lifetechnologies) then selected by using G418 (geneticin).

4. Westernblotting

Total cell lysates were isolated using cell lysis buffer (Cell signaling), which contained 20mM Tris-HCl pH7.5, 150mM NaCl, 1mM Na2EDTA, 1mM EGTA, 1% Triton, 2.5mM sodium pyrophosphate, 1mM beta-glycerophosphate, 1mM Na3VO4, 1μg/mi leupetin and the protease inhibitor cocktail set (Calbiochem). Lysates were separated by sodium dodecyl sulfate gel electrophoresis, and transferred to nitrocellulose membranes for Western blot analysis. Detection was performed using ECL (enhanced electrochemiluminescence) plus (Amersham Bioscience).

5. Cell synchronization

Hela cells were synchronized by treated with AraC (cytosine arabinoside) in 16h for G1/S phase. After cells were released into fresh medium in 4h, cells were then arrested in early mitosis by using nocodazole 100ng/ml for 6h. For metaphase, cells were then treated with MG132 for 30min. And cells were in anaphase and telophase after being released from MG132 30min and 60 min respectively.

6. Immunocytochemistry

Cells were grown on circular glass coverslips plated in 24-well plate. Cells were fixed in ice methanol in 10 min, permeabilized by incubating in PBS containing 0.25% Triton-X in 10min and washed 3 times by PBS, then blocked for 1 hour in 1% BSA. Primary antibody recognizing Aurora A (1:500) was diluted in blocking buffer and incubated overnight at 4oC. Alexa Flour®546 goat anti-mouse antibody was used at recommended concentration and

incubated in 1 hour in the dark at room temperature. This was followed by counterstaining with DAPI and mounting with Gel/mount (Biomeda Corp.). Cells were observed under Axiovert M200 microscope (Zeiss).

7. Immunoprecipitation

Cells were homogenized in 100μl lysis buffer (0.01M HEPES pH7.9, 0.4M NaCl, 0.1mM EDTA, 5% glycerol, 1mM DTT). Lysate were immunoprecipitated with appropriate primary antibody. The immunoprecipitated complexes were subjected to electrophoresis and immunoblotting.

8. In vitro acetylation assay

Freshly prepared recombinant hARD1 and Aurora A were incubated in the reaction mixture [50 mmol/L Tris-HCl (pH 8.0), 0.1mmol/LEDTA, 1mmol/LDTT, 10%glycerol, and 10 mmol/L acetyl-CoA] at 37oC.

9. Cell proliferation assay

Cells were seeded in 96-well plate at a density of 5x103 cells per well and culture for 3 days. The proliferation rates were measured using a Non-Radioactive Cell Proliferation Assay Kit (Promega) according to the manufacturer's instructions. Briefly, 20ul of freshly mixed tetrazolium/phenazine methosulfate was added, and the cells were incubated for 1 hour to allow color development. The absorbance at 490nm was measured to indicate the number of viable proliferating cells.

III. RESULTS

1. ARD1 expression in cell cycle

Recent studies reported that ARD1 is an important factor in cell cycle regulation since knockdown of ARD1 in human cells impacts on cell survival and induces G1 arrest (T Arnesen et al., 2006; Lim et al., 2008; Lim et al., 2006). Cells were arrested in different phases of cell cycle by synchronization method (Fig. 2) and checked the expression level of ARD1 in phases. It is found that ARD1 increased in stages of mitotic phase (Fig. 3).

From this result, we examined the localization of ARD1 in cell cycle phases. ARD1 was found in the cytoplasm but concentrated at one region of the cell in G1/S phase. In prometaphase, ARD1 expressed at the zone close to condensed chromosomes. As the chromosomes align at metaphase plate and segregate to opposite poles in anaphase, ARD1 was observed at two opposite poles of the cell. When cell proceed into telophase, ARD1 was found to be more concentrated at the outer edge of the midzone and became fainter at two poles compared with metaphase and anaphase (Fig. 4).

