Molecules and Cells

Research Article

MicroRNA-196a increases apoptosis in B cells through downregulation of FOXO1

Soyoung Kim[1,†], Mina Han[1,†], Hyun Ju Hwang[1,†], Young-Ho Ahn[2], Ho Joon Im[3], Sang-Hyun Hwang[4], Kyung-Nam Koh[3,*], and Nayoung Kim[1,*]

[1]Asan Institute for Life Sciences and Department of Convergence Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea, [2]Department of Molecular Medicine, College of Medicine, Ewha Womans University, Seoul, Republic of Korea, [3]Department of Pediatrics, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea, [4]Department of Laboratory Medicine, Asan Medical Center, University of Ulsan College of Medicine, Seoul, Republic of Korea
*Correspondence: pedkkn@amc.seoul.kr (K.-N. Koh), naykim@amc.seoul.kr (N. Kim).
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ABSTRACT

MicroRNAs (miRNAs) are key regulators of cancer pathogenesis, and their expression is often dysregulated in cancer cells. The role of miR-196a-5p has been investigated in various types of cancers. However, it is relatively less understood in B-cell malignancies. This study aimed to investigate the role of miR-196a-5p in B cells by using a human diffuse large B-cell lymphoma cell line, SU-DHL-6, and mouse B lymphocytes. The enforced expression of miR-196a in SU-DHL-6 cells increased daunorubicin-mediated apoptosis. Luciferase assay revealed that FOXO1 was a direct target of miR-196a-5p in SU-DHL-6 cells. The mRNA and protein expression of FOXO1 was downregulated in miR-196a-overexpressing SU-DHL-6 cells. In addition, miR-196a-5p was highly expressed in mouse bone marrow cells, compared with that of splenic B cells, and FOXO1 expression was negatively correlated with miR-196a-5p level. miR-196a-5p was upregulated by B-cell receptor stimulation, which was inversely correlated with FOXO1 expression in splenic B cells. Apoptosis was increased when miR-196a-5p was upregulated in murine primary B cells. These results identify miR-196a-5p as a post-transcriptional regulator of FOXO1 and indicate its importance in regulating B-cell malignancies and activation.

Keywords: Apoptosis, B-cell activation, B-cell lymphoma, FOXO1, MicroRNA-196a-5p

INTRODUCTION

MicroRNAs (miRNAs) are small endogenous noncoding RNAs of 20 to 25 nucleotides that could regulate the development, differentiation, and homeostasis of cells by suppressing the translation or inducing the degradation of target mRNAs (Gebert and MacRae, 2019). miRNAs could act as tumor suppressors or oncogenes and could be dysregulated in most human cancers (Peng and Croce, 2016). miR-196 family molecules have been demonstrated to act as oncogenes in various cancer cell types, including non–small-cell lung cancer, hepatocellular carcinoma, cervical cancer, gastric cancer, head and neck cancer, and colorectal cancer (Hou et al., 2014; Liang et al., 2020; Ren et al., 2019; Sun et al., 2012; Xin et al., 2019). miR-196, which is located in the regions of homeobox (HOX) clusters within the mammalian genome, consists of 3 genes, that is, miR-196a-1, miR-196a-2, and miR-196b (Chen et al., 2011). The sequence of a mature miR-196a-1 and miR-196a-2 differs from that of miR-196b by 1 nucleotide (Chen et al., 2011). Accumulating evidence has indicated that miR-196 can also act as a tumor suppressor in several tumors, such as melanoma, chronic myeloid leukemia, and B-cell acute lymphoblastic leukemia (ALL) (Bhatia et al., 2010; Braig et al., 2010; Liu et al., 2013). In particular, miR-196b has a potential tumor-suppressive role in B-cell lineage ALL (Bhatia et al., 2010). However, the roles of miR-196 in B-cell lymphoma and primary B cells are poorly understood.

B-cell malignancies are derived from different stages of B-cell commitment and can be divided into B-cell lymphomas, B-cell leukemias, and plasma cell dyscrasias (Cuenca and Peperzak, 2021). Diffuse large B-cell lymphoma (DLBCL) is an aggressive and common subtype of non-Hodgkin's lymphoma, which originates from mature B cells (Shankland et al., 2012). Despite the response of more than 60% of large B-cell lymphoma patients to standard chemotherapy using R-CHOP (CD20 antibody rituximab with cyclophosphamide, doxorubicin, vincristine, and prednisone), nonrespond patients are often associated with poor outcome (Sehn and Salles, 2021). Therefore, it is important to understand the molecular mechanism of DLBCL pathogenesis.

† Equally contributed.

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Forkhead box O (FOXO) transcription factors are regulated by several signaling pathways, including protein kinase B (AKT) and serum and glucocorticoid kinase, which are deregulated in cancer cells (Fu and Tindall, 2008). In mammals, FOXO1, FOXO3, and FOXO4 are phosphorylated by the activation of the phosphatidylinositol-3-kinase (PI3K)/AKT signaling pathway, which are exported from the nucleus to the cytoplasm, resulting in the suppression of their transcriptional activity (Salih and Brunet, 2008). The nuclear retention of FOXO1 owing to mutations at T24, a phosphorylation site of AKT, is associated with the decreased overall survival in DLBCL patients treated with R-CHOP (Trinh et al., 2013). Furthermore, nuclear FOXO1 exerts a stimulating effect on the proliferation and survival of Burkitt lymphoma (BL) cells, which are derived from mature B cells (Kabrani et al., 2018). FOXO1 is abundantly expressed in some types of B-cell malignancies, which are derived from mature B cells, including DLBCL and BL (Xie et al., 2012). B-cell receptor (BCR) signaling has been associated with the pathogenesis and/or progression of chronic lymphocytic leukemia (CLL), and FOXO1 has been observed to be downregulated by BCR-mediated activation in mature B cells (Hinman et al., 2007; Mraz et al., 2014). These findings suggest that FOXO1 might play a critical role in B-cell malignancies, particularly those that originate from mature B cells. However, it is unclear whether miRNAs are responsible for the FOXO1 expression in mature B cells.

In the present study, the gain-of-function effect of miR-196a-5p was investigated using the DLBCL cell line, SU-DHL-6. Here, we showed that the overexpression of miR-196a-5p suppressed proliferation and promoted apoptosis by directly targeting FOXO1 in DLBCL cells, demonstrating that miR-196a-5p might be a novel therapeutic target for DLBCL. Furthermore, we investigated the role of miR-196a-5p and FOXO1 in healthy primary B cells, where a negative correlation was observed between miR-196a and FOXO1 upon activation. The results indicated that miR-196a-5p could modulate FOXO1 expression to regulate B-cell activation.

MATERIALS AND METHODS

Cell Culture

SU-DHL-6 cells, a human DLBCL cell line, were purchased from ATCC (Manassas, VA, USA) and maintained in RPMI 1640 (Corning, Manassas, VA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA), 100 IU penicillin plus 100 μg/ml streptomycin (Corning), and 1 mM sodium pyruvate (Sigma-Aldrich). HEK293T cells, a human embryonic kidney cell line (ATCC), were maintained in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Logan, UT, USA) supplemented with 10% FBS (Sigma-Aldrich), 100 IU penicillin, and 100 μg/ml streptomycin (Corning).

