Different subcellular localizations and functions of human ARD1 variants

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Abstract. ARD1 is present in various species and has several variants derived from alternative splicing of mRNA. Previously, we reported differential biological functions and cellular distributions of mouse ARD1 (mARD1) variants. However, in comparison to mARD1 variants, human ARD1 (hARD1) variants have been rarely studied. In this study, we characterized a hARD1 variant, hARD1^{131} and investigated its cellular activities. hARD1^{131} mRNA was isolated from HeLa cells and sequenced. Sequence alignment revealed that, compared to hARD1^{235}, the most common form of hARD1, the mRNA sequence encoding hARD1^{131} possesses an altered reading frame due to a 46-bp deletion. Thus, hARD1^{131} and hARD1^{235} differ in their C-terminal regions with a partially deleted acetyltransferase domain at the C-terminus of hARD1^{131}. Moreover, hARD1^{131} and hARD1^{235} showed different subcellular localizations and biological functions. hARD1^{131} was mostly localized in the cell nucleus, whereas hARD1^{235} was primarily localized in the cytoplasm. In addition, hARD1^{235} stimulated cell proliferation by upregulation of cyclin D1, however hARD1^{131} had no influence on cyclin D1 expression and cell growth. Because hARD1^{235} enhances cell proliferation by its autoacetylation activity, we examined the autoacetylation activity of hARD1^{131} and observed that this function was absent in hARD1^{131}. These results suggest that human ARD1 variants have different effects on cell proliferation, which may result from distinct subcellular localizations and autoacetylation activities.

Introduction

ARD1 was originally identified in yeast as an N-acetyltransferase that catalyzes N-terminal acetylation of newly synthesized proteins. In yeast, ARD1 is required for entry into the stationary phase and sporulation during nitrogen deprivation (1). Subsequently, mammalian ARD1 was identified and found to catalyze not only N-terminal acetylation but also lysine acetylation of several proteins including hypoxia-inducible factor-1 α (HIF-1α), β-catenin, myosin light chain kinase, the androgen receptor, tuberous sclerosis 2 (TSC2) and the tubulin complex (2-7). In mammalian cells, ARD1 regulates diverse cellular activities including growth, apoptosis, autophagy and differentiation (7-12). In particular, ARD1 garnered attention as a molecule that plays a critical role in cancer progression (13-15). ARD1 expression is elevated in various human cancers such as lung, breast, prostate, thyroid, and colorectal cancer (16-20). Furthermore, depletion of ARD1 leads to impaired proliferation or induces apoptosis in human cancer cells (3,21). Thus, emerging evidence suggests that ARD1 could be a potential target for cancer therapy.

There are several isoforms of ARD1 derived from alternative splicing of mRNA. Alternative splicing of ARD1 mRNA is a species-specific event, thus isoform compositions differ between humans and mice (22). Previously, we identified three mouse (mARD1^{198}, mARD1^{225}, mARD1^{235}) and two human (hARD1^{131}, hARD1^{235}) ARD1 variants (23). Among these, mARD1^{225}, mARD1^{235} and hARD1^{235} have been well characterized and were found to have different cellular localizations with distinct roles in tumor angiogenesis (2,22-27). However, the cellular expression profiles and biological functions of mARD1^{198} and hARD1^{131} remain unelucidated.

The current study was designed to characterize the hARD1 variant, hARD1^{131} and to investigate how its cellular functions differ from hARD1^{235}, the most common form of hARD1. Our results demonstrate that the human ARD1 (hARD1) variants, hARD1^{131} and hARD1^{235}, have different subcellular localizations and play distinct roles in the regulation of cell proliferation.

Materials and methods

Reagents and antibodies. Anti-GFP and cyclin D1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-acetyl-lysine antibody was purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-tubulin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

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Table I. Design of the primer sets for RT-PCR.

Primer	Sequence (5'-3')	Region (nucleotide)
P1 sense	ATGAACATCCGCAATGCCAGG	1-21
P1 antisense	CTAGGAGGCTGAGTCGGAGGC	688-708
P2 sense	AACTTCAATGCCAAATATGTC	301-321
P2 antisense	TCATGGCATAGGCGTCCTCCC	422-442
P3 sense	AGCGGGACCTCACTCAGATGG	443-463
P3 antisense	CTAGGAGGCTGAGTCGGAGGC	688-708

The sequences of P1, P2 and P3 are conserved in hARD1^{235} and hARD1^{131} mRNA sequences. P1 sense and antisense primers correspond to the regions containing the start and stop codons of hARD1^{235}, respectively. P2 antisense primer corresponds to the region containing the stop codon of hARD1^{131}.

