Molecules and Cells

RNA Editing Enzyme ADAR1 Suppresses the Mobility of Cancer Cells via ARPIN

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https://doi.org/10.14348/molcells.2023.2174
www.molcells.org

Deamination of adenine or cytosine in RNA, called RNA editing, is a constitutively active and common modification. The primary role of RNA editing is tagging RNA right after its synthesis so that the endogenous RNA is recognized as self and distinguished from exogenous RNA, such as viral RNA. In addition to this primary function, the direct or indirect effects on gene expression can be utilized in cancer where a high level of RNA editing activity persists. This report identified actin-related protein 2/3 complex inhibitor (ARPIN) as a target of ADAR1 in breast cancer cells. Our comparative RNA sequencing analysis in MCF7 cells revealed that the expression of ARPIN was decreased upon ADAR1 depletion with altered editing on its 3'UTR. However, the expression changes of ARPIN were not dependent on 3'UTR editing but relied on three microRNAs acting on ARPIN. As a result, we found that the migration and invasion of cancer cells were profoundly increased by ADAR1 depletion, and this cellular phenotype was reversed by the exogenous ARPIN expression. Altogether, our data suggest that ADAR1 suppresses breast cancer cell mobility via the upregulation of ARPIN.

Keywords: ADAR1, ARPIN, breast cancer, metastasis, RNA editing

INTRODUCTION

Adenosine deaminase acting on RNA (ADAR) is one of the key enzymes responsible for the deamination of adenosine on nascent RNA (Quin et al., 2021). Deamination occurs typically in Alu-repeats in intergenic regions or introns that are not directly related to the expression of target genes (Athanasiadis et al., 2004). Deamination is regarded as a "self" mark that prevents the activation of the double-stranded RNA (dsRNA) sensing pathway triggered by exogenous, non-edited RNA (Danan-Gotthold et al., 2016). In this regard, recent studies have implicated the suppression of ADAR1 activity in tumor-targeting immunity in a melanoma model, enhancing immunotherapy efficacy (Ishizuka et al., 2019). However, other research showed that epigenetic therapy induced ADAR1 dependency due to the upregulated repetitive SINE (short interspersed nuclear element) and deamination of the dsRNA generated from this element (Mehdipour et al., 2020).

Despite the critical role of ADAR1 in innate immunity (Lamers et al., 2019), its function is expected to be more diverse. For example, ADAR1 variants are associated with neurological disorders, such as Aicardi-Goutières syndrome type 6 (Rice et al., 2012) and bilateral striatal necrosis (Livingston et al., 2014). Also, ADAR1 mutations are found in rare pigmentary

Received November 9, 2022; revised January 5, 2023; accepted January 15, 2023; published online March 16, 2023

eISSN: 0219-1032

disorders, including dyschromatosis symmetrical hereditaria (Suzuki et al., 2005) and reticulate acropigmentation of Kitamura (Kono and Akiyama, 2019). In recent years, the role of ADAR-mediated RNA editing has been demonstrated in cancer (Baker and Slack, 2022). One study in gastric cancer cells revealed ADAR1-dependent microRNA regulation (Cho et al., 2018) and non-canonical interferon signaling (Jiang et al., 2020). Other reports also support ADAR1's function in the editing-dependent regulation of tumor suppressor or oncogene expression, including Blcap (Riedmann et al., 2008), GLI1 (Lazzari et al., 2017), and AZIN1 (Chen et al., 2013). Few studies also show the role of ADAR1 in tumor metastasis. For example, Takeda et al. (2019) demonstrated that edited AZIN1 enhanced the invasive potential of fibroblasts in colorectal cancer, which promotes tumor invasion and metastasis.

Actin-related protein 2/3 complex inhibitor (ARPIN) is one of the inhibitors of actin-related protein 2/3 (Arp2/3) by competing with nucleation-promoting factors such as WAVE (Lomakina et al., 2016). Arp2/3 generates a branch in the actin cytoskeleton, thereby playing a critical role in cell mobility, adhesion, and vesicular transport (Volkmann et al., 2001). Arp2/3 activity is often upregulated in multiple types of metastatic cancers, including melanoma (Kashani-Sabet et al., 2009), glioblastoma (Liu et al., 2013), gastric (Zheng et al., 2008), and breast cancer (Iwaya et al., 2007). Consistently, ARPIN downregulation in breast cancer and its association with a poor prognosis has been reported (Lomakina et al., 2016). Moreover, the restoration of ARPIN in breast cancer cells was shown to suppress the cancer's aggressiveness (Li et al., 2017). Thus, the regulation of Arp2/3 via ARPIN seems to play a key role in the metastatic conversion of cancer cells. In this report, we show that ADAR1 regulates the expression of ARPIN via its 3'UTR in breast cancer cells. Consequently, this regulation affects the mobility of cancer cells, proposing a novel function of ADAR1 in cancer metastasis.

MATERIALS AND METHODS

Cell cultures and transfection

Human breast cancer cell lines, MDA MB 468, MCF7, and MDA MB 231 cells, and HEK 293T human embryonic kidney cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. The cells were incubated at 37°C and 5% CO_2. To overexpress ARPIN, MCF7 cells were plated in 100 mm plates at 2×10^6 cells, and 5 μg of ARPIN expression vector or control empty vector were transfected with Lipofectamine 3000 (Invitrogen, USA). For the ectopic expression of microRNAs, miR-1285-3p, miR-1285-5p, and miR-619-5p mimic (Qiagen, Germany) were used. For transient knockdown, the cells were transfected with siADAR1 and siARPIN (Genolution, Korea) using Lipofectamine 3000 (Supplementary Table S1 for sequences).