Lentiviral Transduction

The pLVX-Hyg miR-196a-2 lentiviral vector and the *pLVX-Hyg control vector with none-targeting sequences* were constructed for stable overexpression of miR-196a-2, as previously described (Kim et al., 2022). HEK293T cells were seeded at a density of 1 × 10^6 cells/well in 100-mm dishes the day before transfection. Pseudoviral particles were produced using 6 μg of lentiviral vector, 3 μg of psPAX2 (Gag-Pro-Plo), and 1 μg of pMD2. G (Env) in HEK293T cells. The virus-containing medium was collected and filtered using a 0.45-μm syringe filter (Millipore, Burlington, MA, USA). One day prior to infection, 7 × 10^5 SU-DHL-6 cells were seeded in 100-mm dishes. SU-DHL-6 cells were infected for 48 hours with viral particles and selected using hygromycin B (Sigma-Aldrich). Stably transduced cells were maintained in a medium supplemented with 50 μg/ml hygromycin B.

Quantitative Reverse-Transcription PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Life Technologies, San Diego, CA, USA) according to the manufacturer's instructions. After the resuspension of total RNA in nuclease-free water (Hyclone), the amount of RNA was measured using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). HB miR Multi Assay Kit System I (Heim Biotek, Seongnam, South Korea) was used for miRNA quantification. In brief, HB_I RT Reaction Kit (Heim Biotek) was used for the synthesis of complementary DNA (cDNA) according to the manufacturer's instructions. The cDNA was amplified using HB_I Real-time PCR Master Mix Kit with hsa-miR-196a-5p-specific and RNU6B-specific primers (Heim Biotek). RNU6B was used for the normalization of miRNA.

For target gene expression analysis, total RNA was reverse-transcribed into cDNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) and Oligo (dT) 12 to 18 primers (Thermo Fisher Scientific) following the manufacturer's protocol. The cDNA was then amplified using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) with the specific primers. The primer sequences for the target genes were as follows: human FOXO1 forward primer 5′-TCATGGATGGAGATACATT GGATT-3′ and reverse primer 5′-CCAGCTATGTGTCGTTGTC TTGA-3′, human HupO forward primer 5′-CCATTCTATCAT CAACGGGTACAA-3′ and reverse primer 5′-AGCAAGTGGGA AGGTGTAATCC-3′, human HPRT forward primer 5′-CCTGGC GTCGTGATTAGTG-3′ and reverse primer 5′-CAGAGGGCT ACAATGTGATGG-3′, mouse Foxo1 forward primer 5′-CGTGC TTACAGCCTTCTA-3′ and reverse primer 5′-ACCTCCATCGT GACAAAA-3′, mouse Hprt forward primer 5′-TGCCGAGGATT TGGAAAAAGTG-3′ and reverse primer 5′-AGAGGGCCACAA TGTGATGG-3′, and mouse Gapdh forward primer 5′-TTGTCA GCAATGCATCCTGCAC-3′ and reverse primer 5′-ACAGCTTT CCAGAGGGGCCATC-3′. Human HupO, human HPRT, mouse Hprt, and mouse Gapdh were used for the normalization of human or mouse FOXO1 mRNA. qRT-PCR was performed using QuantStudio 5 Real-Time PCR System (Applied Biosystems). The results were analyzed by SDS 2.4 (Applied Biosystems), and relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

Cell Viability Assay

SU-DHL-6 cells stably expressing miR-196a-2 and control cells were seeded in 96-well plates at 5 × 10^3 cells/well in triplicate with 200 μl of medium and treated with 5 nM daunorubicin for 6 days. Alternatively, miR-196a-overexpressing SU-DHL-6 and

SU-DHL-6 wild-type (WT) control cells were seeded at 5 × 10^3 cells/well in 96-well plates with the addition of either 10 nM daunorubicin or 10 μM LY294002 (Tocris) for 3 days. Cell viability was assessed using Cell Counting Kit-8 (CCK-8) (Dojindo Molecular Technologies, Kumamoto, Japan) according to the standard protocols (Ryu et al., 2023). In brief, 100 μl of the cell supernatant was removed, and 10 μl of CCK-8 solution was added to each well. Following incubation for 3 hours at 37°C in a CO_2 incubator, the absorbance was measured at 450 nm using a Sunrise microplate reader (Tecan, Männedorf, Switzerland).

Caspase 3/7 Activity Assay

SU-DHL-6 cells stably expressing miR-196a-2 and nontargeting control cells were seeded in 24-well plates at 5 × 10^4 cells/well with 200-μl medium and treated with 5 nM daunorubicin for 6 days. Caspase 3/7 activity was determined using Cell Meter Caspase 3/7 Activity Apoptosis Assay Kit (AAT Bioquest, Sunnyvale, CA, USA) following the manufacturer's instructions. In brief, FITC-conjugated TF2-DEVD-FMK was added to the cell suspension. Following incubation for 3 hours at 37°C in a CO_2 incubator, the cells were resuspended with assay buffer. The FITC level was measured using a CytoFLEX flow cytometer (Beckman Coulter Life Sciences, Brea, CA, USA). At least 50,000 cells were counted for each sample. The data were analyzed by FlowJo software (Tree Star, Inc, Ashland, OR, USA).

Cell Cycle Analysis

SU-DHL-6 cells stably expressing miR-196a-2 and control cells were seeded at 7 × 10^5 cells/well in 6-well plates and treated with 5 or 10 nM daunorubicin for 2 days. The cells were washed with phosphate-buffered saline (PBS) (Biosesang, Yongin-si, South Korea) and fixed in 70% ice-cold ethanol (Merck, Darmstadt, Germany) at −20°C. Following overnight incubation, the cells were washed with PBS and resuspended with DNA extraction buffer containing Triton X-100 and Na_2HPO_4. DNA was stained with 0.5 mg/ml propidium iodide (PI) (Biolegend, San Diego, CA, USA) in the presence of 5 μg/ml RNase A (Thermo Fisher Scientific) at room temperature for 15 minutes. Cell cycle analysis was performed using CytoFLEX (Beckman Coulter Life Sciences). The data were analyzed by FlowJo software (Tree Star, Inc).

Cell Death Assay

Cell death assay using annexin V and PI or 7-AAD was performed, and the results were analyzed as previously described (Lee et al., 2018). In brief, miR-196a-expressing SU-DHL-6 cells (clone #2) were seeded at 5 × 10^5 cells/well in 6-well plates and treated with either 10 nM daunorubicin or 10 μM LY294002 (Tocris). After incubation for 3 days, the cells were stained with both annexin V-APC (BD Biosciences, San Jose, CA, USA) and PI (BioLegend) to detect the early and late stages of cell death. Fluorescence was measured using the CytoFLEX flow cytometer (Beckman Coulter Life Sciences) and analyzed using FlowJo software.

Construction of the Luciferase Reporter Plasmids

The 3′-UTR containing the predicted binding site was amplified from the genomic DNA of HEK293T cells by PCR. The forward primer with an *Xho*I restriction site (5′-TATTCTCGAG CCCAA TGTGTGCAGGTTATG-3′) and the reverse primer with a *Not*I restriction site (5′-ATGCGGCCGCAGGTCCAAGGCTGTTCA ATG-3′) were used to amplify the FOXO1 3′-UTR (Hou et al., 2014). The PCR products were then cloned into the psiCHECK-2 Vector (Promega, Madison, Wis, USA), and the inserted sequences in the constructs were validated by DNA sequencing analysis (Bioneer, Daejeon, South Korea).

