HHS Public Access

Author manuscript

Biochem Biophys Res Commun. Author manuscript; available in PMC 2016 September 11.

Published in final edited form as:

Biochem Biophys Res Commun. 2015 September 11; 465(1): 47–52. doi:10.1016/j.bbrc.2015.07.123.

ADCY7 supports development of acute myeloid leukemia

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Abstract

Acute myeloid leukemia (AML) is the most common adult acute leukemia. Despite treatment, the majority of the AML patients relapse within 5 years. *In silico* analysis of several available databases of AML patients showed that the expression of adenylate cyclase 7 (ADCY7) significantly inversely correlates with the overall survival of AML patients. To determine whether ADCY7 supports AML development, we employed an shRNA-encoding lentivirus system to inhibit *adcy7* expression in human AML cells including U937, MV4-11, and THP-1 cells. The ADCY7 deficiency resulted in decreased cell growth, elvated apoptosis, and lower c-Myc expression of these leukemia cells. This indicates that G protein-coupled receptor signaling contributes to AML pathogenesis. Our study suggests that inhibition of ADCY7 may be novel strategy for treating leukemia.

Introduction

Acute myeloid leukemia (AML) is the most common adult acute leukemia. Treatments for AML yield poor outcomes, especially for the elderly patients. Even with continuous treatment, most AML patients relapse within 5 years [1]. The medical need for new therapies for AML is underscored by the fact that no new therapies for AML have been approved in more than 30 years.

Specific surface receptors and coupled signaling molecules play key roles in interaction between extrinsic environment and leukemia cells. It is known that signaling mediated by

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Competing interest disclosure
The authors declare no competing financial interests.

Author contributions
C.L., J.X., and C.C.Z. contributed to experimental design, performed experiments, interpreted data, and contributed to writing of the manuscript. Z.L., C.C., R.Z., Y. F., and X.H. performed experiments and interpreted data.

tyrosine kinase receptors [2], cytokine receptors [3], chemokine receptors [4], Notch [5], Wnt receptors [6,7], Smoothened [8], member of the TGF-β receptor family [9], G protein-coupled receptors (GPCRs) [10], adhesion molecules, integrins [11,12,13,14,15,16], and other molecules regulate leukemia development. Some of these receptors mediate signaling that differs in leukemia cells from that in normal hematopoietic cells, which should enable the development of anti-leukemia-specific strategies [16,17,18,19,20].

We have developed a systematic strategy to identify factors important for leukemia development [21]. We first use clinical databases to identify plasma membrane proteins that have correlations with the clinical outcomes of AML patients. We then validate the functions of candidate proteins in leukemia models and compare these functions to those in normal cells. The signaling pathways identified provide candidate targets for development of therapeutic approaches. Using this approach, we previously identified several ITIM-containing receptors that support AML development [21,22].

Here we describe our finding that the expression of membrane protein ADCY7 is correlated with negative clinical outcomes in leukemia patients. Using a loss-of-function approach, we discovered that ADCY7 supports the survival of human AML cells, and c-Myc expression is decreased upon ADCY7 knockdown. ADCY7 is an effector of $G\alpha_{12}$ and $G\alpha_{13}$ signaling transmitted from surface GPCRs [23]; therefore, our results show that G protein-coupled signaling resulting from the interaction between the microenvironment and leukemia cells plays a key role in leukemia pathogenesis. ADCY7 is a promising target for AML treatment.