Cell culture. HeLa and 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37˚C and 5% CO_2 in a humid atmosphere.

Plasmid constructions and transfection. To construct expression vectors for human ARD1 variants, ARD1 cDNA was amplified by PCR and sub-cloned into a GFP-tagged pCS2+ vector for cell expression, and a pGEX-4T vector for bacterial induction of the recombinant protein. Transfection was carried out using Lipofectamine (Life Technology, Carlsbad, CA, USA) or Polyfect (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions.

Immunoblotting and immunoprecipitation. Cells were harvested and proteins were extracted using protein lysis buffer (10 mM HEPES at pH 7.9, 40 mM NaCl, 0.1 mM EDTA, 5% glycerol, 1 mM DTT and protease inhibitors). The concentration of extracted protein was measured using a BCA assay. Total cell lysates were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Pharmacia Bioscience, Piscataway, NJ, USA). The membrane was probed with a primary antibody followed by a secondary antibody conjugated to horseradish peroxidase, and protein was visualized using the ECL system (Intron Biotechnology, Gyeonggi-do, Korea).

In vitro acetylation assay. Recombinants of GST-hARD1 variants were freshly prepared as previously described (21). ARD1 recombinants were incubated in reaction mixture (50 mM Tris-HCl at pH 8.0, 0.1 mM EDTA, 1 mM DTT, 10% glycerol and 10 mM acetyl-CoA) at 37˚C for 1 h.

Immunofluorescence staining and microscopy. Cells were placed on cover slips then incubated with Hoechst 33342 (Molecular Probes, Eugene, OR, USA) for nucleus staining. Axiovert M200 microscopes (Carl Zeiss, Jena, Germany) were used for immunofluorescence imaging.

Reverse transcription-PCR analysis. Total RNA was extracted using an RNA extraction kit (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized from 2 μg of RNA using an oligo(dt) primer. Primers used for PCR reactions were as follows: human ARD1, 5'-ATGAACATCCGCAATGCGAG-3' (forward) and 5'-CTCATATCATGGCTCGAGAGG-3' (reverse); cyclin D1, 5'-CTGGCCATGAACTACCTGGA-3' (forward) and 5'-GTC ACACTTGATCACTCTGG-3' (reverse); GAPDH, 5'-ACCAC AGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCCT GTTGCTGTA-3' (reverse). The PCR reaction was performed for 25 cycles to allow ARD1, cyclin D1 and GAPDH amplification.

Cell proliferation assay. The rate of cell proliferation was measured using a Non-Radioactive Proliferation Assay kit (Promega) according to the manufacturer's instructions. Briefly, cells were seeded into 96-well plates and cultured for three days. Subsequently, 20 μl of substrate solution was added and the cells were incubated for 1 h to allow color development. The absorbance at 492 nm was measured to determine the number of proliferating cells.

Statistical analysis. Results are presented as means ± SD and P-values were calculated by applying the two-tailed Student's t-test to data derived from three independent experiments. Differences were considered statistically significant when $P<0.05$.

Results

Expression of hARD1 variants. During the cloning of human ARD1 using mRNA prepared from HeLa cells, we observed that cDNA from one clone (no. 4) was shorter than cDNA from other clones (Fig. 1A). Sequence analysis revealed that the clone with the shorter cDNA sequence (clone no. 4) was hARD1^{131} (GenBank accession no. BC063377), whereas the other clones were all hARD1^{235} (GenBank accession no. NM_003491). As shown in Fig. 1B, the nucleotide sequence from residue 342 to 387, which is located in exon 6 and exon 7 of ARD1 mRNA, was deleted in hARD1^{131}. Therefore, the nucleotide sequence of hARD1^{131} is 46 bp shorter than that of hARD1^{235}.