Mutagenesis

Mutagenesis of the target sequence of hsa-miR-196a-5p in the FOXO1 3′-UTR was performed by PCR. The primer sequences for PCR were as follows: 5′-TTGTACACTCTGTTTTCTGCGG AACTGACGGA-3′ (forward) and 5′-AAACAGAGTGTACAAAC AAAAGTTTAACTTAT-3′ (reverse). Mutant sequences of the FOXO1 3′-UTR were confirmed by DNA sequencing analysis (Bioneer).

Dual-Luciferase Reporter Assay

SU-DHL-6 cells stably expressing miR-196a-2 and nontargeting control cells were seeded in triplicate at a density of 10,000 cells/well in 96-well plates the day before transfection. The cells were transfected with 50 ng of the psiCHECK-2_FOXO1_3′-UTR-WT- or mutant (Mut) construct using Lipofectamine 3000 reagent (Life Technologies, Carlsbad, CA, USA). Luciferase activity was measured 48 hours post transfection using the Dual-Luciferase Reporter Assay System (Promega) and Victor3 luminometer (PerkinElmer, Waltham, MA, USA) according to the manufacturer's protocol. The firefly luciferase activity was normalized relative to the Renilla luciferase activity.

Western Blotting

Total cellular protein was extracted from cells using RIPA lysis buffer (Biosesang) containing a protease/phosphatase inhibitor cocktail (Thermo Fisher Scientific). After centrifugation at 20,000 g at 4°C for 15 minutes, total protein was quantified using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific) and denatured with 5× sodium dodecyl sulfate (SDS) sample buffer (Elpis Biotech, Daejeon, South Korea) at 95°C for 5 minutes. Total protein (20 μg per sample) was electrophoresed on 10% SDS-PAGE gels and transferred to 0.45-μm polyvinylidene fluoride (PVDF) membranes (Millipore), followed by blocking with 5% bovine serum albumin (BSA; Bovogen Biologicals, Essendon, Australia) in Tris-buffer saline (Elpis Biotech) containing 0.1% Tween 20 (Duchefa Biochemie, Haarlem, Netherlands) (TBST) for 2 hours at room temperature. The membranes were then incubated with primary antibodies against anti-FOXO1 (1:2,000 dilution; Cell Signaling Technology, Danvers, MA, USA) or anti-β-actin (1:5,000 dilution; Bioss, Woburn, MA, USA) overnight or for 1 hour at 4°C. After washing with TBST 3 times, the membranes were incubated with horseradish peroxidase–conjugated goat anti-rabbit secondary antibodies (1:10000 dilution; Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hour at room temperature. Protein signals were visualized via using Pierce ECL

western blotting substrate (Thermo Fisher) and detected using Image Quant LAS 4000 (GE Healthcare, Milwaukee, WI, USA). Band intensities were analyzed using Image Studio Lite software Ver 5.2 (LI-COR Biosciences, Lincoln, NE, USA).

Detection of Phospho-AKT by Flow Cytometry

Cells were treated with 100 μM pervanadate, an inhibitor of phosphatases, which was used as a phosphorylation inducer (Imbert et al., 1994) for 30 minutes. The cells were immediately fixed in a 4% paraformaldehyde solution. After washing with PBS containing 2% FBS, the cells were permeabilized with BD Phosflow Perm Buffer III (BD Biosciences) for 30 minutes on ice. Then, they were incubated with anti-AKT1 (Clone 55/PKBa/AKT; BD Biosciences), anti-phospho-AKT (pAKT) antibody (Clone M89-61; BD Biosciences), or IgG1κ isotype control for 1 hour, following FcR blocking (Miltenyi Biotec). Flow cytometry was performed using CytoFLEX (Beckman Coulter Life Sciences). The data were analyzed by FlowJo software (Tree Star, Inc).

Preparation of Murine Bone Marrow (BM) and Splenic (SP) B Cells

Tibias and femurs were obtained from 6 to 12-week-old C57BL/6 mice (OrientBio, Gyeonggi-do, South Korea). All the animal procedures were approved by the Institutional Animal Care and Use Committee (IACUC Approval No. 2017-12-280). BM was collected by flushing tibias and femurs with chilled PBS. The cell suspension was passed through a 100-μm Falcon nylon cell strainer (Corning) and then centrifuged for 5 minutes at 450 *g* to remove the supernatant. For the lysis of red blood cells (RBCs), cells were incubated for 5 minutes at room temperature with 1 ml of RBC lysis buffer (Biolegend, San Diego, CA, USA). The cells were resuspended in complete DMEM.

Splenocytes from C57BL/6 mice were isolated by straining spleens through a 100-μm Falcon nylon cell strainer (Corning) to create a single-cell suspension in complete DMEM. RBCs were lysed by RBC lysis buffer as described above. SP B cells were obtained by negative selection using Pan B Cell Isolation Kit, mouse (Miltenyi Biotec) according to the manufacturer's instructions. The purity of $CD19^+$ B-cell populations was above 90%, as measured by flow cytometry (Supplementary Fig. S1). In some experiments, isolated B cells were stimulated with 5 μg/ml goat anti-mouse IgM F(ab')2 (Southern Biotech, Birmingham, AL, USA) at 37°C for 5 or 24 hours (Lee et al., 2022; Wortis et al., 1995).

Statistical Analyses

Statistical analysis was performed by Student's *t*-test for paired data or 2-way analysis of variance (ANOVA) test using GraphPad Prism Ver. 6.0 (GraphPad Software, San Diego, CA, USA) or GraphPad InStat Ver. 3.10 (GraphPad Software). $P < .05$ was considered statistically significant.

RESULTS

Overexpression of miR-196a-5p Increases Apoptosis in Human B-Cell Lymphoma Cells

To investigate the effect of miR-196a gain-of-function in human B-cell lymphoma cells, a SU-DHL-6 cell line with low basal miR-196a levels was used. SU-DHL-6 cells were transduced with miR-196a-expressing vectors for stable overexpression using a lentiviral system, and transduction efficiency was confirmed by qRT-PCR (Fig. 1A). As daunorubicin can induce cell death against DLBCL cells (Luo et al., 2018), SU-DHL-6 cells were treated with 5 or 10 nM daunorubicin for 6 days. CCK-8 assay revealed that the enforced expression of miR-196a-2 markedly suppressed the proliferation of SU-DHL-6 cells, similar to the effect of daunorubicin treatment (Fig. 1B). SU-DHL-6 cells were treated with daunorubicin for 6 days, and TF2-DEVD-FMK, an indicator of caspase 3/7 activity, was indirectly measured by flow cytometry to investigate the effect of miR-196a-5p on the apoptosis of SU-DHL-6 cells (Zhang et al., 2015). Caspase 3/7 activity was significantly enhanced by the enforced expression of miR-196a-5p in SU-DHL-6 cells, indicating increased apoptosis (Fig. 1C and D). Notably, it was further increased by daunorubicin treatment (Fig. 1C and D), suggesting that the expression of miR-196a-5p could increase the susceptibility of DLBCL cells to daunorubicin.