Materials and methods

Cell culture

Human leukemia cell lines were grown in RPMI-1640 medium (Hyclone, South Logan, UT, USA) supplemented with 10% FBS (Gibco, Gaithersburg, MD). HEK293T cells were cultured in high glucose Dulbecco's modified Eagle's medium (Hyclone) supplemented with 10% FBS. All cell lines were maintained at 37 °C in 5% CO_2.

shRNAs and primers

Three sets of shRNAs were used in our study. The first two sets of shRNAs were designed based on an online program (http://rnaidesigner.lifetechnologies.com/rnaiexpress/). The third shRNA sequence was validated in a previous study [24]. RT-PCR primers were designed online (http://www.idtdna.com/site). The oligonucleotide sequences of the shRNAs used are:

Scrambled shRNA: 5′-GATATGTGCGTACCTAGCAT-3′

ADCY7 shRNA1: 5′-GGAATGGAGTCTGTTTAGA-3′

ADCY7 shRNA2: 5′-CACTTTATCGGTGACAAGTTA-3′

ADCY7 shRNA3: 5′-GGCAGGCAACTTTAGCACA-3′

Lentiviral shRNA constructs, lentiviral production, and cell transduction

Lentivirus infection was performed essentially as we described [[22,25]][22,26,27]. The lentiviral vector Pll3.7 was used to express shRNAs designed to target *adcy7* mRNA. For the lentiviral supernatant production, HEK-293T cells maintained in DMEM supplemented with 10% FBS and antibiotics were grown on 10-cm culture plates to ~60% confluence. For transfection, Polyjet (SignaGen, Rockville, MD, USA) was used according to the manufacture's protocol. For *adcy7* inhibition, 5.5 μg of the envelop plasmid *pMD2G*, 2 μg of the packaging plasmid *PsPAX2*, 7.5 μg of the shRNA lentiviral construct were dissolved in 0.5 mL of DMEM medium (serum and antibiotic free), Polyjet was added, and the solution was incubated for 15 min before addition to HEK293T cells. After 5 hours, the supernatant was discarded and replaced with 10 mL of DMEM with 10% FBS without antibiotics. After 48 hours, the supernatant was collected into a 15-mL tube, and 10 mL of medium were added to the plate. This was repeated 72 hours later. The lentivirus-containing supernatant was filtered through a 20-μM pore filter, and quickly frozen in liquid nitrogen for storage at −80 °C. U937, MV4-11, and THP-1 cells were cultured in 6-well plates (5×10^5 cells/well), and 16 hours later were transduced with virus by addition of 1 mL of lentiviral supernatant diluted in 1 mL of DMEM complete medium and 4 μL of protein sulfate. Cells were centrifuged at 2,000 rpm (Heraeus Biofuge Stratos centrifuge, ThermoScientific, Waltham, MA, USA) for 120 min at 37 °C. Cells were then cultured at 37 °C in 5% CO_2 for 5 hours. Culture medium was discarded, and cells were cultured in complete 1640 medium with 10% FBS and antibiotics for 19 hours. This procedure was repeated for a second infection.

Cell growth assays

GFP^+ shRNA-encoding lentivirus-infected cells were sorted by flow cytometry two days post-infection and 20,000 cells were plated in 1.55-cm wells. Cell numbers were determined on days 2, 4, and 6 in triplicate wells. The experiment was repeated three times with similar results.

Apoptosis assay

The apoptosis assay was performed using the Annexin V-PE apoptosis detection kit (eBioscience, San Diego, CA, USA) as we described previously[10, 12]. Fluorescence signals from at least 10,000 cells were collected by FACS (Beckman, CA, USA) to determine the percentage of apoptotic cells.

Quantitative real-time RT-PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was applied to total RNA extracted from cells or patient samples. Reverse transcription was performed using M-MLV reverse transcriptase cDNA Synthesis Kit (Takara Bio, Otsu, Shiga, Japan). Real-time RT-PCR was carried out on ABI 7900HT Fast Real-Time PCR System (Foster City, CA, USA) with SYBR-Green PCR Master Mix (Toyobo, Kita-ku, Osaka, Japan). A comparative CT method ($2^{-\Delta\Delta CT}$) was used to quantify gene expression. *β-actin* was used as the internal control for normalization. The primers for *β-actin* were as follows: *β-actin*-F 5′-AACACCCCAGCCATGTACG-3′; *β-actin* -R 5′-ATGTCACGCACGATTTCCC-3′.