To confirm the expression of hARD1^{131} in human cells, we performed RT-PCR using mRNA isolated from 293T and HeLa cells. As described in Table I and Fig. 1B, three kinds of primer sets named P1, P2 and P3 were designed for the PCR analysis: P1 and P2 contained the deleted mRNA

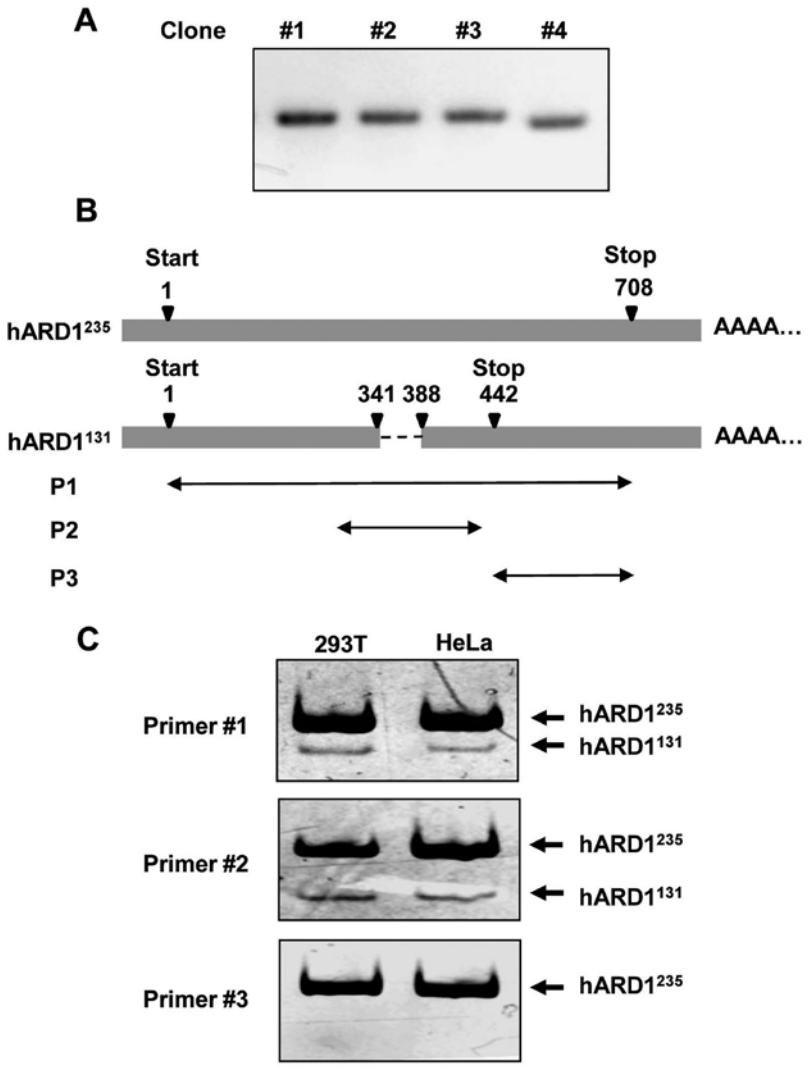

Figure 1. Expression of hARD1 variants in human cell lines. (A) hARD1 sequence was amplified from cDNA reverse transcribed from HeLa cell mRNA. Four clones were analyzed by electrophoresis on agarose gels. (B) cDNA alignment of hARD1^{235} and hARD1^{131}. Primers sets P1, P2 and P3 were designed for the detection of hARD1^{235} and hARD1^{131}. (C) Total RNA was exacted from 293T and HeLa cells and RT-PCR was performed using primer sets P1, P2 and P3. Expression of hARD1^{235} and hARD1^{131} was analyzed by electrophoresis on DNA polyacrylamide gels.

region, whereas P3 did not. As predicted, when P1 and P2 were used in PCR, two bands corresponding to hARD1^{131} and hARD1^{235} were detected by polyacrylamide gel electrophoresis. However, only one band, corresponding to hARD1^{235}, was detected following PCR with the P3 primer set. These data confirm the expression of the hARD1^{131} splice variant in human cells (Fig. 1C).

Sequence comparison of hARD1 variants. Next, we compared the coding sequences of hARD1^{131} and hARD1^{235}. The coding sequence of hARD1^{235} terminates at amino acid 708, thus it encodes a 235 amino acid proteins. However, in hARD1^{131}, deletion of 46 bp in hARD1^{131} results in a frame shift after amino acid 113, resulting in premature termination at amino acid 131 (Fig. 2A and B). ARD1 is predicted to have an acetyltransferase domain located between amino acid residues 45 and 130, in which an acetyl-CoA binding domain is positioned between amino acid residues 82 and 87. Compared with hARD1^{235}, hARD1^{131} protein possesses a conserved acetyl-CoA binding domain. However, 20% of the acetyltransferase domain was deleted in hARD^{131} and it was found to have a different C-terminal region (Fig. 2B).