Since the overexpression of miR-196a increased cell death in SU-DHL-6 cells, we investigated whether miR-196a could induce the cell cycle arrest of DLBCL cells. SU-DHL-6 cells were treated with 5 or 10 nM of daunorubicin for 2 days, and the cell cycle was analyzed by PI staining (Supplementary Figs. S2 and 1E). We observed that miR-196a tended to increase the percentage of sub-G1-phase cells, suggesting that miR-196a-5p augmented daunorubicin-induced apoptosis in DLBCL cells. The G0-/G1-phase cells were increased, whereas that of the S-phase cells were decreased by miR-196-1 overexpression. Importantly, the percentage of G2-/M-phase cells was significantly decreased in miR-196a-5p-expressing SU-DHL-6 cells following daunorubicin treatment, suggesting G2 arrest by daunorubicin. It should be noted that the caspase assay and cell cycle analysis were performed on Days 6 and 2, respectively, which might explain the slight differences in the results. The results suggest that daunorubicin could increase cell death in miR-196a-5p-expressing DLBCL cells, compared with that of control cells.

FOXO1 Is a Direct Target of miR-196a-5p in B Cells

The target genes of miR-196a-5p in DLBCL cells were searched using target prediction websites, such as miRTarBase and TargetScan. FOXO1 was selected among target gene candidates that have a conserved binding site with a seed sequence of miR-196a-5p in the 3′-UTR region. Luciferase assay was performed to determine whether FOXO1 is a direct target gene of miR-196a-5p. Stable miR-196a-5p-overexpressing SU-DHL-6 or mock control cells were transfected with psiCHECK-2 constructs containing either the wild-type (WT) or mutant (Mut)

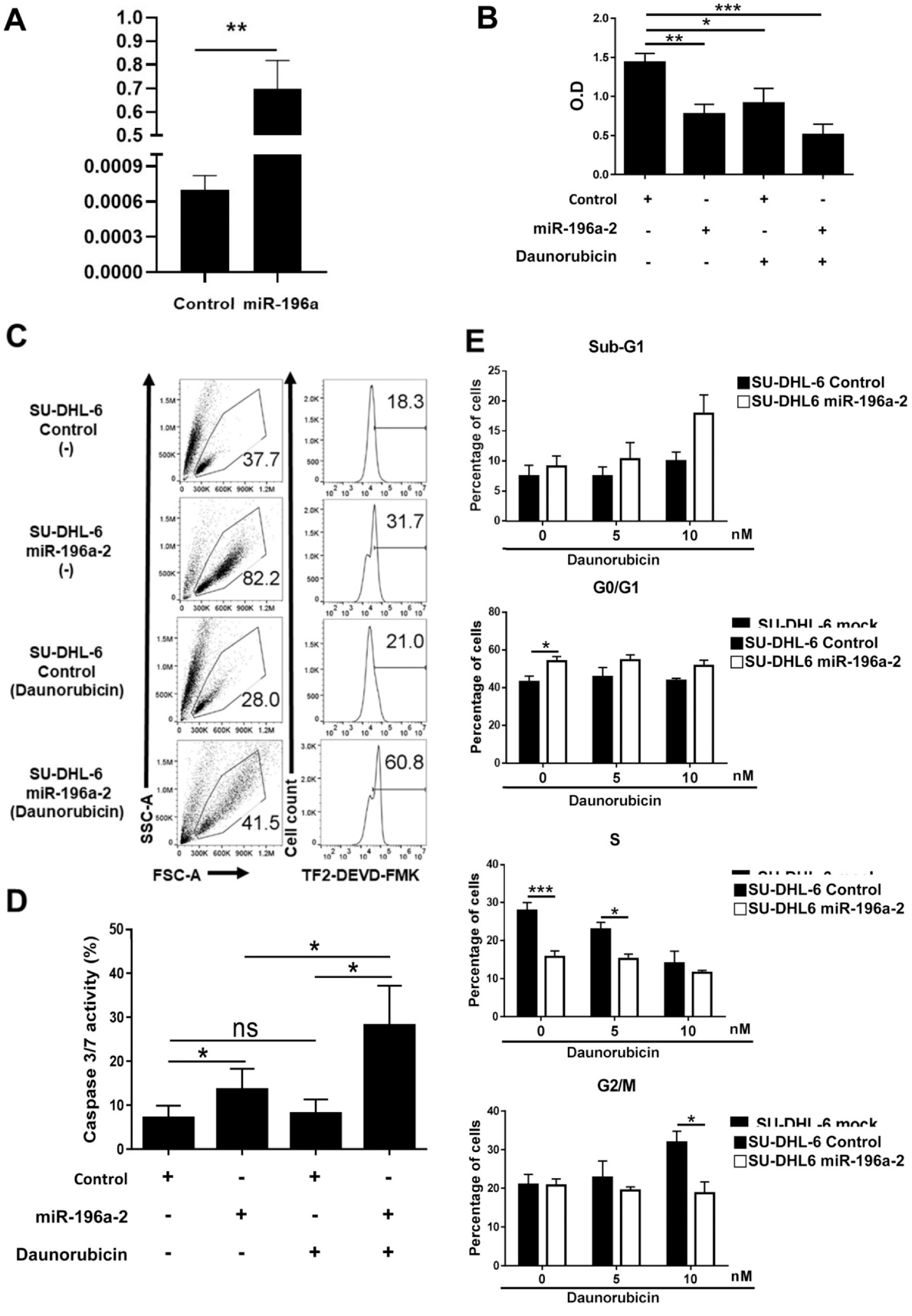

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Fig. 1. Enforced expression of miR-196a increases apoptosis in human DLBCL cells. (A) miR-196a-5p overexpression is induced in SU-DHL-6 cells. Relative miR-196a-5p expression levels in SU-DHL-6 cells stably overexpressing miR-196a-5p were measured by qRT-PCR. miR-196a-5p level was normalized to RNU6B level. Bars represent the means ± SEM from 4 independent experiments. A paired *t*-test was used. (B) SU-DHL-6 cells stably overexpressing miR-196a-5p or mock control cells were cultured for 6 days with 5 nM daunorubicin. The viability of SU-DHL-6 cells was assessed using CCK-8. Bars represent the means ± SEM from 8 to 12 independent experiments. Two-way ANOVA was performed. (C and D) SU-DHL-6 cells stably overexpressing miR-196a-5p or mock control cells were treated with 5 nM daunorubicin for 6 days. Caspase 3/7 activity was assessed in SU-DHL-6 cells by flow cytometry with TF2-DEVD-FMK staining. (C) Representative histograms of TF2-DEVD-FMK expression in the SU-DHL-6 cells are shown. (D) The results of (C) are summarized. Bars represent the mean ± SEM from 3 to 5 independent experiments. A paired *t*-test was performed. (E) SU-DHL-6 cells stably overexpressing miR-196a-5p or mock control cells were cultured for 2 days with 5 or 10 nM daunorubicin. The cell cycle was assessed by flow cytometry with PI staining. The representative flow cytometric histograms are shown in Supplementary Fig. S2. Bars represent the means ± SEM from 3 to 5 independent experiments. Two-way ANOVA was performed. **P* < .05, ***P* < .01, ****P* < .001.