Gene expression and survival analysis

We analyzed publicly available gene expression datasets from human AML studies as described [21]. Data were obtained from the TCGA AML database (https://tcga-data.nci.nih.gov/tcga/, n = 187), the GSE6891 database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE6891, n = 520), and the GSE10358 database (n = 91). Expression was normalized to total mRNA. Patients were separated into two groups based on whether they had higher or lower than the average expression levels of the ADCY7 to perform Kaplan-Meier survival analysis (GraphPad Prism, version 5.0, GraphPad, San Diego, CA, USA).

Statistical analyses

Data are expressed as means ± SEM. Data were analyzed by Student's *t*-test and were considered statistically significant if $p < 0.05$. The survival rates of the two groups were analyzed using a log-rank test and were considered statistically significant if $p < 0.05$. p values are represented as precise p values or generally as *p < 0.05.

Results

High expression of ADCY7 correlates with poor survival of AML patients

To identify genes encoding membrane protein genes that potentially support leukemia development, we performed an *in silico* analysis of the relationship between gene expression and the overall survival of AML patients using data from three independent databases: the TCGA AML database (https://tcga-data.nci.nih.gov/tcga/; n = 187), the GSE6891 database (n = 520), and the GSE10358 database (n = 91). Expression of genes encoding several membrane proteins, including IL2RA, GPR56, and ACDY7, inversely correlated with the overall survival of AML patients. Of these, IL2RA and GPR56 are known to play key roles in supporting leukemia development [10,28]. These published studies validated the effectiveness of our approach and suggest that the correlational data are predictive of functional roles in leukemia promotion.

ADCY7 is an adenylate cyclase with 12 membrane-spanning domains that catalyzes the formation of cyclic AMP from ATP. The *adcy7* gene, located at 16q12-16q13 of the human genome, was previously cloned from a erythroleukemia cell line [29]. Regulation of signaling through $G\alpha_{12}$ and $G\alpha_{13}$ pathways converges on ADCY7 [23].

We compared the overall survival of AML patients with low versus high CCDC6, and observed that, in each dataset, the expression of *adcy7* inversely correlated with the overall survival of AML patients (Fig. 1A–C). *adcy7* mRNA was detected in cells from all subclasses of AML (Fig. 1D) and in mouse granulocyte/monocyte progenitors (GMPs) upon MLL-AF9 transformation (Fig. 1E). Because MLL-AF9 transformed GMPs are enriched for AML stem cells [30], this result suggests that ADCY7 is highly expressed by leukemia stem cells.

Gene expression analysis indicated that *adcy7* mRNA is highly expressed in human monocytic and monoblastic AML cell lines U937, MV4-11, and THP-1 and in B

lymphoblastic leukemia (B-ALL) cell lines 697, KOPN-8, and RCH-ACV (Fig. 2). This high expression of *adcy7* in monocytic and monoblastic AML cells is consistent with its elevated expression in MLL-rearranged leukemia cells (Fig. 1E).

ADCY7 is essential for the growth of human acute leukemia cells

To study the potential function of ADCY7 in human leukemia, we inhibited the expression of ADCY7 by introducing lentivirus-encoded shRNAs designed to inhibit expression from *adcy7* into U937, MV4-11, and THP-1 cells. The three shRNAs tested all efficiently decreased expression of ADCY7. shRNA3 had the greatest efficiency and was used to silence *adcy7* in a previous report [24]; treatment of cells with this shRNA reduced *adcy7* mRNA to 45% of the endogenous level (Fig. 3A).

