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MicroRNA-155 Downregulation Promotes Cell Cycle Arrest and Apoptosis in Diffuse Large B-Cell Lymphoma

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Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin's lymphoma in the adult population, and treatment of DLBCL is still unfavorable. Therefore, there is an urgent requirement to investigate the molecular mechanisms underlying DLBCL tumorigenesis. To study the potential function of microRNA-155 (miR-155) involved in the regulation of lymphoma, we monitored lymphoma cell behavior including proliferation, cell cycle, and apoptosis using CCK-8 and flow cytometry analysis. Real-time PCR was used to detect the expression levels of miR-155 in 118 lymphoma patients' tissues, and Western blot was also used to analyze the expression level of proteins correlated with cell cycle and apoptosis in lymphoma cells. miR-155 expression levels were higher in lymphoma tissues compared with adjacent tissues. Downregulation of miR-155 inhibited lymphoma cell progress by arresting cell cycle in the G_0/G_1 phase and promoting apoptosis. Cell cycle-correlated proteins (cyclin B1, cyclin D1, and CDK4) were inhibited by downregulation of miR-155. Apoptosis-correlated proteins level (Bax/Bcl-2 and caspase 3 activity) were increased by downregulation of miR-155. In addition, a significant inverse correlation between the level of miR-155 and transforming growth factor-β receptor 2 (TGFBR2) was observed, which has been demonstrated to be a novel tumor suppressor gene. A further in vivo tumor formation study in nude mice indicated that downregulation of miR-155 in lymphoma cells delayed the progress of tumor formation. These findings indicate that miR-155 may serve as a useful potential target for the treatment of lymphoma.

Key words: Lymphoma; MicroRNA-155 (miR-155); Transforming growth factor-β receptor 2 (TGFBR2); Cell cycle; Apoptosis

INTRODUCTION

Diffuse large B-cell lymphoma (DLBCL) is the most common non-Hodgkin's lymphoma in the adult population, accounting for 40% of lymphoid neoplasms and accounting for more than 80% cases of aggressive lymphomas in the world (1). It is a clinically, morphologically, and genetically heterogeneous disease with different genetic abnormalities, molecular grounds, clinical characteristics, therapy responses, and prognosis (2,3), in part because of the diversity of its normal cell counterparts (4). Although recent advances in combination chemotherapy improved overall survival rates, a large number of DLBCL patients ultimately develop refractory disease, and the response to treatment remains variable. The precise mechanism underlying the cause of DLBCL remains unknown because of the heterogenic nature of DLBCL; however, dysregulation of cell cycle and apoptosis plays an important role in lymphogenesis (5). Hence, a better understanding of lymphoma biology, identification of additional biologic markers, and novel molecular targets are crucial for the improvement of the current diagnostic and treatment tools and clinical outcome of patients with DLBCL.

MicroRNAs (miRNAs) are single-stranded noncoding RNAs of approximately 19 to 25 nucleotides. They influence the stability of mRNA transcription and protein translation through base pairing with the 3′-untranslated region (UTR) of the target mRNA (6,7). At present, 711 miRNAs have been identified in the human genome and

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have important regulatory functions including cell cycle, apoptosis, and differentiation. Recently, many miRNAs have been found to be involved in cancer, acting either as oncogenes or tumor suppressors (8). To date, a large number of miRNA signatures characterizing lymphomas were identified, and the role of miRNAs in the development, classification, and regulation of target genes is under intensive investigation (9,10). miR-155 has been demonstrated to function in hematopoiesis and the immune response (11), acting as an oncogenic miRNA in many malignancies. It has been shown that miR-155 is upregulated in pediatric Burkitt's lymphoma, DLBCL, primary mediastinal B-cell lymphoma, and Hodgkin's lymphoma (12,13). Furthermore, primary DLBCL samples and cell lines exhibit a positive correlation between miR-155 upregulation and NF-κB activation (14). In vivo research demonstrated that anti-miR-155-mediated inhibition of miR-155 in low-grade B-cell lymphomas emphasizes the potential of therapeutic targeting of miR-155 in lymphoma malignancies (15). The identified targets of miR-155 include FOXO3a (16), SOCS1 (17), RhoA (18), SHIP1 (19), etc., suggesting that miR-155 may play an oncogenic role through diverse mechanisms. However, the role of miR-155 overexpression in lymphomagenesis is largely unknown.