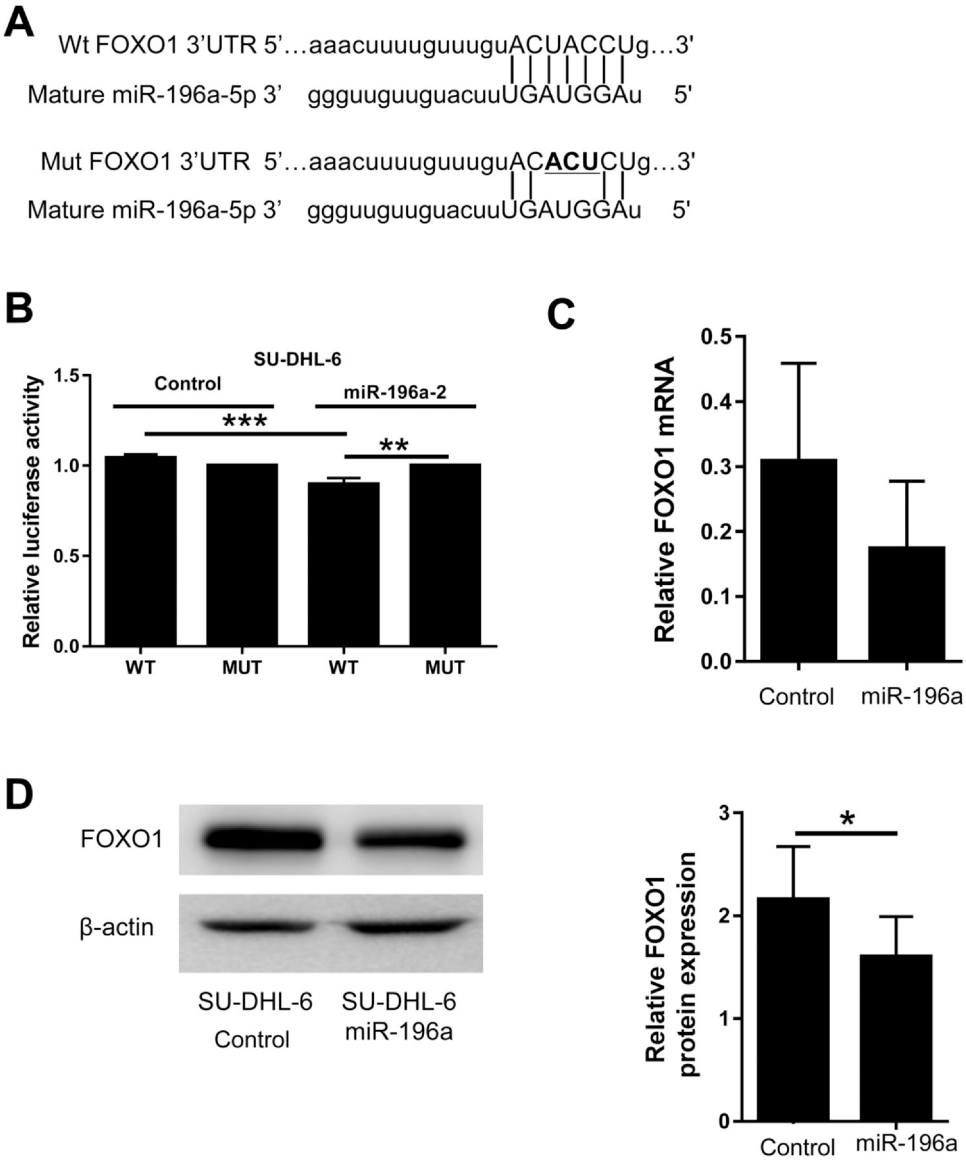

Fig. 2. FOXO1 is a direct target of miR-196a. (A) miR-196a-5p and its putative binding sequence in the 3′-UTR of FOXO1. The mutant seed sequence is indicated by an underline. (B) Luciferase assay for direct miR-196a-5p binding to the 3′-UTR of FOXO1. Luciferase reporter constructs containing the wild-type (WT) or mutated form of the FOXO1 3′-UTR were transfected into SU-DHL-6 cells stably overexpressing miR-196a-5p or mock control cells. Bars represent the relative luciferase activity against the control activity. Normalization was performed with Renilla luciferase. Bars represent the means ± SEM from 7 independent experiments. A paired *t*-test was used. **P* < .05. (C) Relative mRNA expression levels of FOXO1 in SU-DHL-6 cells stably overexpressing miR-196a-5p or mock control cells were measured by qRT-PCR. FOXO1 level was normalized to HuPo and GAPDH levels. Bars represent the mean ± SEM from 3 independent experiments. (D) Relative protein expression levels of FOXO1 in SU-DHL-6 cells stably overexpressing miR-196a-5p or mock control cells were measured by western blotting. β-Actin was used as the loading control. Bars represent the mean ± SEM from 5 independent experiments. A paired *t*-test was used. **P* < .05. WT, wild type; MUT, mutant.