Subcellular localization of hARD1 variants. In a previous study, we reported that splice variants of mouse ARD1 have a different subcellular localization (22). Therefore, we speculated that hARD1^{131} might have a distinct localization compared to hARD1^{235}. To investigate ARD1 location in human cells, we constructed GFP-tagged plasmids containing hARD1^{131} and hARD1^{235}, which were subsequently transfected into HeLa and 293T cells. The localization of GFP-hARD1 variants and control GFP protein was analyzed by fluorescence microscopy. As shown in Fig. 3, GFP-hARD1^{235} was observed predominantly in the cytoplasm, despite hARD1 containing a putative nuclear localization sequence (NLS) (Fig. 2B). however, hARD1^{131} was specifically located in the cell nucleus. These results demonstrate that hARD1 variants have distinct subcellular localizations, and suggest that the roles of hARD1^{131} and hARD1^{235} might differ within the cell.

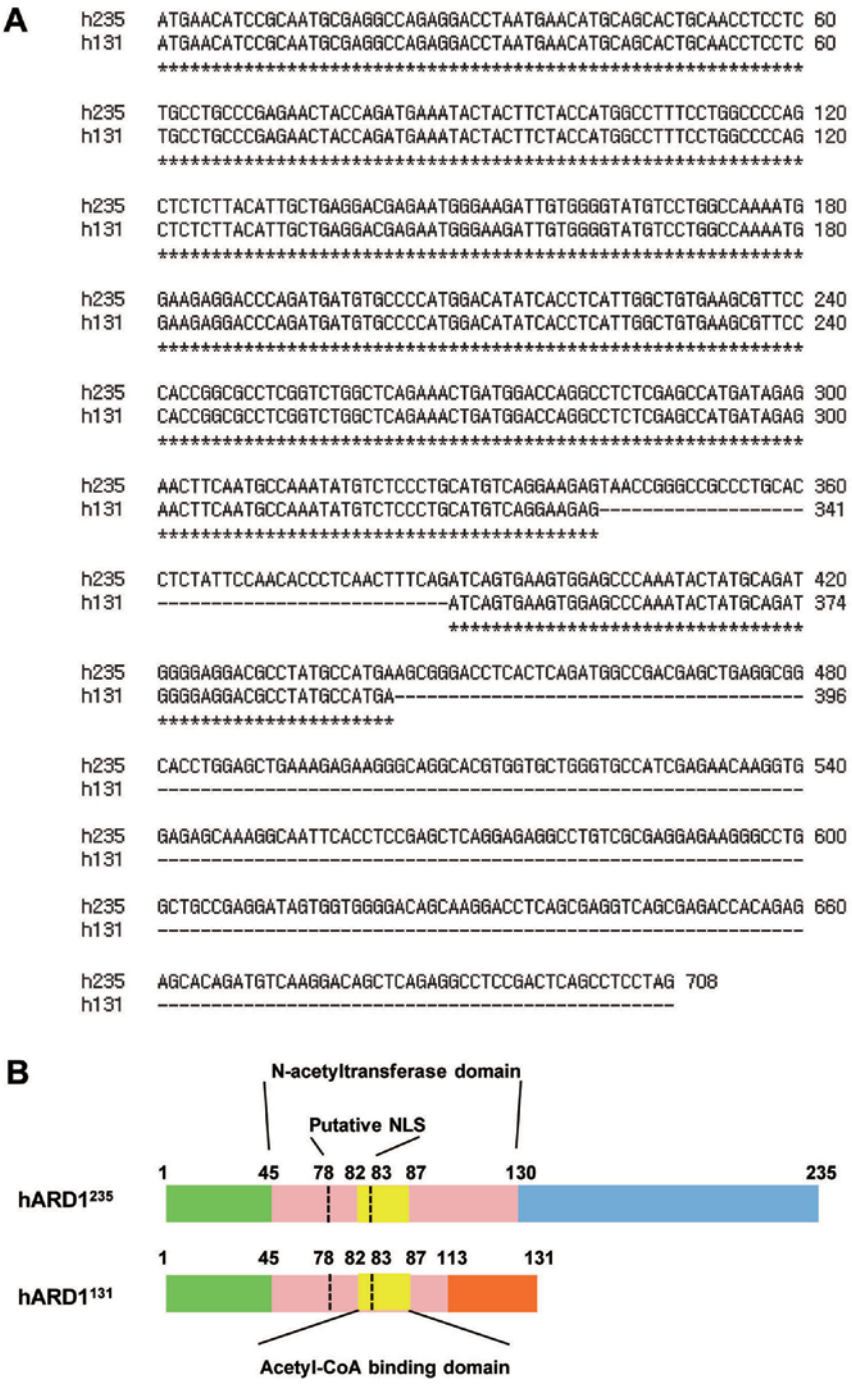

Figure 2. Sequence comparison of human ARD1 (hARD1) variants. (A) Sequence alignment of hARD1^{235} and hARD1^{131}. Identical residues are indicated by asterisk. (B) Schematic representation of hARD1^{235} and hARD1^{131} amino acid sequences. The acetyltransferase domain at amino acid 45-130, the putative NLS at amino acid 78-83 and the acetyl-CoA binding domain at amino acid 82-87 are indicated.

Different functions of hARD1 variants in the regulation of cell proliferation. Several studies have reported that hARD1^{235} regulates the cell cycle and stimulates cell proliferation (3,21). Thus, we aimed to determine whether hARD1^{131} could promote cellular growth in a similar way to hARD1^{235}. Consistent with previous studies, compared to control cells, cell proliferation was significantly accelerated in hARD1^{235}-expressing HeLa cells. However, hARD1^{131} had no effect on cell growth, suggesting that hARD1 variants have different roles in the regulation of cell proliferation (Fig. 4A and B). In a previous study, cyclin D1 was found to mediate ARD1-induced cell growth, therefore we examined the expression levels of cyclin D1 mRNA and protein in hARD1^{235} and hARD1^{235} transfected cells (3,21). Consistent with enhanced cellular growth, cyclin D1 mRNA and protein expression were significantly increased in hARD1^{235}, but not hARD1^{131} transfected cells (Fig. 4C and D). These results suggest that the diverse roles of hARD1 variants in the regulation of cell proliferation may be due to their different effects on cyclin D1 expression.

Previously, we showed that the autoacetylation activity of hARD1^{235} was required for enhanced cell proliferation (21). Thus, we compared the autoacetylation activities of hARD1^{131}