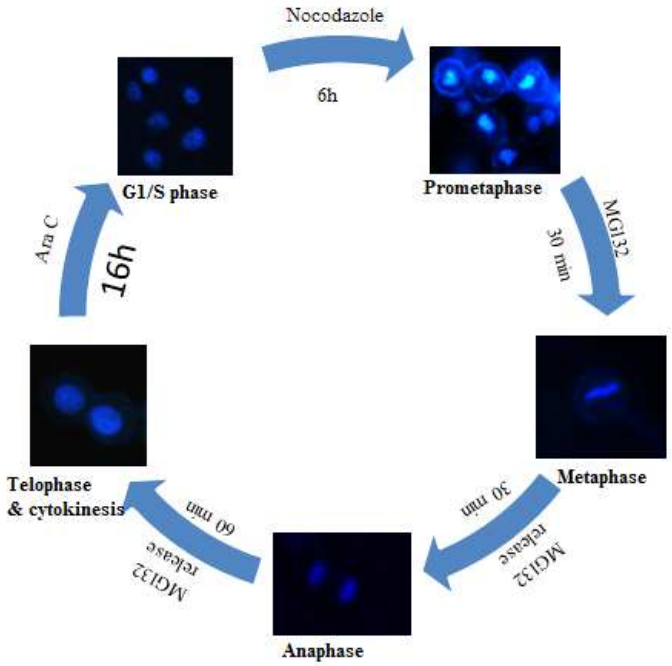

Figure 2. Cell synchronization procedure.

Cells were treated with Arabinoside cytosine for 16h. After cells were released into fresh medium in 4h, cells were treated with nocodazole 100ng/ml for 6h. Then, MG132 was used for 30min. Last, cells were released in fresh medium after 30min and 60 min.

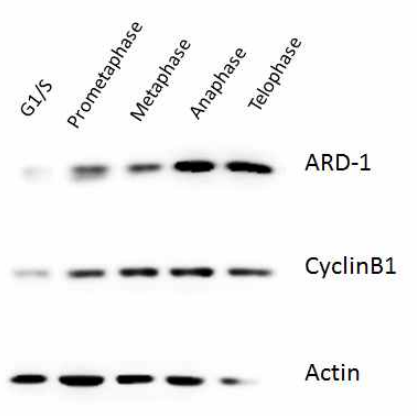

Figure 3. ARD1 expression in cell cycle.

Cell lysates were prepared after cell synchronization and immunoblotted with indicated antibodies.

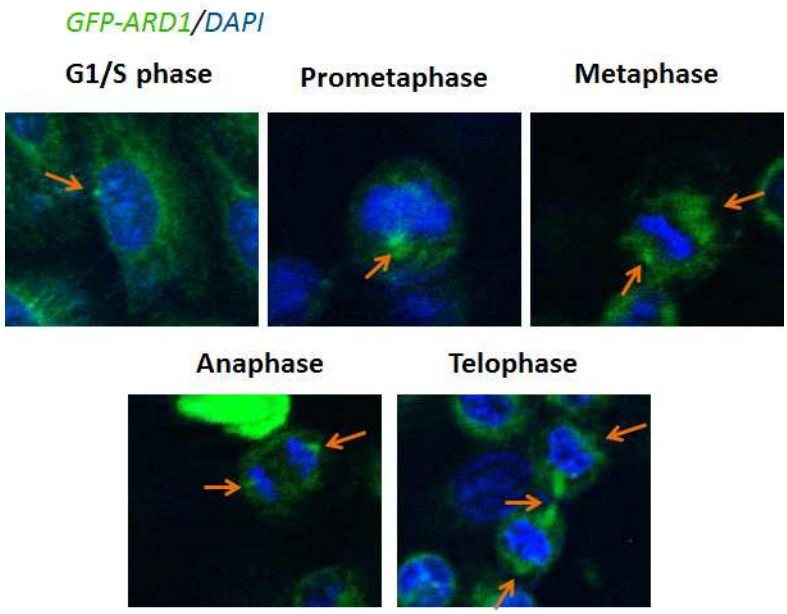

Figure 4. ARD1 localization in cell cycle.

Hela cells expressed GFP-ARD1 were seeded on 24-well plate and fixed with methanol, counterstained with DAPI and mounted.

2. Interaction of ARD1 with Aurora kinase A

As the ARD1 localization pattern suggested the centrosome pattern, we stained overexpressed ARD1 Hela cells with Aurora kinase A, which localizes to centrosomes and regulates centrosome formation as well as separation (Dutertre, Descamps, & Prigent, 2002). ARD1 was observed to be colocalized with Aurora kinase A at centrosomes in prometaphase, metaphase, anaphase and telophase (Fig. 5).