A previous study found a reduced expression of transforming growth factor-β receptor 2 (TGFBR2) in DLBCL (20), which was subsequently supported by the findings from Rai et al. (21) Although many miRNAs [e.g., miR-21 (9), miR-155 (21), miR-28 (22), miR-34a (23)] have been reported to be involved in DLBCL carcinogenesis, no evidence was given for their associations with TGFBR2 downregulation.

In the present study, we investigated the effect of miR-155 on the proliferation, cell cycle, and apoptosis of DLBCL cell lines. Furthermore, we aimed to determine whether miR-155 could directly bind to TGFBR2. The results suggest that miR-155 serves as an oncomiR and a regulator of TGFBR2 expression in DLBCL.

MATERIALS AND METHODS

Patients and Tissue Samples

Paired tumor and adjacent human lymphoma samples were obtained from 118 patients who underwent surgery at Shenzhen People's Hospital. The percentage of tumor cellularity was at least 80% composed of viable-appearing tumor cells on histological assessment. The pathological stage and grade were appraised by an experienced pathologist. Tumor and adjacent tissues were immediately snap frozen in liquid nitrogen and stored at −80°C until total RNA was extracted. Ethical approval for the study was provided by the independent ethics committee, Shenzhen People's Hospital. Written informed consents were obtained from all participants in this study.

Cell Culture

The human lymphoma cell lines (SUDHL-6, SUDHL-4, OCI-Ly3, and Rose) and normal B-cell lines were obtained from the Academia Sinica Cell Bank (Shanghai, China). SUDHL-6, SUDHL-4, and Rose were grown in 90% RPMI-1640 medium supplemented with 10% fetal calf serum (FBS) and 100 U/ml penicillin and 0.1 mg/ml streptomycin. OCI-Ly3 and B-cell lines were maintained in 90% Iscove's modified medium supplemented with 10% human serum (NABI Biopharmaceuticals, Boca Raton, FL, USA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine (Invitrogen, Carlsbad, CA, USA). All the cells were incubated in a humidified atmosphere at 37°C in 5% CO_2.

Vector Construction and Transfection

The pre-miR-155 sequence and anti-miR-155 sequence were purchased from Neuron Biotech Co. Ltd., Shanghai. Oligonucleotides encoding antisense RNA were directed against human miR-155 (forward: 5′-GCATTTTGTTTA TTTTAAAGACT-3′, reverse: 5′-GTGTATATTCTTCA AATCTTTCT-3′). The sequences were cloned into the pLVX-AcGFP-C1 lentiviral vector. Negative control miRNA (50 nM) (mock), miR-155 mimics, or miR-155 inhibitor (anti-miR-155) was then cotransfected into HEK-293T cells with lentiviral packaging vectors by using Lipofectamine 2000 (Invitrogen Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instruction. Viruses were collected 48 h after transfection and used to infect OCI-Ly3 and Rose cells at a multiplicity of infection (MOI) of 20 in the presence of 8 μg/ml polybrene (Sigma-Aldrich, St. Louis, MO, USA). Assay was performed 48 h after infection.

Cell Proliferation Assay

Cell proliferation was assessed by Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) assay. In brief, we plated the cells in 96-well plates at an initial density of 5×10^3 cells/well. Cells were subsequently infected with mock, miR-155 mimics, or anti-miR-155 following culture overnight. At 0, 24, 48, and 72 h, 10 μl of CCK-8 solution was added to each well of the plate. The plate was incubated for 1 h. Cell proliferation was determined by scanning with a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA) at 450 nm.

Cell Cycle and Apoptosis Assay

Cells were collected after mock, miR-155 mimics, or anti-miR-155 infection for 48 h. For cell cycle assay, cell

cycle distribution was analyzed using propidium iodide (PI; Sigma-Aldrich) staining and flow cytometer (BD Biosciences, San Diego, CA, USA). For cell apoptosis assay, cells were stained with annexin V-fluorescein isothiocyanate (FITC)/PI, and apoptosis rates were analyzed using a flow cytometer.

Luciferase Reporter Assays

The 3′-UTR of human TGFBR2 gene insert was introduced downstream of the luciferase reporter gene in the pGL3 promoter vector (Promega, Madison, WI, USA). Luciferase activity assays were performed with the Dual-Luciferase Reporter System (Promega). HEK-293T cells were cotransfected with luciferase reporter plasmids and miR-155 mimics or mock. Luciferase activities were measured 48 h after transfection.