Stable knockdown of ADAR1 using shRNA

To deplete ADAR1 in human breast cancer cell lines (MCF7, MDA MB 468, and MDA MB 231), a shADAR1 (TRCN 0000050788; Sigma, USA) viral vector was used. To generate the virus, HEK 293T cells were plated at 100 mm at a density of 2×10^6 cells and co-transfected with a viral vector (shADAR1) and packaging plasmid (pMD2.G, psPAX2) using Lipofectamine 3000. After infection, puromycin (1-2 μg/ml) was used to select stable clones of ADAR1 knockdown (ADAR1-KD).

Quantitative real-time PCR (qRT-PCR) analysis and validation of RNA editing

Real-time PCR analysis was performed to quantify mRNA and microRNA (miRNA). Total RNA was isolated using TRIzol according to the manufacturer's instructions. cDNA synthesis was performed with 1 μg of RNA by Primer Script 1st strand kit (Takara, Japan). For miRNA analysis, the miScriptII RT Kit (Qiagen) was used. Relative expression was calculated by the 2-(ΔΔCt) method (Livak and Schmittgen, 2001). qRT-PCR primer sequences are given in Supplementary Table S1. To confirm the A to G RNA editing identified from RNA sequencing (RNAseq), Sanger sequencing of the editing site was carried out. For this, the PCR amplicon of cDNA or genomic DNA (gDNA) of the editing region was prepared using the KOD FX Neo Kit (Toyobo, Japan). The location of the editing site was validated using the human reference genome (UCSC hg19/GRCh37).

Protein extraction and western blotting

For the detection of target proteins by western blot, MCF7 and MCF7 ADAR1-KD cell lines were lysed using rapid immunoprecipitation assay (RIPA) lysis buffer with a protease inhibitor cocktail and phosphatase inhibitor (Roche, Switzerland) tablets. After 10 min of incubation on ice, the supernatant was obtained by centrifugation at 13,000 rpm for 10 min. The protein concentration was measured with a BCA protein assay kit (Bio-Rad Laboratories, USA). The protein samples were separated in 10% and 12% SDS-PAGE gels and transferred onto nitrocellulose membranes. The blot was probed with anti-β-actin (1:2,000; Santa Cruz Biotechnology, USA), anti-ADAR1 (1:2,000; Santa Cruz Biotechnology), anti-ARPIN (ab235421, 1:1,000; Abcam, UK), E-cadherin (1:1,000; Cell Signaling Technology, USA), N-cadherin (1:1,000; Cell Signaling Technology), Slug (1:1,000; Cell Signaling Technology), and Snail (1:10,000; Cell Signaling Technology) antibodies. The intensity of the band signals was analyzed using National Institutes of Health (NIH) image J 1.47v software.

Immunocytochemistry (ICC)

For ICC, the cells were seeded on 35 mm dishes. After 24 h, the cells were washed in phosphate-buffered saline (PBS) and fixed with 5% formaldehyde in DMEM at 37°C for 10 min, followed by incubation with 4% formaldehyde in PBS at 37°C for 10 min. The cells were then permeabilized with 0.5% triton × 100 for 20 min and incubated for 24 h with an anti-ARPIN (1:50) (ab150113; Abcam) antibody. After washing, an Alexa 488-conjugated anti-mouse IgG antibody (ab150113; Abcam) was probed, followed by an anti-Rhodamine phalloidin (R415, 1:100; Invitrogen) antibody. Fluorescence microscope images were obtained using an inverted microscope (Carl Zeiss, Germany).

Migration/invasion assay

Cell migration and invasion were measured using transwell chambers with a 6.5 mm diameter (polycarbonate filters of 8.0 μm pore size). In brief, the cells were resuspended in serum-free medium and plated on the upper chamber, while the lower chamber was filled with DMEM containing 10% FBS. The cells were incubated for 24 h at 37°C. For the invasion assay, the upper chamber was loaded with Matrigel (BD, USA) for 20 min and incubated at 37°C before cell seeding. Migration and invasion were measured by counting cells on the lower side of the filter after H&E staining using NIH Image J 1.47v software.

Luciferase reporter assay

For the reporter assay, the cells were plated on 24-well plates. After 24 h, the cells were co-transfected with 100 ng of pMIR ARPIN 3'UTR plasmid or pGL3 ARPIN promoter plasmid plus 10 ng TK luc (Renilla luciferase, internal control) per well. After 48 h, the cells were lysed, and luciferase activity was measured using the Dual-Luciferase reporter assay system (Promega, USA) and GloMax Luminometer (Promega) according to the manufacturer's protocol.

Analysis of RNA editing events from RNAseq data

RNAseq data of shGFP or shADAR1 clonal cells from MCF7 cells was generated by the Illumina HiSeq™ 2500 platform. Initial alignment was done using the UCSC reference genome hg19 by STAR software. The A to G editing was detected by the process previously described (Cho et al., 2018). Candidates for editing were filtered by Alu and non-Alu regions, site of A to G editing events, and alteration frequency of editing by comparing shGFP clone cells and shADAR1 clone cells.

Statistical analysis

Error bars are presented as the SEM. Statistical significance was determined by a two-tailed Student's *t*-test. For all the measurements, *P* values less than 0.05, 0.01, and 0.001 were marked as *, **, and ***, respectively.