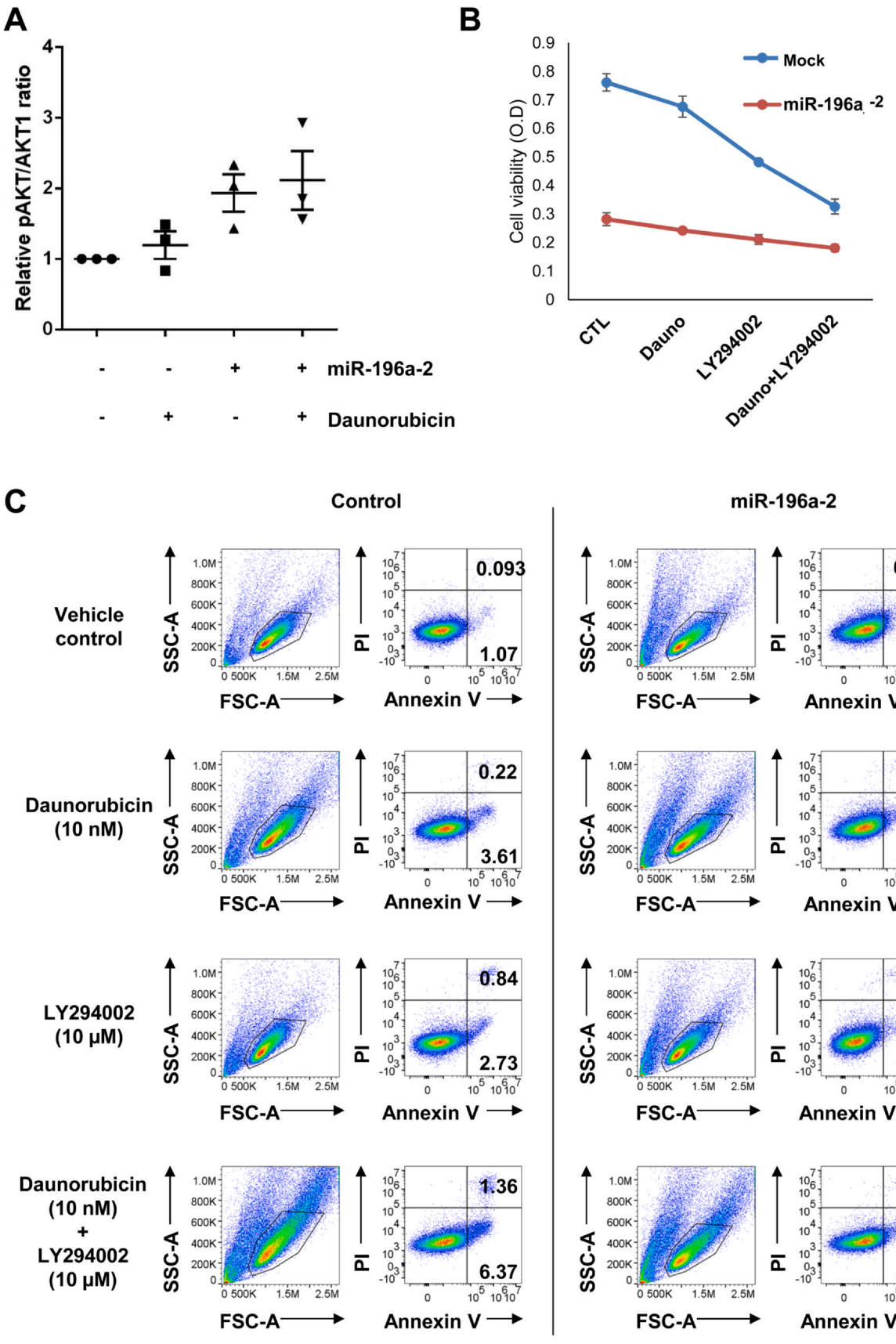

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Fig. 3. Effect of miR-196a on the PI3K/AKT pathway is assessed. (A) Flow cytometric results of AKT1 and phospho-AKT (S473) expression in miR-196a-overexpressing SU-DHL-6 or mock control are summarized. The cells were treated with 10 nM daunorubicin for 72 h. The representative histograms are shown in Supplementary Figure S2. The relative phospho-AKT/AKT1 ratio was presented as the mean ± SEM (n = 3). (B) CCK-8 assay was performed with miR-196a-overexpressing SU-DHL-6 and control cells treated with either 10 nM daunorubicin or 10 μM LY294002 (PI3K inhibitor) for 72 hours. (C) Flow cytometry was performed with annexin V/PI staining following daunorubicin (10 nM) or LY294002 (10 μM) treatment for 72 hours.

form of the miR-196a-5p-binding site in 3′-UTR (Fig. 2A). Luciferase activity was significantly reduced in miR-196a-5p-overexpressing SU-DHL-6 cells, compared with mock control cells transfected with the WT form of the 3′-UTR (Fig. 2B). Transfection with the mutated form of the 3′-UTR abrogated the effect of miR-196a-5p overexpression on reduced luciferase activity, suggesting that miR-196a-5p directly binds to the putative binding site in the 3′-UTR of FOXO1 (Fig. 2B). qRT-PCR and western blot analysis revealed that miR-196a-5p overexpression led to a reduction in FOXO1 mRNA and protein in SU-DHL-6 cells (Fig. 2C and 2D). Taken together, these results suggest that FOXO1 could be a direct target of miR-196a-5p in DLBCL cells.

Apoptosis Induction by miR-196a-5p Is Independent of the PI3K/AKT Pathway

It is known that FOXO1 is phosphorylated by the activation of the PI3K/AKT signaling pathway (Fu and Tindall, 2008) and that daunorubicin activates the PI3K/AKT pathway (Laurent and Jaffrezou, 2001). Therefore, we examined active AKT expression in miR-196a-5p-overexpressing SU-DHL-6 cells. The results showed that the increased miR-196a-5p expression and consequently reduced FOXO1 expression increased the phosphorylation of AKT slightly (Fig. 3A and Supplementary Fig. S3), however the difference was not statistically significant. Treatment of a PI3K inhibitor (LY294002) in combination with daunorubicin further decreased the cell viability of mock-transfected SU-DHL-6 cells as expected, but not in miR-196a-5p-expressing cells (Fig. 3B). The results were confirmed by annexin V/PI cell death assay (Fig. 3C). Therefore, cell death induction in miR-196a-5p-overexpressing B-cell lymphoma cells was not related to the PI3K/AKT pathway, implying that FOXO1 downregulation was not associated with the PI3K/AKT pathway either.

miR-196a-5p Is Inversely Correlated With FOXO1 in Murine Primary B Cells, Where Apoptosis Is Induced Upon Activation

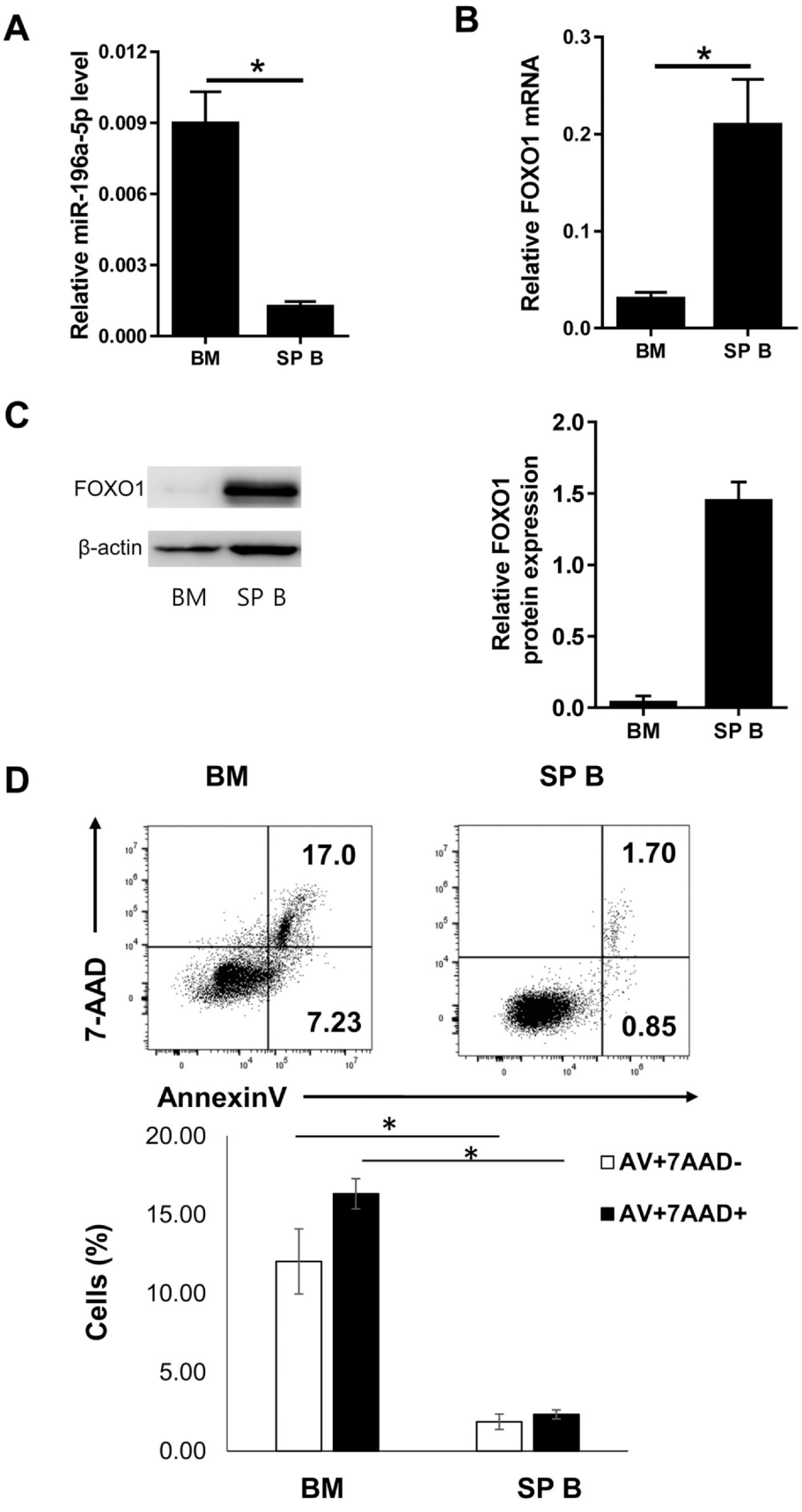
The miR-196 family is abundantly expressed in stem and progenitor cell populations and downregulated in cells at differentiated stages (Petriv et al., 2010). Consistently, qRT-PCR results revealed that miR-196a-5p was highly expressed in BM cells compared with SP B cells from wild-type healthy mice (Fig. 4A). As previous studies have shown that FOXO1 could regulate B-cell development and differentiation (Dengler et al., 2008; Szydłowski et al., 2014), we hypothesized that miR-196a might modulate the FOXO1 expression in healthy B cells as well. The FOXO1 expression was significantly lower in BM cells than in SP B cells at the mRNA and protein levels (Fig. 4B and C). Accordingly, apoptosis was significantly higher in BM cells (Fig. 4D).