The ADCY7 deficiency resulted from shRNA 1–3 decreased the *in vitro* growth of U937 cells to 66%, 76%, and 58% respectively of that of control cells treated with a scrambled shRNA at 6 days after lentivirus infection (Fig. 3B). To determine the underlying mechanism by which ADCY7 supports the growth of these leukemia cells, we compared the cell cycle status and apoptosis of U937 cells treated with these shRNAs or scrambled control shRNA. No significant difference was observed in cell cycle distribution in these two treatment conditions (data not shown). However, ADCY7-deficient cells infected with shRNA3 had significantly increased levels of early apoptosis compared to cells treated with the control shRNA (Fig. 3C–D; 15.1% early apoptotic cells in scramble control-treated cells vs. 28.7% in ADCY7-deficient cells at day 3 of culture). shRNA 1 and 2 also had similar effects on apoptosis induction (data not shown). Moreover, similar effects of inhibition of *adcy7* expression on apoptosis were observed in MV4-11 and THP-1 cells (Fig. 3E–F). These results indicate that ADCY7 supports leukemia cell growth by suppressing apoptosis.

The inhibition of *adcy7* expression decreased *c-Myc* mRNA expression in both U937 cells and MV4-11 cells (0.76 ± 0.10% and 0.65 ± 0.15% of amounts in scrambled control-treated cells, respectively, Fig. 4). By contrast, the expression of *p21, p53, and survivin* was not significantly altered in cells deficient in ADCY7 compared to control cells (data not shown). Our results clearly indicate that ADCY7 is essential for survival of these human AML cell lines, and suggest that ADCY7 acts through a pathway involving c-Myc.

Discussion

In this study, we demonstrated that the expression of an adenylate cyclase ADCY7 negatively correlates with the overall survival of AML patients. The gene encoding ADCY7 was upregulated upon the transformation of cells with an MLL-fusion oncogene. Importantly, using a loss-of-function approach, we showed that ADCY7 supports leukemia development by decreasing apoptosis of AML cells. This is the first demonstration of ADCY7 function in cancer development and in the hematopoietic system.

The gene encoding ADCY7 was cloned from a erythroleukemia cell line [29] and is one of the nine membrane-spanning mammalian adenylate cyclases that catalyze intracellular cAMP production. ADCY7 is reportedly a downstream effector of the $G\alpha_{12}/G\alpha_{13}$ pathways [23] and has been shown to modulate affective neural circuitry [31]. ADCY7 also has a sex-

specific role in depression and alcohol dependence [32,33,34]. Only 6% of *adcy7*-knockout mice live to adulthood [32], suggesting that ADCY7 is essential in embryonic development. The function of ADCY7 in most tissues in the body is unclear, however.

GPCR- and $G\alpha_{12}/G\alpha_{13}$-mediated signaling has been linked to AML development. GPR56, a GPCR that is coupled with $G\alpha_{12}/G\alpha_{13}$ signaling and regulates cell adhesion and migration, maintains hematopoietic stem cell (HSC) pools by coordinating interactions between HSCs and the bone marrow osteosteal niche. GPR56 also supports cell adhesion and survival of $EVI1^{high}$ AML cells through RhoA signaling [10]. Because ADCY7 is coupled with $G\alpha_{12}/G\alpha_{13}$, signaling that is downstream of GPR56, it is possible that ADCY7 plays a role similar to that of GPR56 in AML development. Our study supports this hypothesis. Because ADCY7 is expressed by all subclasses of AML cells evaluated, it has potential as a treatment target for various types of AML. Further investigations into the potential connection between ADCY7 and GPR56 and other GPCRs are warranted. If a definitive signaling flow between a particular group of GPCRs and ADCY7 is established, targeting the upstream GPCR may be an effective strategy for treating AML.

Adenylate cyclases catalyze cAMP production. cAMP stimulates growth of many cell types but inhibits growth of others. cAMP inhibits survival of certain lymphoma and chronic lymphocytic leukemia (CLL) cells [35]. By contrast, cAMP protects acute promyelocytic leukemia (APL) cells against anthracycline-induced apoptosis and thus promotes progression of this type of AML [36]. Here we showed that the deficiency of ADCY7 in AML cells decreased the expression of c-Myc. This is in line with the reported regulation of c-Myc expression by the cAMP cascade in thyrocytes [37]. Future work will be needed to determine whether cAMP is a key second messenger downstream of ADCY7. If this is the case, identification of the downstream effectors to which it transmits the signaling will help clarify the roles of cAMP in different subtypes of leukemia.