RESULTS

The stable knockdown of ADAR1 in MCF7 cells profoundly reduces A to G editing in Alu regions

To understand the role of ADAR1 in breast cancer, we first checked ADAR1 expression among breast cancer cell lines (Supplementary Fig. S1). Compared to MCF10A (untransformed breast cell line), we found high expression of ADAR1

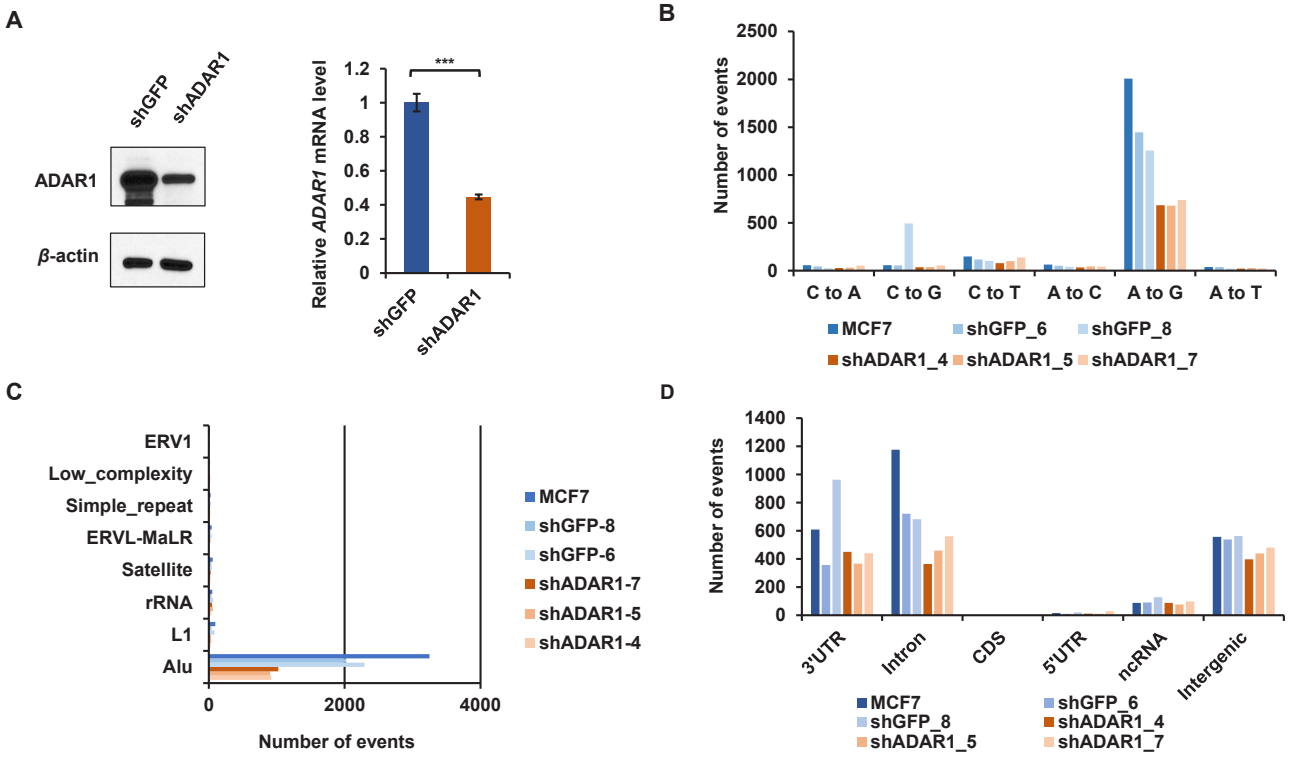

Fig. 1. RNA editing in Alu repeats is primarily affected by the depletion of ADAR1 in MCF7 cells. (A) shRNA-mediated stable knockdown of ADAR1 in MCF7 cells. Western blot on the left panel shows decreased ADAR1 protein expression, and the graph on the right indicates reduced RNA level by ADAR1 shRNA. β-Actin was used as a loading control. (B) Marked decrease of A to G editing events in the whole transcriptome via the depletion of ADAR1. Number of A to G alteration events in control (blue bars) or cells with shADAR1 (brown bars), indicating a decreased A to G events by the ADAR1 depletion. (C) Analysis of editing events in repetitive elements shows an evident decrease in editing events in Alu repeats. (D) The number of editing events grouped by genomic locus. Among the editing sites, intronic and 3'UTR regions are markedly affected by ADAR1 depletion. ***$P < 0.001$.

RNA in cancer cell lines, with BT-20 showing maximum ADAR1 expression (Supplementary Fig. S1A). Similar to results reported previously (Kung et al., 2021), we also observed a high level of ADAR1 protein expression in breast cancer cells (Supplementary Fig. S1B). We then suppressed ADAR1 expression in several cell lines by lentivirus-mediated shRNA targeting ADAR1 (ADAR1-KD hereafter). While BT-20 cells showed extensive cell death upon ADAR1 depletion, we successfully obtained stable knockdown clones in MCF7 cells. Western blot data in Fig. 1A shows reduced ADAR1 expression in the ADAR1-KD cells. Real-time PCR also showed a ~60% reduction of ADAR1 RNA expression (Fig. 1A, right panel). Using the ADAR1-KD cells, we performed RNAseq to identify editing targets of ADAR1. The results are summarized in Figs. 1B-1D. As previously reported (Wang et al., 2013), A to G conversion was dominant in the control (Fig. 1B, blue bars) and markedly decreased in three independent clones of ADAR1-KD cells (Fig. 1B, brown bars). The reduction of editing was evident in Alu repeats (Fig. 1C), which are known genomic regions of extensive editing (Daniel et al., 2014). In addition, we also observed decreased editing of the 3'UTR and intronic region upon ADAR1 depletion (Fig. 1D). These results indicate ADAR1 depletion suppressed A to G RNA editing in MCF7 cells.