Aurora kinase A plays key role in cell cycle regulation whereas ARD1 has been revealed to be related to cell growth. Moreover, ARD1 and Aurora kinase A colocalize at centrosome during mitosis (Fig. 5). These data implied the relation between ARD1 and Aurora kinase A. In order to uncover the interaction of ARD1 and Aurora kinase A, immunoprecipitation assay was performed in overexpressed ARD1 Hela stable cells and in transient transfection HEK 293T cells. As a result, Aurora kinase A was found to be a binding partner of ARD1 (Fig. 6).

It is the fact that ARD1 is an N-acetyltransferase protein (Park & W.Szostak, 1992). We questioned if ARD1 had acetylation activity on Aurora kinase A. In vitro acetylation assay was performed to identify the acetylation reaction between ARD1 and Aurora kinase A with the presence of acetyl-CoA. Consequently, Aurora kinase A was seen to be acetylated in a time-dependent manner (Fig. 7)

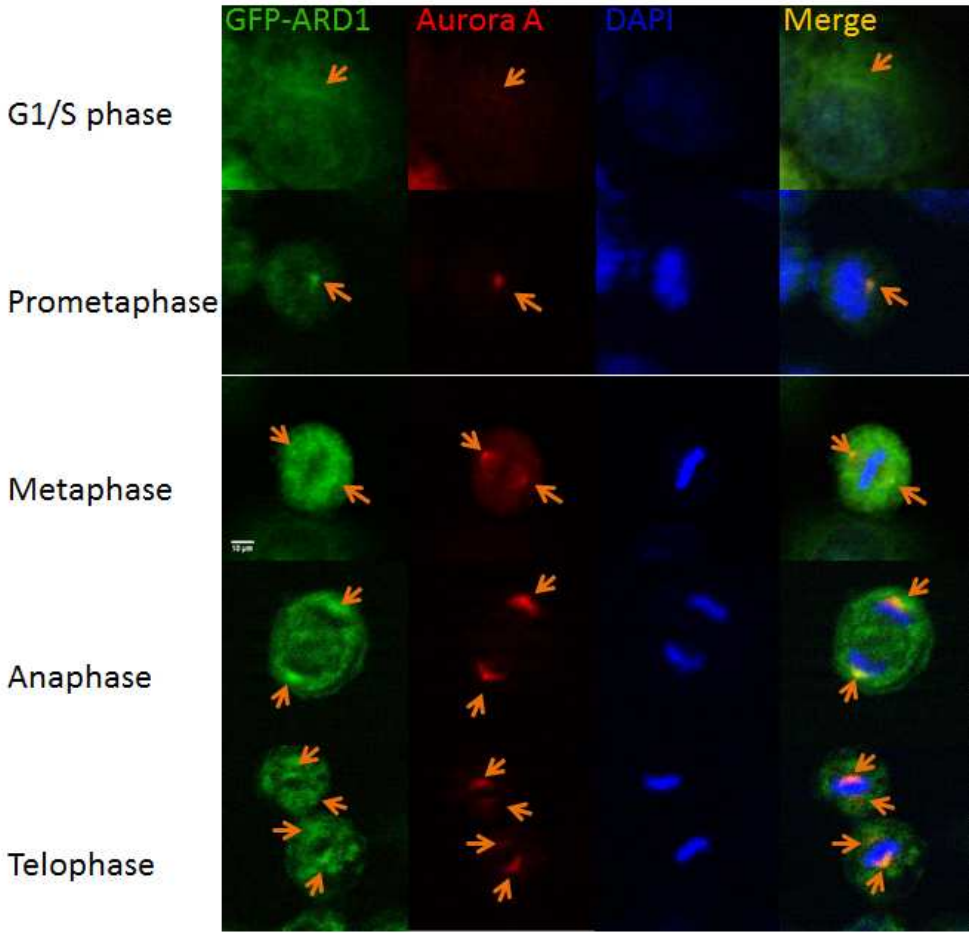

Figure 5. Localization of ARD1 and Aurora kinase A in cell cycle.

Hela cells expressed GFP-ARD1 were seeded on 24-well plate and fixed with methanol, permeabilized, followed by blocking with 1% BSA in PBS and overnight treatment of anti-Aurora A antibody at 4°C.