Acknowledgments

We would like to acknowledge the support of this project by NIH grant 1R01CA172268, Leukemia & Lymphoma Society Awards LLS#1024-14 and LLS#6024-14-01, the CPRIT RP140402 award, and the Taishan Scholar Program.

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Highlights

1. The expression of ADCY7 negatively correlates with the overall survival of AML patients.
2. ADCY7 supports growth of human AML cells by inhibiting apoptosis.
3. Knockdown of ADCY7 decreases c-Myc expression in human AML cells.

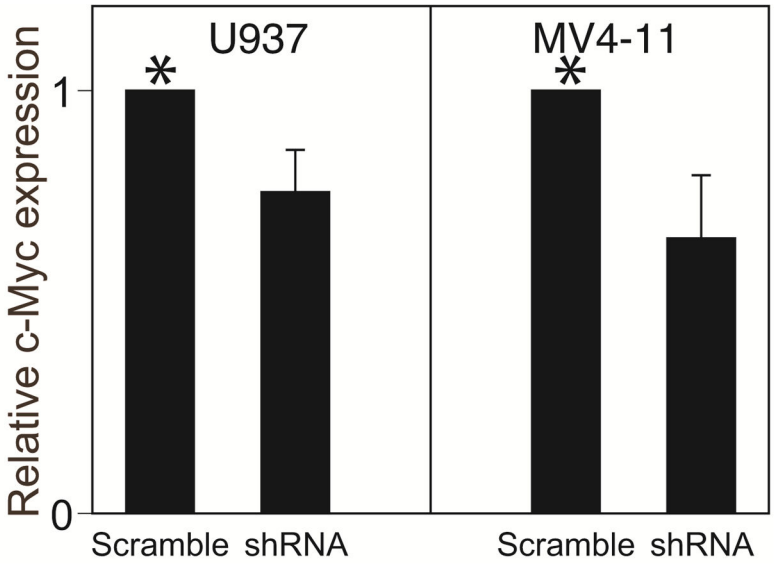

Figure 1. *adcy7* mRNA expression negatively correlates with overall survival of AML patients

(A–C) *adcy7* mRNA expression data from (A) the TCGA AML database (n = 187), (B) the GSE6891 database (n = 520), and (C) the GSE10358 database (n = 91) were normalized to total mRNA expression. Patients were separated into two groups based on whether expression of *adcy7* was higher of lower than the average expression levels, and % survival vs. time was plotted. (D) An *in silico* analysis of human *adcy7* mRNA expression in human AML subclasses M1 through M7. Data were obtained from the TCGA AML database. (E) An *in silico* analysis of *adcy7* mRNA expression in normal GMPs ($Lin^{-}Kit^{+}Sca\text{-}1^{-}CD34^{+}CD16/32^{+}$ cells) and MLL-AF9-infected GMP cells. Data were obtained from GDS3839/1456307_s_at/Adcy7.