Table 1. List of ADAR1 target genes edited on 3'UTR

Gene	Chr	Editing			A to G editing alteration frequency					
		Site	Start	End	MCF7	shGFP-1	shGFP-2	shADAR1-1	shADAR1-2	shADAR1-3
RSL1D1	chr16	3'UTR	11929175	11929176	0.769	0.4	0.583333	0.1875	0	0
METTL7A	chr12	3'UTR	51325236	51325237	0.500	0.5	0.352941	0	0	0
METTL7A	chr12	3'UTR	51324118	51324119	0.600	0.304348	0.444444	0	0	0
RSL1D1	chr16	3'UTR	11928845	11928846	0.500	0.5	0.578947	0	0	0.238095
METTL7A	chr12	3'UTR	51324201	51324202	0.556	0	0.461538	0	0	0
ARPIN	chr15	3'UTR	90444707	90444708	0.476	0	0.5	0	0	0

Chr, chromosome.

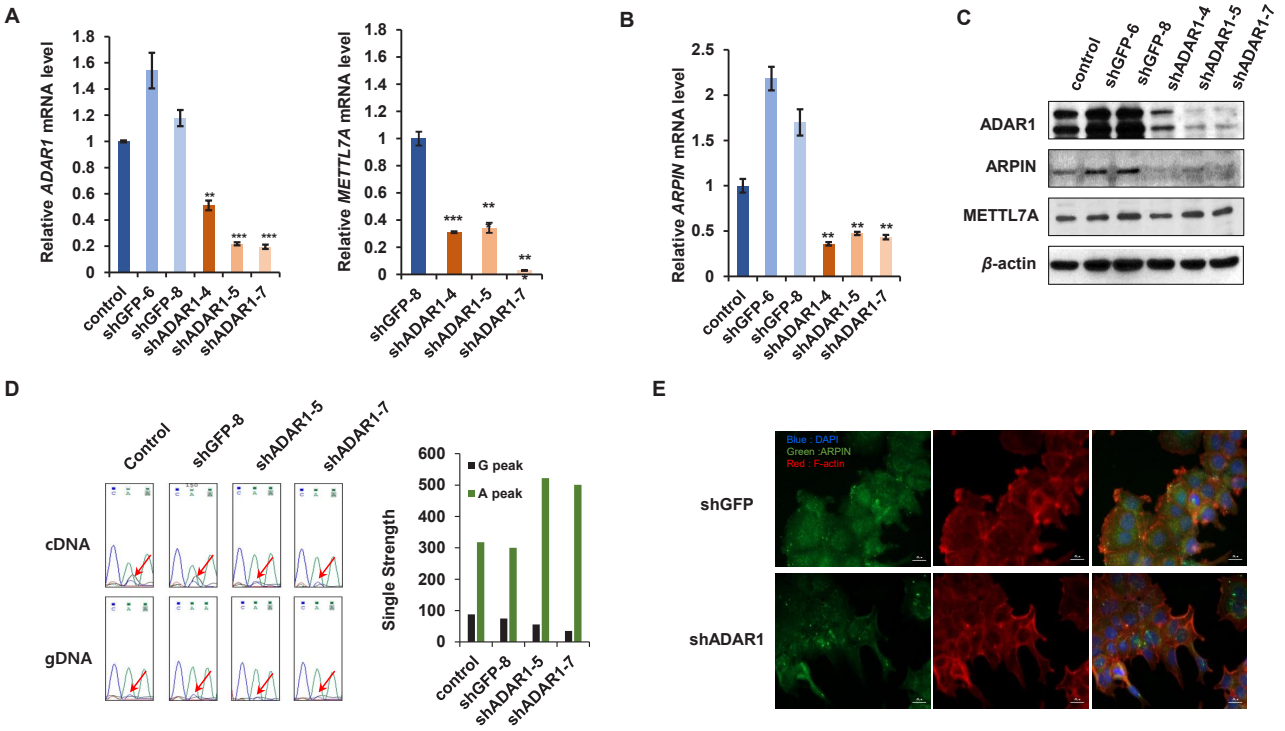

Fig. 2. RNAseq analysis identifies ARPIN downregulation in an ADAR1-dependent manner. (A and B) The RNA level of ADAR1 (A) or ARPIN (B) upon the depletion of ADAR1. (C) Western blot results show the protein level of ADAR1, METTL7A, and ARPIN. β-Actin was used as a loading control. (D) Sanger sequencing results of an editing site in the 3'UTR of ARPIN, from control (MCF7 and shGFP-8) or ADAR1-depleted cells (shADAR1-5, shADAR1-7). The graph on the right shows the intensities of A and G peaks on the ARPIN UTR, measured by the area of peaks shown on the left. (E) Immunofluorescence images of ARPIN (green) and F-actin (red) upon the depletion of ADAR1. Note the decreased ARPIN (bottom left) and more spiky actin filaments in the shADAR1 cells (bottom middle). Magnification, 40×. $^{**}P < 0.01$, $^{***}P < 0.001$.