Given that BCR signaling is essential for B-cell development and B-cell malignancies (Liu et al., 2020), we further investigated whether miR-196a-5p is associated with B-cell activation by BCR cross-linking. Purified SP B cells were treated with anti-IgM F(ab')2 antibodies for 5 or 24 hours. Following stimulation for 24 hours, the miR-196a-5p level in SP B cells was upregulated, whereas FOXO1 was significantly downregulated (Fig. 4E-G). These results indicate that BCR engagement could regulate miR-196a-5p to modulate FOXO1 expression in healthy primary B cells. Late apoptotic cells (AV^+7AAD^+) were significantly increased after 24 hours (Fig. 4H), suggesting that miR-196a-5p might regulate B-cell homeostasis through FOXO1 modulation upon activation.

DISCUSSION

In this study, we investigated the roles of miR-196a in human B-cell lymphoma cells and murine primary B cells. Accumulating evidence has demonstrated that various onco-miRNAs and tumor suppressor miRNAs are deregulated in B-cell malignancies, including human B-cell non-Hodgkin lymphoma, and the modulation of these miRNAs has therapeutic potential (Fernando et al., 2012; Fuertes et al., 2020). miR-196b has been reported to be downregulated in B-cell ALL cells and exerts a tumor-suppressive function by targeting oncogenic c-myc (Bhatia et al., 2010). Nevertheless, the roles of miR-196a are poorly understood in mature B-cell-derived malignancies and healthy primary B cells.

In the present study, we showed that miR-196a-5p was expressed at extremely low levels in SU-DHL-6 cells, a DLBCL cell line. In agreement with our study, a recent report has demonstrated that miR-196a-3p is markedly downregulated in DLBCL tissues and DLBCL cell lines, such as Farage and OCI-Ly3 cells, compared with the normal control (Fu et al., 2021). Since these results implicate miR-196a as a tumor suppressor miRNA in DLBCL cells, the function of miR-196a in DLBCL cells was investigated by examining the proliferation and apoptosis of miR-196a-overexpressing SU-DHL-6 cells. Notably, the overexpression of miR-196a-5p in combination with daunorubicin significantly increased cell death. The effect of miR-196a and anticancer drugs on other B-cell lymphoma cells was also shown with 5-aza-2′-deoxycytidine (5-AZA) (Supplementary Fig. S4), implying that the pro-apoptotic effect of miR-196a-5p could be consistent with a wide range of anticancer drugs. The overexpression of miR-196a attenuated the cell cycle by blocking G1-/S-phase transition and enhanced apoptosis of SU-DHL-6 cells in combination with daunorubicin as well. The overexpression of miR-196a reduced the expression of Cyclin D1 mRNA (Supplementary Fig. S5), supporting the effect on the cell cycle by miR-196a. Cyclin D1 is required for G1-/S-phase



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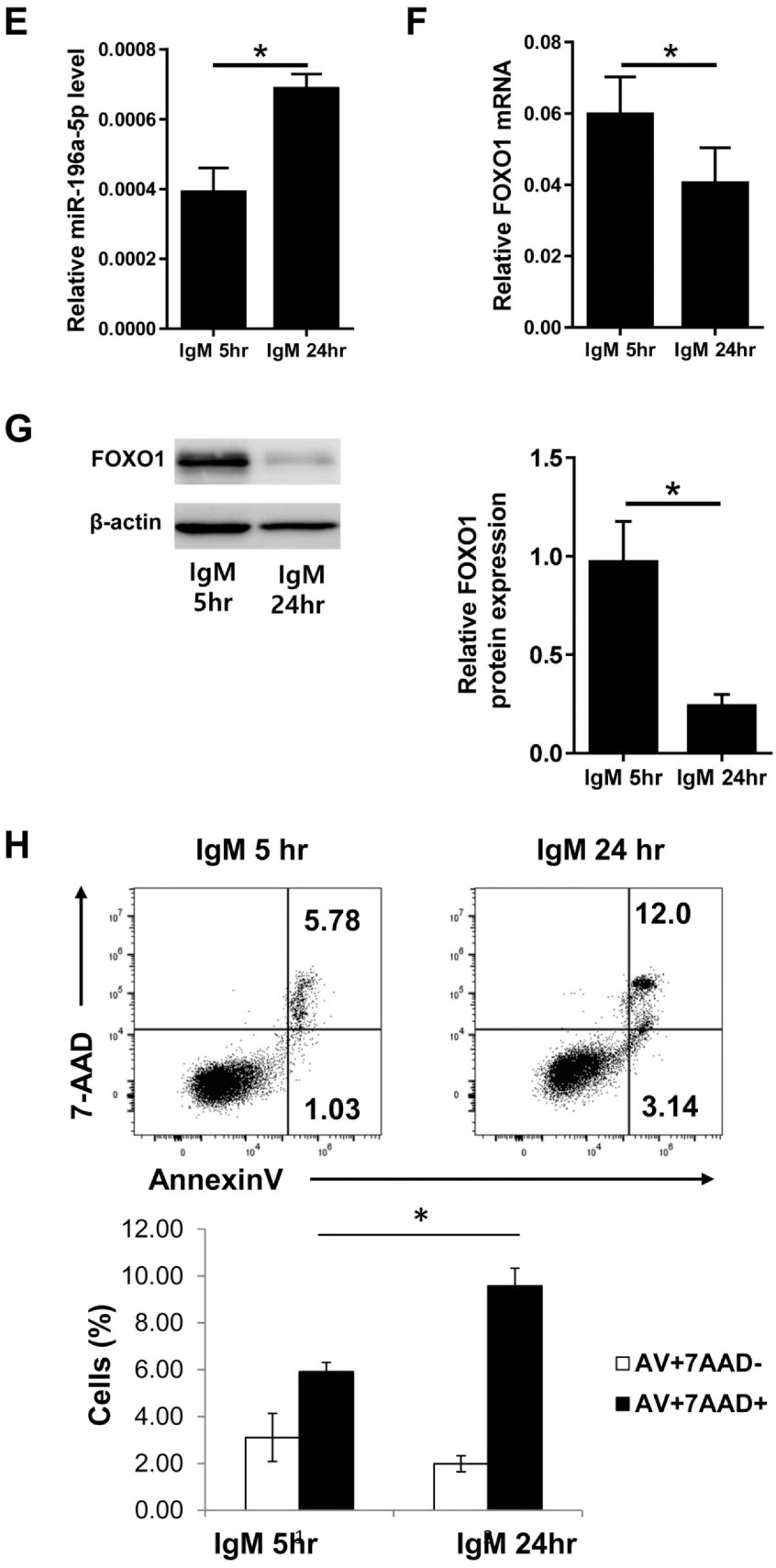