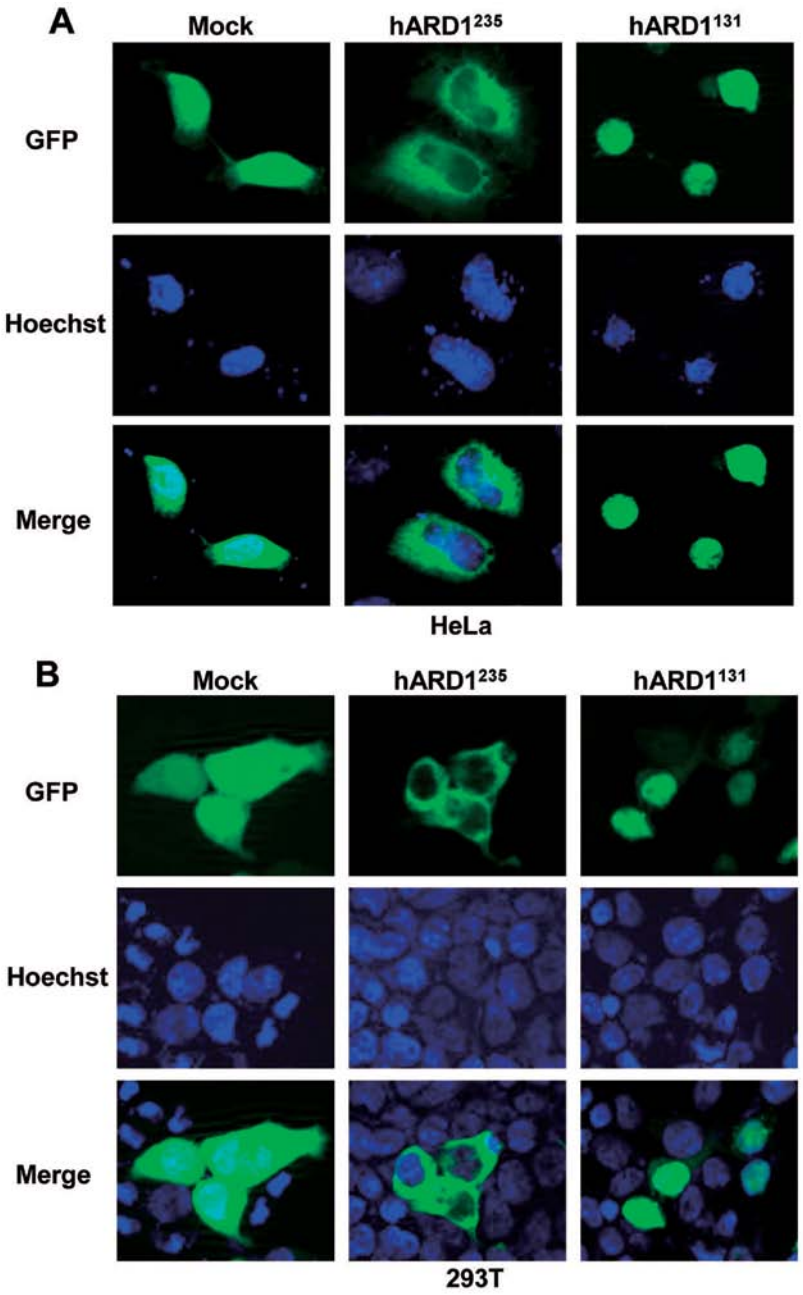

Figure 3. Subcellular localization of hARD1 variants in human cell lines. GFP-tagged plasmids for hARD1 variants were transfected into HeLa cells (A) and 293T cells (B). Nucleus-staining was performed with Hoechst. Localization of ARD1 variants was analyzed and photographed by a fluorescence microscope.

and hARD1^{235} using an *in vitro* acetylation assay. While the hARD1^{235} recombinant acetylated itself, hARD1^{131} was not acetylated *in vitro*, indicating a lack of autoacetylation activity in this splice variant (Fig. 4E). These results suggest that, unlike hARD1^{235}, hARD1^{131} has no autoacetylation activity, and is therefore unable to upregulate cyclin D1 expression and promote cell proliferation.

Discussion

Alternative mRNA splicing is a common process in the regulation of gene expression by which a single gene codes for multiple proteins. This process contributes to protein diversity, and different proteins produced from alternative splicing often have distinct cellular functions (28,29). The current study characterized alternative splice variants of human ARD1 and demonstrated differential biological functions and cellular distributions of these variants.

Previously, we identified two hARD1 variants, hARD1^{235} and hARD1^{131} (23). However, human cells dominantly express hARD1^{235}, and hARD1^{131} expression has not been detected using RT-PCR or western blots in previous studies (22,23). In this study, the expression of hARD1^{131} was clearly detected using RT-PCR and polyacrylamide gel electrophoresis, which can separate DNA well beyond the resolving capabilities of an agarose gel (Fig. 1C). The basal expression level of hARD1^{131} was relatively lower than that of hARD1^{235}. Therefore, we could not exclude the possibility that the cellular activity