ADAR1 knockdown reduces ARPIN expression in breast cancer cells

Using the RNAseq data from the ADAR1-KD cells, we next aimed to identify novel editing targets whose expression is correlated with ADAR1 levels (Supplementary Tables S2 and S3). To find such candidates, we first focused on the ADAR1-dependent editing event on the UTR that is commonly recognized by microRNA or RNA binding proteins and affects gene expression. The data in Table 1 represents top genes that are highly edited on specific sites of 3'UTRs and whose editing is markedly inhibited by ADAR1 depletion. We examined the expression of METTL7A and ARPIN in three ADAR1-KD clones and found downregulated METTL7A (Fig. 2A, right) and ARPIN (Fig. 2B) mRNA levels. Further analysis by western blot showed reduced ARPIN protein levels and unchanged METTL7A levels (Fig. 2C). Therefore, we focused on ARPIN regulation by ADAR1. We first validated the editing event on the 3'UTR of ARPIN by Sanger sequencing, corresponding to G peaks in the cDNA of control cells (Fig. 2D, MCF7 control and shGFP-8), that is diminished in two of ADAR1-KD clones (Fig. 2D, shADAR1-5 and shADAR1-7). The genomic DNA sequence confirmed there was no A to G alteration DNA level. The ADAR1-dependent editing on the 3'UTR of ARPIN was also validated in MDA-MB-231-shADAR1 cells (Supplementary Fig. S2). Additionally, the reduced level of ARPIN expression upon ADAR1 depletion was confirmed by immunofluorescence, indicating that ARPIN (Fig. 2E, green signal) colocalization with F-actin (red signal) was markedly reduced in the ADAR1-depleted cells with their membranes becoming more spiky-shaped.

UTR and promoter analysis of ARPIN gene reveals that ADAR1 depletion downregulates ARPIN via the 3'UTR

To understand the mechanism by which ADAR1 regulates ARPIN expression, we first constructed a luciferase reporter for the 3'UTR of ARPIN, where the editing was observed. Due to the large size of the 3'UTR, we divided it into two parts (Supplementary Fig. S3A) and found the second region of the UTR showed significantly less activity upon ADAR1 depletion (Fig. 3A, Supplementary Figs. S3B and S3C), consistent with the reduced RNA and protein expression (Fig. 2). The ADAR1-dependent suppression of ARPIN-3'UTR activity was further confirmed by a transient knock-down of ADAR1 (Fig. 3B, Supplementary Fig. S3D) and by the ectopic expression of ADAR1 that partially rescued UTR activity (Fig. 3C).

Since microRNA are a major player in UTR-mediated expression regulation (Chang and Sharan, 2012), we questioned if the editing on the ARPIN 3'UTR can change critical miRNA-mRNA interaction sites (so-called seed-regions). However, the sequence alignment of ARPIN-3'UTR with the