Fig. 4. (*continued*)

Fig. 4. miR-196a expression is inversely correlated with FOXO1 expression in BM and SP B cells and induces apoptosis upon activation. (A) Relative expression levels of miR-196a-5p in fresh mouse BM and SP B cells were measured by qRT-PCR. The miR-196a-5p level was normalized to the RNU6B level. Bars represent the means ± SEM from 4 independent experiments. A paired *t*-test was performed. (B) Relative expression levels of FOXO1 mRNA in fresh mouse BM and SP B cells were measured by qRT-PCR. FOXO1 level was normalized to Hprt and Gapdh levels. Bars represent the mean ± SEM from 5 independent experiments. A paired *t*-test was performed. (C) Relative expression levels of FOXO1 protein in fresh mouse BM and SP B cells were measured by western blotting. β-Actin was used for normalization. Western blot images represent 2 independent experiments. Relative band intensities are summarized in the graph. (D) Cell death was assessed by annexin V/7-AAD staining. Upper panels are representative flow cytometric plots. The results are represented as the mean ± SD in the lower panel. One-way ANOVA was performed. **P* < .05. (E) Enriched SP B cells were seeded at 2×10^6 cells/ml and stimulated with 5 μg/ml F(ab')2-anti-mouse IgM antibodies for the indicated time period. Relative expression levels of miR-196a-5p in SP B cells were measured by qRT-PCR following IgM stimulation. The miR-196a-5p level was normalized to the RNU6B level. Bars represent the mean ± SEM from 5 to 6 independent experiments. A paired t-test was performed. **P* < .05. (F) Relative expression levels of FOXO1 mRNA in SP B cells were assessed by qRT-PCR, following IgM stimulation. FOXO1 mRNA levels were normalized to Hprt and Gapdh levels. Bars represent the mean ± SEM from 5 to 6 independent experiments. A paired t-test was performed. **P* < .05. (G) Relative expression levels of FOXO1 protein in SP B cells were assessed by western blotting, following IgM stimulation. β-Actin was used for normalization. Western blot images represent 3 independent experiments. Bars represent the mean ± SEM from 3 independent experiments. A paired t-test was performed. **P* < .05. (H) Cell death was assessed by annexin V/7-AAD staining. Upper panels are representative flow cytometric plots. The results are presented as mean ± SD in the lower panel. One-way ANOVA was performed (n = 3). **P* < .05.

transition (Hinz et al., 1999). Thus, our study supports that miR-196a plays a tumor-suppressing role in DLBCL.

Through luciferase assay, we identified FOXO1 as a direct target of miR-196a in DLBCL cells. FOXO1 expression was downregulated in miR-196a-5p-expressing SU-DHL-6 cells at the RNA and protein levels. It is known that the FOXO1 transcription factor acts as a tumor suppressor in various types of cancers, including B-cell lymphomas (Kabrani et al., 2018; Xie et al., 2012). Several studies demonstrated that both the inactivation and overactivation of FOXO1 induces antiproliferative and pro-apoptotic activities in B-cell precursor ALL cells and B-cell leukemic cells, suggesting that the tight regulation of FOXO1 is crucial in B-cell malignancies (Gehringer et al., 2019; Wang et al., 2018). Furthermore, FOXO1 expression is upregulated in primary B-CLL and associated with a poor prognosis (Cosimo et al., 2019). miR-196a has been reported to promote cancer cell progression by *directly* targeting FOXO1 (Hou et al., 2014; Song et al., 2020; Yang et al., 2017). Nevertheless, the current results indicate that miR-196a may play a tumor-suppressive role by regulating FOXO1 in DLBCL.

FOXO1 is a critical regulator of early B-cell development and the function of peripheral B cells (Dengler et al., 2008). Here, qRT-PCR and western blot results revealed that miR-196a is downregulated in murine primary mature B cells compared with hematopoietic stem cells, and its expression was negatively correlated with FOXO1. The present study also showed that miR-196a, upregulated by BCR engagement in SP B cells, was negatively correlated with FOXO1 expression. In agreement with previous studies, the activation of mature B cells through BCR cross-linking downregulated FOXO1 at the mRNA and protein levels (Hinman et al., 2007; Yusuf et al., 2004). The deletion of FOXO1 in SP B cells could increase expression of pro-apoptotic factor Bim following BCR stimulation (Dengler et al., 2008). Taken together, these results demonstrate that miR-196a might induce BCR-mediated apoptosis through FOXO1. We have shown that the cell death induced by miR-196a overexpression and consequent FOXO1 downregulation was not associated with PI3K/AKT pathway modulated by daunorubicin. Further investigation is required to understand the cell death mechanisms by miR-196a in B cells.

In summary, the enforced expression of miR-196a arrested the cell cycle partially through inhibition of G1-/S-phase transition, and it promoted apoptosis in DLBCL cells. Daunorubicin further increased the apoptosis of miR-196a-5p-expressing DLBCL cells. miR-196a-5p expression was inversely correlated with FOXO1 expression in mouse primary BM and SP B cells and was upregulated in SP B cells by BCR cross-linking. The current findings suggest that regulation of FOXO1 through miR-196a-5p could play a role in B-cell differentiation and survival, and modulation of the miR-196a-5p/FOXO1 axis might be a potent therapeutic target for B-cell malignancies.

AUTHOR CONTRIBUTIONS

S. K. and M. H. performed the experiments, analyzed the results, and wrote the paper. H. J. H. and Y.-H. A. performed the experiments. H. J. I. conceptualized the research. S.-H. H., and K.-N. K. conceptualized the research and obtained the grants. N. K. conceptualized and supervised the research, analyzed the results, and wrote the paper.

CREDIT AUTHORSHIP CONTRIBUTION STATEMENT

Kyung-Nam Koh: Writing – review and editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization. **Nayoung Kim:** Writing – review and editing, Writing – original draft, Validation, Supervision, Project administration, Methodology, investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Soyoung Kim:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Mina Han:** Methodology, Investigation, Formal analysis, Data curation. **Ho Joon Im:** Supervision, Resources, Project administration. **Sang-Hyun Hwang:** Project administration, Methodology, Funding acquisition. **Hyun Ju Hwang:** Methodology, Investigation, Formal analysis, Data curation. **Young-Ho Ahn:** Writing – review and editing, Resources, Methodology.

DECLARATION OF COMPETING INTERESTS

The authors declare no competing financial interests.

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APPENDIX A. SUPPLEMENTARY MATERIAL

Supplemental material associated with this article can be found online at: doi:10.1016/j.mocell.2025.100223.

AUTHORS' ORCID

Soyoung Kim: 0000-0002-3181-1290
Mina Han: 0000-0003-3921-1393
Hyun Ju Hwang: 0000-0002-6937-4153
Young-Ho Ahn: 0000-0002-1084-0367
Ho Joon Im: 0000-0001-8799-4068
Sang-Hyun Hwang: 0000-0002-8130-6671
Kyung-Nam Koh: 0000-0002-6376-672X
Nayoung Kim: 0000-0001-8598-0068

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