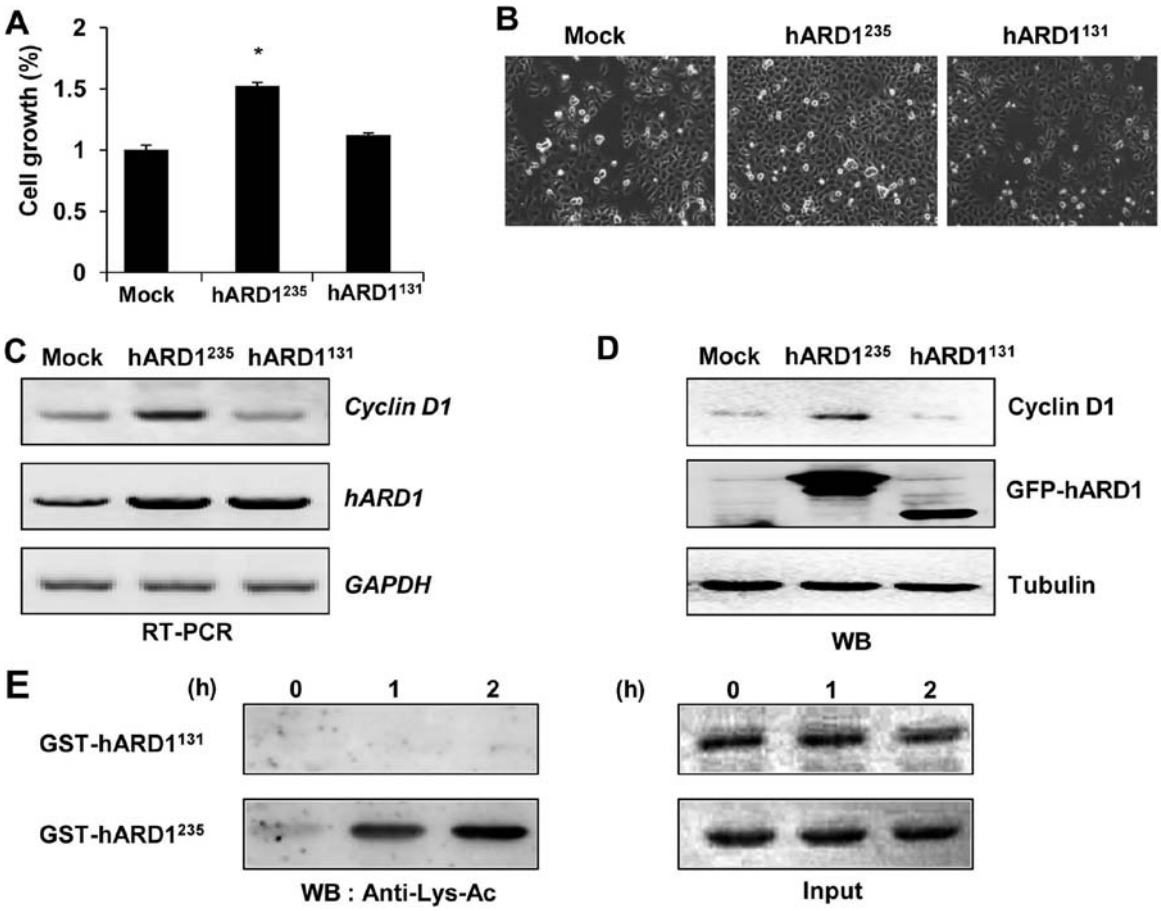

Figure 4. Differential functions of human ARD1 (hARD1) variants in the regulation of cell proliferation. (A) HeLa cells were transfected with GFP-tagged hARD1^{235} and hARD1^{131}, then cell growth was analyzed $^{*}P<0.05$ vs. control. (B) After transfecting cells with plasmids containing hARD1^{235} and hARD1^{131}, cells were grown for 3 days and photographed. (C, D) GFP-tagged hARD1^{235} and hARD1^{131} plasmids were transfected into HeLa cells. Cyclin D1 mRNA and protein levels were analyzed by RT-PCR and western blot, respectively. (E) Purified recombinants for GST-hARD1^{235} and GST-hARD1^{131} were each subjected to the *in vitro* acetylation assay for 1 and 2 h. Acetylated proteins were detected using an anti-acetyl lysine (Lys-Ac) antibody. Total proteins were stained with Coomassie Brilliant Blue (Input).

of hARD1^{131} might be small or performed preferentially by hARD1^{235}.

Interestingly, hARD1^{131} has a unique subcellular localization compared to hARD1^{235} (Fig. 3). Although the NLS is conserved at amino acid residues 78-83 in all ARD1 variants, the different subcellular localizations of hARD1 variants could be explained by structural difference in C-terminal regions (Fig. 2).

The subcellular localization of a protein corresponds to its biological function. As an N-acetyltransferase, hARD1^{235} cooperates with NATH-1 for N-terminal acetylation of newly synthesized proteins in the cytoplasm (14). However, the nuclear localization of hARD1^{131} suggests that it might have functions other than N-acetylation. Indeed, N-terminal acetyltransferase activity is associated with cellular growth. However, hARD1^{131} had no effect on cellular growth (Fig. 4A and B). Moreover, autoacetylation activity, which is essential for the ability of hARD1^{235} to stimulate cell proliferation, was also absent in the hARD1^{131} variant (Fig. 4E). These results suggest novel functions of hARD1^{131} that differ from that of hARD1^{235}, and suggest the necessity for further experiments to investigate the diverse cellular functions of hARD1^{131}.

In summary, the present study revealed that hARD1 variants have different cell proliferative activities that might be associated with their different subcellular localizations and enzymatic activities. To further our understanding of hARD1 isoforms, future studies will focus on elucidating the specific roles played by hARD1 variants and their relationships under various physiological conditions.

Acknowledgements

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