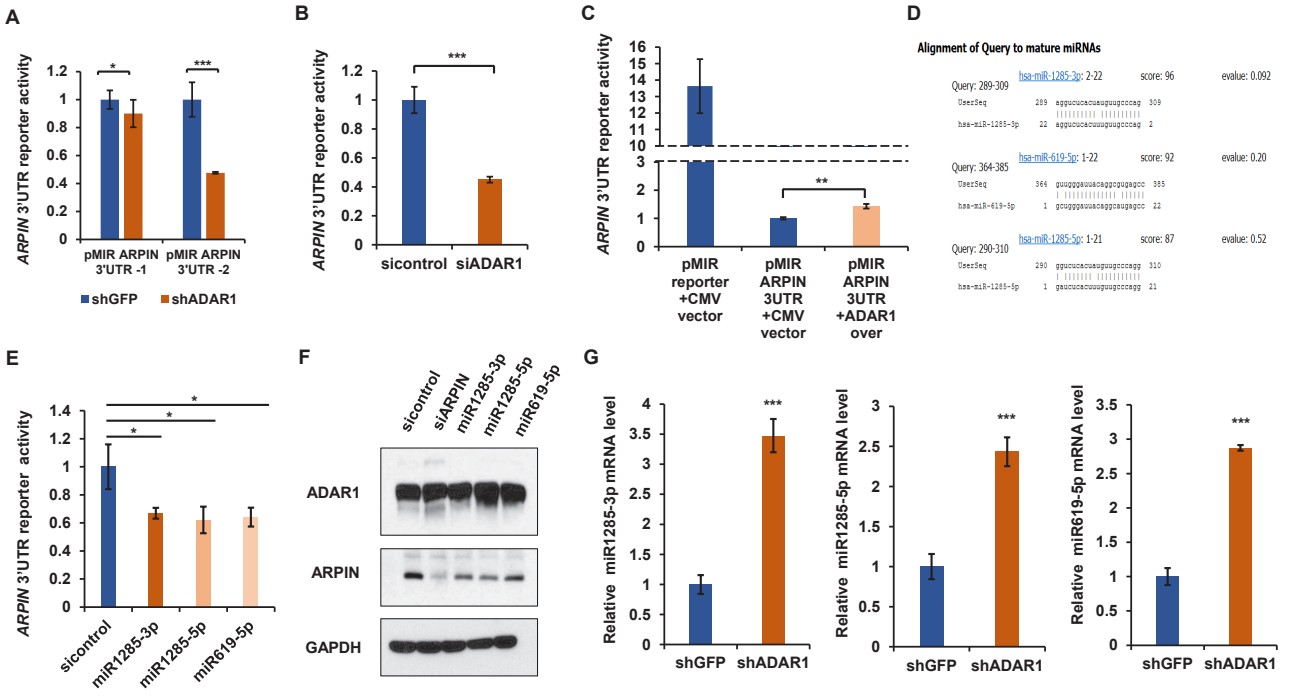

Fig. 3. ARPIN downregulation by ADAR1 is mediated by the overexpression of miR-1285-3p, miR-1285-5p, and miR-619-5p targeting the 3'UTR of ARPIN. (A) Luciferase reporter activity of the 3'UTR of ARPIN in control (shGFP in blue) or ADAR1-depleted cells (shADAR1 in brown). (B) Luciferase reporter activity of the ARPIN 3'UTR upon the transient knockdown of ADAR1 (in brown). (C) Luciferase reporter activity of the ARPIN 3'UTR after the ectopic expression of ADAR1 (ADAR1 over, in brown). Dotted horizontal lines indicate y-axis discontinuity due to the high activity of the empty reporter vector. (D) The sequence alignment of miRNAs predicted to interact on the 3'UTR of ARPIN. (E) Luciferase reporter activity of the ARPIN 3'UTR upon the expression of three candidate miRNAs. (F) Western blot analysis of ARPIN and ADAR1 upon the overexpression of three miRNAs. (G) Real-time PCR for three miRNAs in control (shGFP) or ADAR1-depleted cells (shADAR1). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

miRNA database showed no miRNA binding site changed by the editing, suggesting that the UTR regulation is not mediated by editing of the miRNA binding region. Alternatively, we also examined if ADAR1 affects the promoter activity of ARPIN. A luciferase-based reporter for ARPIN promoter was introduced in MCF7 or HEK293 cells in combination with ADAR1 siRNA. The data in Supplementary Fig. S4 show that ADAR1 depletion by siRNA does not change ARPIN promoter activity. These data suggest that ADAR1 regulates ARPIN expression via UTR regulation, not by its promoter.

To understand how ADAR1 depletion downregulates ARPIN via its 3'UTR, we screened putative miRNAs whose expression is upregulated by ADAR1 depletion and predicted to bind to the ARPIN 3'UTR by conducting small RNAseq in ADAR1-KD cells. We found four miRNAs (Table 2) that are upregulated in ADAR1-depleted cells and predicted to bind to the ARPIN 3'UTR. Interestingly, three miRNAs, namely miR-1285-3p, miR-1285-5p, and miR-619-5p, interact with nearby sequences of the editing site (Fig. 3D), and the ectopic expression of these three miRNAs indeed suppressed the ARPIN 3'UTR reporter (Fig. 3E). Additionally, a western blot after the ectopic expression of three microRNAs showed reduced ARPIN expression (Fig. 3F). The increased expression of the three miRNAs by ADAR1 depletion was confirmed by real-time PCR (Fig. 3G). These results suggest that ARPIN is suppressed in ADAR1-depleted cells by the upregulated three miRNAs acting on the 3'UTR of ARPIN.

Table 2. ADAR1-correlated miRNAs targeting the ARPIN 3'UTR

Mature miRNA	shGFP	shADAR1
hsa-miR-1285-3p	113	230
hsa-miR-1285-5p	4	12
hsa-miR-4512	0	1
hsa-miR-619-5p	13	29

ADAR1 knockdown increases the migration/invasion potential of cancer cells with altered epithelial-mesenchymal transition (EMT) marker expression

As ARPIN functions to inhibit actin polymerization (Veltman, 2014), we next questioned whether the ADAR1-dependent ARPIN downregulation changes the migratory potential of cancer cells. A migration and invasion assay on ADAR1-KD cells (Fig. 4A) revealed increased migration (Fig. 4B) or invasion (Fig. 4C) upon ADAR1 depletion. These results were confirmed in MDA-MB-468 cells (Supplementary Fig. S5). Based on these data, we further examined if the depletion of