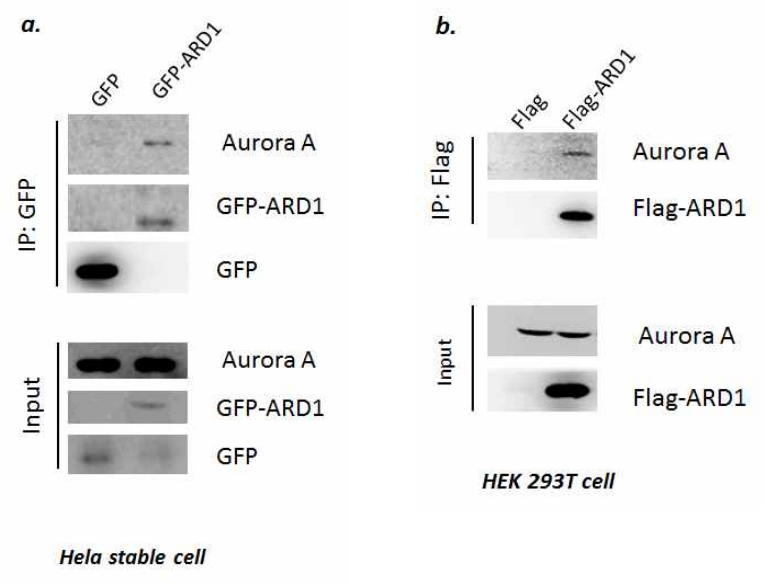

Figure 6. Binding of Aurora kinase A and ARD1.

a. Cell lysates of Hela cells expressed GFP and GFP-ARD1 were immunoprecipitated with anti-GFP antibody overnight at 4°C and immunoblotted with indicated antibodies.

b. HEK 293T cells were transient transfected with pCMV FLAG (control) or pCMV FLAG-ARD1. Cell lysates were immunoprecipitated with anti-GFP antibody overnight at 4°C and immunoblotted with indicated antibodies.

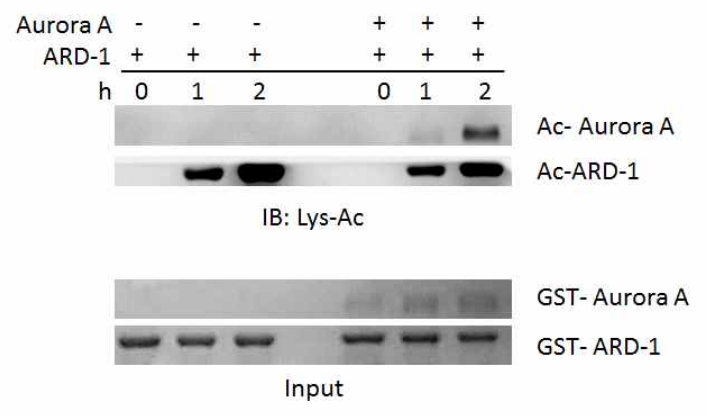

Figure 7. Acetylation of Aurora kinase A by ARD1

Purified recombinant GST-ARD1, and GST-Aurora A were subjected to the in vitro acetylation assay for the indicated times. Acetylated proteins were detected by anti-acetyl-lysine (Lys-Ac) antibody. Coomassie brilliant blue staining shows the quantification of the input proteins.

3. ARD1 WT and ARD1 mutants in cell proliferation

It was reported that R82A and Y122F mutants of ARD1 impacted on the acetylation activity of ARD1. Furthermore, K136R mutation led to the abolition of autoacetylation activity of ARD1 (Seo et al., 2010). To detect the differences of ARD1 WT and its mutants, we checked the colocalization of ARD1 WT and mutants with Aurora kinase A. The results showed that the colocalization signals of mutants with Aurora kinase A seemed weaker in comparison to ARD1 WT (Fig. 8).

Cell proliferation assay proved that ARD1 WT increased cell growth while the mutants didn't show that effect or even decreased the cell proliferation (Fig. 9).