Reverse Transcription and Real-Time PCR

miRNAs were isolated from tissues or cells using the miRNeasy Mini Kit (Qiagen, Valencia, CA, USA). Total RNA was extracted using the TRIzol reagent (Invitrogen). Reverse transcription polymerase chain reaction (PCR) was performed using the PrimeScript RT Reagent Kit (TaKaRa, Dalian, China). Real-time PCR was performed on ABI 7500 (Applied Biosystem, Foster City, CA, USA) thermal cycler using a standard SYBR Green PCR Kit (Finnzymes Oy, Espoo, Finland). Real-time PCR was performed to detect mRNA levels of indicated genes. The 5S RNA was used as an internal control for miR-155. The primer sequences (sense/antisense) used are as follows: miR-155, 5′-GATCAAAGTCTTCAAATATGCCT AAAGG-3′ and 5′-TGAACAAGCCAAAACCTGC-3′; 5S, 5′-CCATACCACCCTGGAAACGC-3′ and 5′-TACT AACCGAGCCCGACCCT-3′. The ΔΔCt method for relative quantification of gene expression was used to determine miRNA expression levels.

Western Blot

Total proteins were isolated from tumor and adjacent lymphoma tissues or lymphoma cell lines. Protein concentration was measured by BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Protein (30 μg) was subjected to electrophoresis using SDS-PAGE and transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, Shanghai, China). The blots were blocked with 5% skim milk, followed by incubation with antibodies specific against TGFBR2 (Abcam, Cambridge, MA, USA), GAPDH (Fermentas, Pittsburgh PA, USA), cyclin B1 (Abcam), cyclin D1 (Santa, Paso Robles, CA, USA), CDK4 (Abcam), Bax (Santa), Bcl-2 (Santa), or caspase 3 (Cell Signalling Technology Inc., Danvers, MA, USA). Blots were then incubated with goat anti-mouse or anti-rabbit secondary antibody (Beyotime, Shanghai, China) and visualized using enhanced chemiluminescence (ECL; Thermo Scientific, Shanghai, China).

Enzyme-Linked Immunosorbent Assay

Secretions of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, and IL-10 were determined by enzyme-linked immunosorbent assay (ELISA). Cells were plated in 96-well plates and treated as indicated; the relative content of each secreted inflammatory cytokines in the supernatant was measured by ELISA according to the manufacturer's protocol.

Animal Experiments

Care of laboratory animals and animal experimentation were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All animal studies were approved by the animal ethics committee of the hospital. Twelve male BALB/c nude mice aged 4–5 weeks old were purchased from Shanghai Laboratory Animal Company (SLAC; Shanghai, China). OCI-Ly3 cells (1×10^7) stably expressing anti-miR-155 or negative control (mock) (six per group) were injected subcutaneously to the right flanks of BALB/c nude mice. The tumor size was determined every 4 days after the tumor formed (around 1–2 weeks) as previously described (24). Tumor volume was measured and calculated using the following formula: V (mm^3) = 0.5 × larger diameter × smaller diameter^2. Thirty-six days later, the mice were sacrificed and photographed, and the tumors were weighted on a digital balance.

Statistical Analysis

The data are presented as mean ± SD. Statistical significance was determined by the unpaired, two-tailed Student's *t*-test and one-way analysis of variance (ANOVA). Overall survival in relation to miR-155 expression was evaluated by the Kaplan–Meyer survival curve and log-rank nonparametric test. A value of $p<0.05$ was considered to be statistically significant.

RESULTS

miR-155 Upregulation in Human Lymphoma Tissues Associated With Poor Survival

To study the biological role of miR-155 in human lymphoma, we first used real-time PCR to detect the expression levels of miR-155 in tumor tissues of lymphoma patients. We collected 118 lymphoma tumor tissues, and their adjacent tissues were used as corresponding controls. Table 1 summarizes the clinicopathologic characteristics of the 118 patients enrolled in the study. As shown in Figure 1A, miR-155 expression levels were significantly upregulated in lymphoma tumor tissues compared with the adjacent tissues. We next explored the