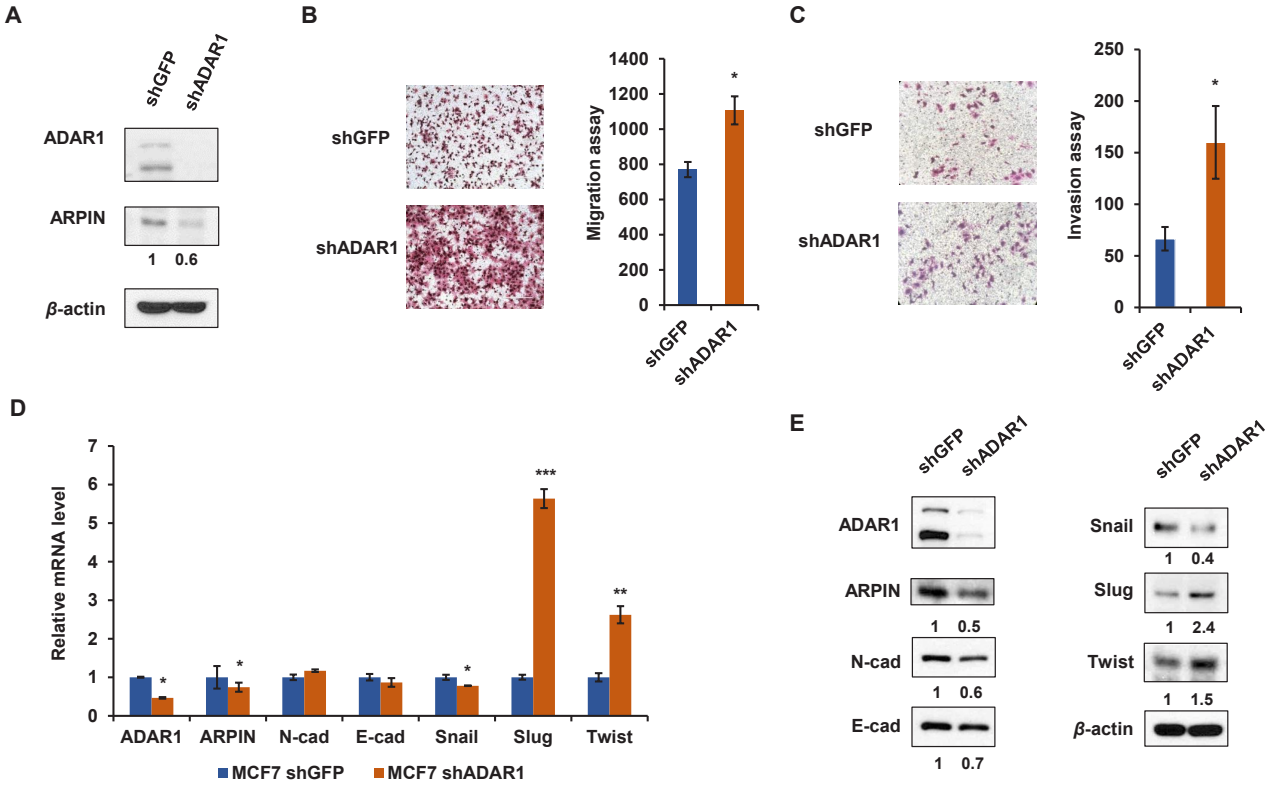

Fig. 4. Depletion of ADAR1 increases cancer cell mobility with epithelial-mesenchymal transition (EMT) marker alteration. (A) Western blot results show decreased ADAR1 and ARPIN levels in shADAR1 cells. (B and C) Representative images (on the left) of migration (B) or invasion (C) assays in control (shGFP) or ADAR1-depleted cells (shADAR1). The graphs on the right indicate migrated or invaded cell counts. (D) Real-time PCR analysis of EMT markers in control (shGFP in blue) or ADAR1-depleted cells (shADAR1 in brown). (E) Western blot results of EMT markers in control (shGFP) or ADAR1-depleted cells (shADAR1). Numbers indicate relative band intensity. β-Actin was used as a loading control. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

ADAR1 changes the expression of EMT markers. Real-time PCR for representative EMT markers indicated increased expression of Slug and Twist (Fig. 4D). Western blot analysis for these EMT markers confirmed the notable increase in Slug and Twist (Fig. 4E). These results demonstrate that the depletion of ADAR1 promotes breast cancer cell mobility with increased EMT marker expression.

Ectopic expression of ARPIN restores the increased mobility of ADAR1-depleted cancer cells

Since we observed decreased ARPIN expression along with increased EMT markers (both expected to confer cell mobility) in ADAR1-KD, we next questioned how much the ADAR1 regulation of ARPIN affects the mobility of cancer cells. We ectopically expressed ARPIN in combination with shADAR1 and examined the migration and invasion of cancer cells. Representative pictures in Fig. 5A indicate that ARPIN expression restores the increased migration (Fig. 5A, left) or invasion (Fig. 5A, right) triggered by ADAR1 depletion. The cell counts of migrated or invaded cells (Fig. 5B) confirmed this observation. Western blot analysis showed ADAR1 depletion, ARPIN downregulation (and restoration by overexpression), and confirmed Slug expression that is upregulated by shADAR1 and restored by ARPIN expression (Fig. 5C). Figure 5D shows the RNA levels of ADAR1 and Slug consistent with the data in Fig. 5C.

DISCUSSION

The role of ADAR in cancer metastasis is emerging in ADAR functional studies. Recent studies indicated that ADAR1 promotes tumorigenesis and metastasis in some cancers. A previous report showed ITGA2 upregulation by ADAR1 in hepatocellular carcinoma cells (Yu et al., 2019). This regulation enhances tumor cell adhesion to the extracellular matrix, enhancing the metastatic potential. In colorectal cancer, the activation of AZIN1, a well-known target of ADAR1, was reported to promote the invasiveness of cancer-associated fibroblasts (Takeda et al., 2019) as well as cancer stemness (Shigeyasu et al., 2018).

ADAR1 has also been reported to suppress melanoma metastasis via reduced editing of miR-455-5p and differential regulation of CPEB1 (Shoshan et al., 2015). Consistently, the downregulation of ADAR1 in melanoma was also shown to increase invasiveness via ITGB3 expression (Nemlich et al., 2018). These studies also revealed that miR-22 and the PAX6 transcription factor are responsible for editing-independent regulation. These results propose that a cancer type-specific regulation of target genes (either editing dependent or independent) by ADAR1 intricately controls metastasis. Our study described here provides a novel insight into the function of ADAR1 in metastasis via the regulation of ARPIN and cell mobility.

In terms of miRNA regulation, ADAR1 forms a heterodimeric complex with Dicer, which is involved in miRNA bio-