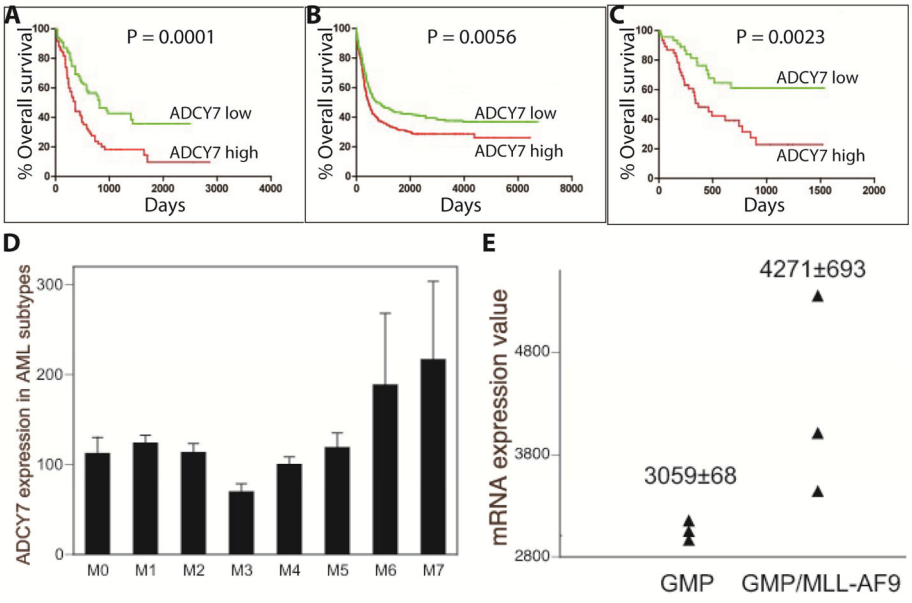

Figure 2. ADCY7 is highly expressed in human AML cell lines

adcy7 mRNA expression (relative fold changes to that in NALM-6 cells) as determined by real-time RT-PCR plotted for human monocytic and monoblastic AML and B-ALL cell lines. Technical replicate n = 3.

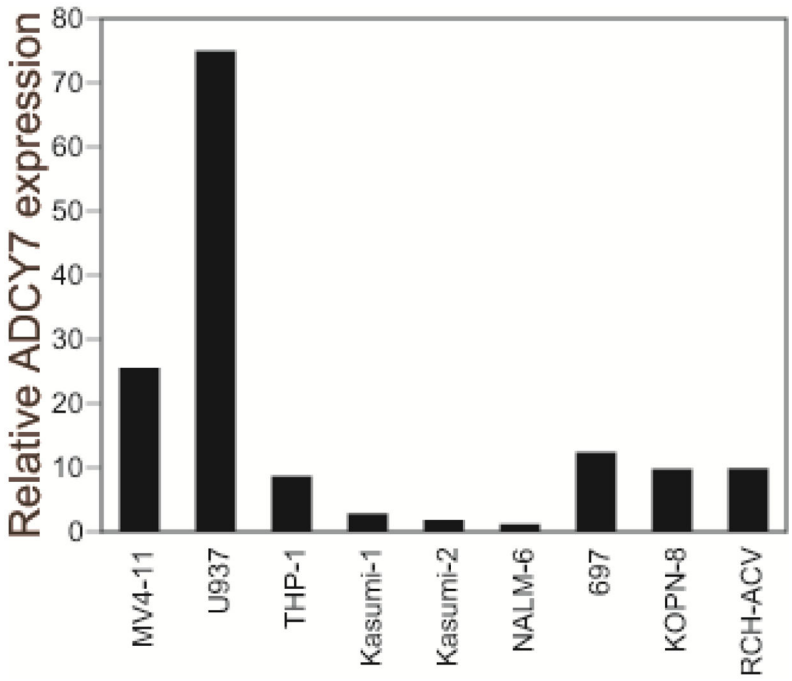

Figure 3. Inhibition of *adcy7* expression significantly decreased cell growth and induced apoptosis of U937, MV4-11, and THP-1 AML cells

(A) Endogenous *adcy7* expression was inhibited in 293T cells transfected with shRNAs targeting *adcy7* mRNA but not with a scrambled control shRNA as determined by real-time RT-PCR at 48 hours after lentiviral infection (n = 3). (B) Inhibition of *adcy7* expression by three different shRNAs significantly decreased growth of U937 cells at 6 days post-infection when compared to growth of cells that express a scrambled control shRNA (n = 4). (C–D) ADCY7 deficiency induced apoptosis of U937 cells at 72 hr post treatment with shRNA3 or a scrambled control shRNA. (C) Representative flow cytometry plots. (D) Plots of percent apoptotic cells (n = 3, *, $p < 0.05$, Student's *t*-test). (E) MV4-11 and (F) THP-1 AML cells were treated with shRNA3 or a scrambled control shRNA. Percent of apoptotic cells were determined at 96 hr (n = 3, *, $p < 0.05$, Student's *t*-test).