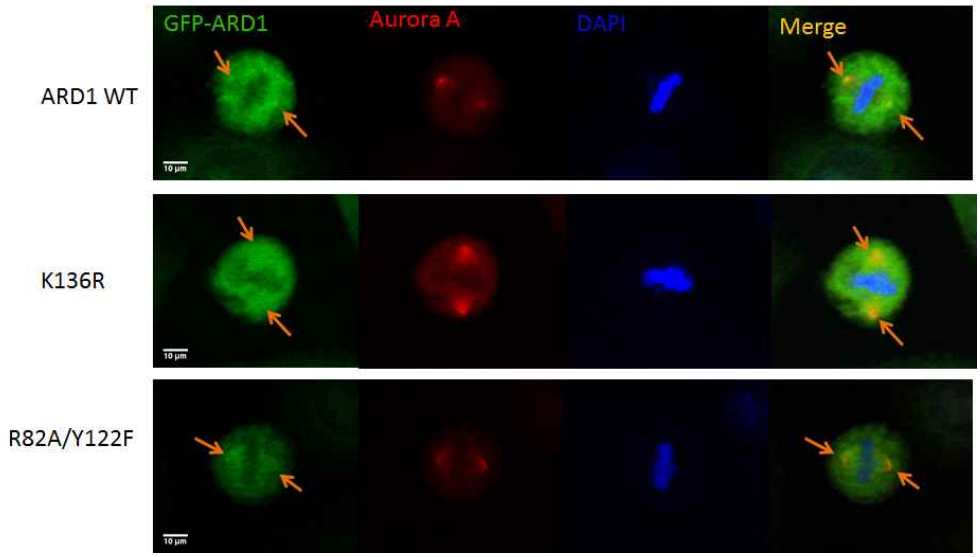

Figure 8. Differences of ARD1 WT and mutants in localization

Hela cells expressed GFP-ARD1, GFP-K136R, GFP-R82A/Y122F were seeded on 24-well plate and fixed with methanol, permeabilized, followed by blocking with 1% BSA in PBS and overnight treatment of anti-Aurora A antibody at 4°C.

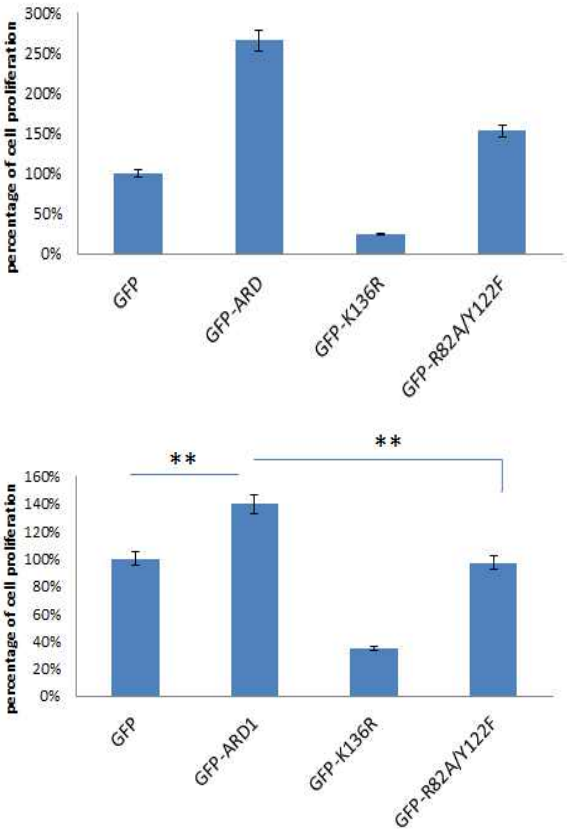

Figure 9. ARD1-induced cell proliferation.

The growth of Hela and Raw 264.7 stable cells were determined by MTS assay after 3 days.

IV. DISCUSSION

Many studies demonstrate the effects of acetylation activity and autoacetylation activity of ARD1 on cell growth and the impact of ARD1 absence in cell survival. Besides, there are several researches studying the localization of ARD1 in cells. However, the expression of ARD1 in cell cycle and localization of ARD1 in phases of cell cycle is still uncovered. Thus, we examined the level of ARD1 in cell cycle phases by cell synchronization. Previous studies show that cells were arrested in G1 when ARD1-siRNA was applied (Lim et al., 2006). Our study found that ARD1 protein level increased in mitotic phase (Fig. 3). Interestingly, ARD1 had centrosome-like localization in different phases, especially in metaphase and anaphase (Fig. 4). Therefore, ARD1 is obviously required for mitosis entry and plays an important role in this phase.