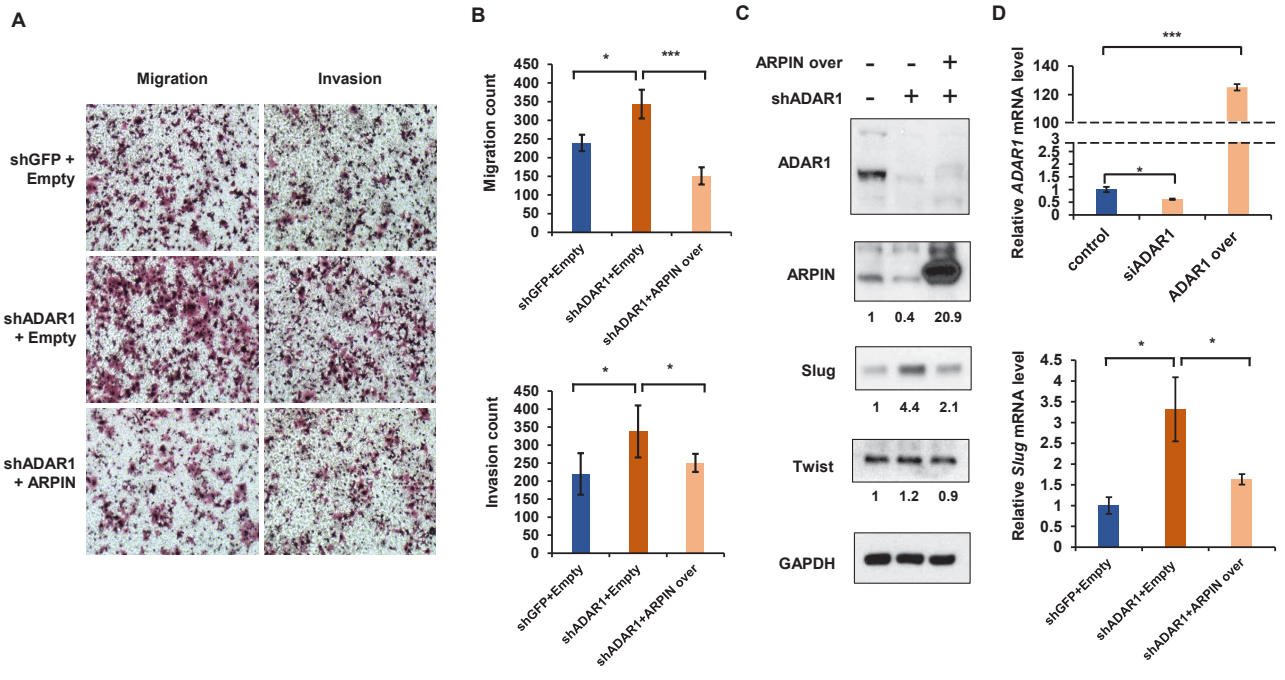

Fig. 5. Reduced migration and invasion of ADAR1-depleted MCF7 cells were restored by the overexpression of ARPIN. (A) Representative images of migration (left) or invasion (right) assays for the control (shGFP), ADAR1-depleted (shADAR1), and ADAR1-depleted cells with ARPIN overexpression (+ARPIN). (B) Quantitation of migration (top) or invasion (bottom) from the images shown in (A). (C) Western blot analysis of ADAR1, ARPIN, and two EMT (epithelial-mesenchymal transition) markers in the cells tested in (A). GAPDH was used as a loading control. (D) Real-time PCR data for ADAR1 and Slug was measured from the cells tested in (A). $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

synthesis, to induce pre-miRNA cleavage and increase target gene silencing (Ota et al., 2013). Therefore, miRNA expression should be downregulated upon knockdown of ADAR1. However, in our results, the three miRNAs (miR-1285-3p, miR-1285-5p, miR-619-5p) were upregulated. Interestingly, a previous report showed that nuclear ADAR1 interacts with Drosha, and many miRNAs, including miR-21, were upregulated by ADAR1 knockdown (Nemlich et al., 2013). Thus, certain miRNAs may be upregulated upon ADAR1 knockdown due to increased pri-miRNA processing by the Drosha-DGCR8 complex.

The regulation of Arp2/3 in actin branching has been extensively studied. For example, transgelin-2 (TAGLN2) was shown to suppress actin branching by blocking Arp2/3 (Kim et al., 2018). When the Arp2/3 complex generates branched actin filaments, it requires nucleation promoting factors, such as Wiskott–Aldrich syndrome protein (WASP), WASP family verprolin-homologous protein (WAVE; also known as SCAR),and NWASP (neural WASP) (Rotty et al., 2013). Among these, ARPIN is reported to antagonize the WAVE complex (Dang et al., 2013), and further study indicated its downregulation is associated with poor prognosis in breast cancer (Lomakina et al., 2016). Actin binding proteins, such as Arp2/3, also have been shown to be involved with actin in cancer cell invasion and migration (Izdebska et al., 2020). Thus, ARPIN may affect cancer cell migration that usually occurs via EMT. Among the known EMT markers, our results show that Slug and Twist increase upon ADAR1 knockdown (Fig. 4E). Additional data in Fig. 5C show that the overexpression of ARPIN in the ADAR1-depleted cells restored Slug and Twist levels, suggesting that the upregulation of these two markers is caused by reduced ARPIN levels.

Our data revealed that the RNA editing enzyme ADAR1 negatively regulates ARPIN via the 3'UTR. Hence, the anti-metastatic role of ADAR shown here coincides with previous studies in melanoma (Nemlich et al., 2018; Shoshan et al., 2015). Since ADAR1 is frequently overexpressed in multiple cancers (Gallo et al., 2017), further study will address how the tumor orchestrates ADAR1 function to evade immune surveillance and maintain the metastatic potential simultaneously.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

ACKNOWLEDGMENTS

This study was supported by the National Research Foundation of Korea (NRF2021R1 A6A1A03040260, NRF2021R1A2C2005472) and Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (KHIDIHI21C00710).

AUTHOR CONTRIBUTIONS

M.J.P. performed the main experiments and primary data analyses. H.J.C. and E.J. assisted with the *in vitro* functional study and provided technical support for the cell imaging studies. E.J.L. conducted the cell culture and managed the reagent supply. K.K. contributed to the RNAseq analysis. B.H.M. helped with manuscript preparation and discussion. S.C. conceived the idea, supervised the research progress, and wrote the manuscript. All the authors have read and approved the final manuscript.

CONFLICT OF INTEREST

The authors have no potential conflicts of interest to disclose.

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