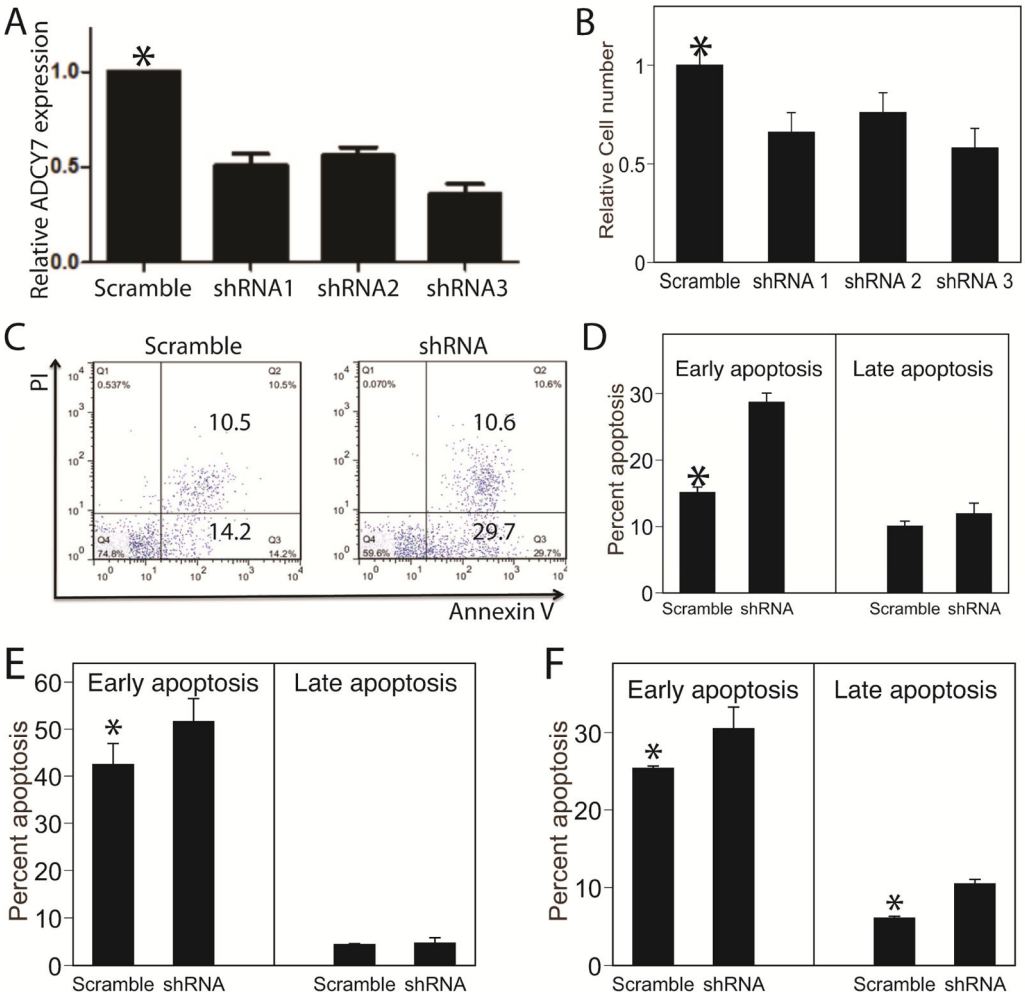

Figure 4. Lack of ADCY7 significantly decreased c-Myc mRNA expression in AML cells

Expression of c-Myc was quantified in U937 cells and MV4-11 cells at 96 hr post transfection with shRNA3 or a scrambled control shRNA by real-time RT-PCR (n = 6, *, $p < 0.05$, Student's *t*-test).