Among the proteins related to centrosomes, Aurora kinase A stands out as a key protein in centrosomes maturation. Aurora kinase A regulates centrosome formation, separation and influence mitotic spindle assembly by interacting directly or indirectly with many checkpoint proteins associated with cell cycle regulation (Fu, Bian, Jiang, & Zhang, 2007; Stéphanie Dutertre, 2002). The finding of the interaction of ARD1 and Aurora kinase A at centrosomes during mitotic phase (Fig. 5,6) reveals the necessary of ARD1 activity in regulating cell progress into mitosis.

Most of studies on Aurora kinase A research on its phosphorylation activity since it belongs to a kinase family. However, ARD1 is renowned as an N-acetyltransferase protein. Therefore, we hypothesized that Aurora kinase A could be acetylated. As expected, the in vitro acetylation assay revealed the acetylated Aurora kinase A bands after incubating with ARD1 and acetyl-CoA

for 1h and 2h. And this experiment showed Aurora kinase A was acetylated in the time-dependent manner (Fig. 7). Thus, the interaction between ARD1 and Aurora kinase A could be associated with acetylation activity.

Previously, K136R and R82A/Y122F were shown to abolish the autoacetylation activity of ARD1 and thereby reduced cell proliferation (Seo et al., 2010). Consistent with this study, Hela stable cell lines and Raw264.7 stable cell lines that overexpressed ARD1 WT and mutants indicated that ARD1 WT promoted cell growth but not K136R and R82A/Y122F mutations (Fig. 8). Furthermore, immunocytochemistry results showed the fainter signal of colocalization of ARD1 mutants with Aurora kinase A compared with the one of ARD1 WT and Aurora kinase A (Fig. 9). These results also suggest the importance of autoacetylation of ARD1 in cell cycle regulation.

Taken together, our data imply the essential role of ARD1 in cell cycle, particularly in mitosis. This role is possibly represented via acetylation activity on Aurora kinase A, which is integral in centrosome function.

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국문초록

ARD1 (N-acetyltransferase arrest defective 1)은 효모에서 처음으로 N-α-acetylation 활성을 갖는다고 알려졌고, NatA acetyltransferase의 소단위체로 분류되었다. 또한 선행연구에 따르면 ARD1은 포유류 세포에서 N-α-acetylation과 N-ε-acetylation 활성을 갖는다고 보고되었다. ARD1은 유방암, 전립선암, 폐암, 자궁경부암에서 과발현 된다고 알려져 있고, ARD1은 세포증식에서 중요한 역할을 한다고 알려져 있다. 또한, ARD1의 autoacetylation 활성은 세포주기에서 중요한 역할을 수행한다고 알려져 있다. 그러나, 세포주기에서 ARD1의 생물학적 기전은 잘 알려져 있지 않다. 한편, Aurora kinase A (AURKA)는 Aurora kinase protein family중 하나이며, Aurora A는 serine/threonine-protein kinase 6로서 중심체의 성숙과 분리, 유사분열 시 방추사 조립에 관여한다고 보고되었다. 따라서, Aurora A는 세포주기 조절에서 중요한 역할을 수행함을 알 수 있다. 최근 연구에서 Aurora A의 과발현은 중심체의 이수성과 과잉, 방추사의 결함과 관련이 있다고 보고되었고 유방암, 전립선암, 난소암, 자궁경부암, 대장암 등에서 과발현 되는 것으로 보고되었다. 본 논문에서는 ARD1이 유사분열과정 중 중심체에 위치하는 것을 확인하였고, 면역침강법을 통해 ARD1이 Aurora kinase A와 결합하는 것을 확인하였다. 또한, *in vitro* actylation assay를 통해 Aurora A가 아세틸화되는 것을 확인하였다. 그리고, wild-type ARD1이 과발현 된 세포는 정상적으로 증식되는 것을 확인하였으나 이와 달리 mutant ARD1이 과발현 된 세포는 정상적으로 증식되지 않음을 확인하였다. 이를 통해, ARD1의 아세틸화 활성이 Aurora A를 통해 세포주기를 조절한다는 것을 규명하였다.

주요어: ARD1, Aurora kinase A, acetyltransferase, acetylation, cell cycle, centrosome

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