Table 1. Clinicopathologic Characteristics of Patients With Human Lymphoma

Characteristic	Total No. of Patients ($N=118$)
Age	
<55	59 (50.0%)
≥55	59 (50.0%)
Sex	
Female	51 (43.2%)
Male	67 (56.8%)
Performance status	
<2	78 (66.1%)
≥2	32 (27.1%)
Unknown	(6.78%)
Extranodal involvement	
Yes	62 (52.3%)
No	56 (47.5%)
Tumor type	
Non-Hodgkin's B-cell lymphoma	39 (33.1%)
DLBCL	79 (66.9%)
Stages	
I	40 (33.9%)
II	28 (23.7%)
III	35 (29.7%)
IV	15 (12.7%)
International prognostic index risk	
Low	50 (42.4%)
High	45 (38.1%)
Unknown	23 (19.5%)

association of miR-155 with clinical characteristics of the patients with lymphoma. Evaluation of miR-155 expression with different clinicopathological features revealed that miR-155 expression correlated with performance status, tumor type, and international prognostic index risk (Table 2). However, we did not find any association between miR-155 expression levels and other clinical pathological features including patients' age, sex, extranodal involvement, and stages (Table 2). We also compared the survival time of patients in Shenzhen People's Hospital. The human lymphoma patients were divided into two groups according to the median level of miR-155 expression. The survival time of human lymphoma patients showed that lower miR-155-expressing patients notably lived longer than higher miR-155-expressing patients (Fig. 1B).

miR-155 Downregulation Inhibits Human Lymphoma Cell Proliferation

In an effort to understand the gene regulatory mechanism of miR-155, initially we examined its expression levels in different DLBCL cell lines including SUDHL-6, SUDHL-4, OCI-Ly3, and Rose. RNA was isolated from each cell line, reverse transcribed into cDNA, and analyzed by real-time PCR for the expression of miR-155. These analyses showed that miR-155 expression was relatively higher in OCI-Ly3 and Rose cells in comparison to several other cell lines including SUDHL-6 and SUDHL-4, especially in normal B cells (Fig. 2A). Anti-miR-155 and miR-155 mimics were then infected into OCI-Ly3 and Rose cells, respectively (Fig. 2B and C). At 0, 24, 48, and 72 h after infection, cell proliferation was analyzed using the CCK-8 assay. Figure 2D and E shows 59.24 and 45.51% cell proliferation inhibition 72 h after anti-miR-155 infection in OCI-Ly3 and Rose cells, respectively, compared with the mock sequence (mock) in both cell lines. Additionally, cell proliferation was promoted 9.12% and 12.40% 72 h after miR-155 mimics infection in OCI-Ly3 and Rose cells, respectively, compared with the mock sequence (mock) in both cell lines.

miR-155 Downregulation Arrests Cell Cycle in Lymphoma Cell Lines

To further validate the cell proliferation inhibition of anti-miR-155, the cell cycle was analyzed in OCI-Ly3 and Rose cells (Fig. 3). Cell cycle analysis showed that downregulation of miR-155 by infection of cells with anti-miR-155 notably increased the rate of G_0/G1 phase cells and reduced the S phase cell population in both cell lines. On the contrary, upregulation of miR-155 slightly reduced the G_0/G_1 phase population and increased the rate of S phase cells in lung cancer cells. However, miR-155 changes had no effects on the cell population of the G_2/M phase. These results indicate that downregulation of miR-155 in lymphoma cells may inhibit cell proliferation by arresting cell cycle progression in the G_0/G_1 phase.

miR-155 Downregulation Promotes Cell Apoptosis in Lymphoma Cell Lines

We then evaluated the apoptotic function of miR-155 in OCI-Ly3 and Rose cells by annexin V-FITC/PI staining assay. As shown in Figure 4, flow cytometry analysis revealed that downregulation of miR-155 in OCI-Ly3 and Rose cells significantly increased cell apoptosis by 9.65-fold and 11.20-fold compared to the corresponding mock. Upregulation of miR-155 in the OCI-Ly3 and Rose cells reduced cell apoptosis by 69.77 and 62.50%, respectively.

miR-155 Regulates Expression Levels of Proteins Correlated With Cell Cycle and Apoptosis

To investigate the molecular mechanism of miR-155 regulating cell cycle and apoptosis of lymphoma cell lines, three proteins correlated with cell cycle (cyclin B1, cyclin D1, and CDK4) and apoptosis (Bax, Bcl-2, and caspase 3) were evaluated by Western blot. As shown in Figure 5A and B, downregulation of miR-155 inhibited

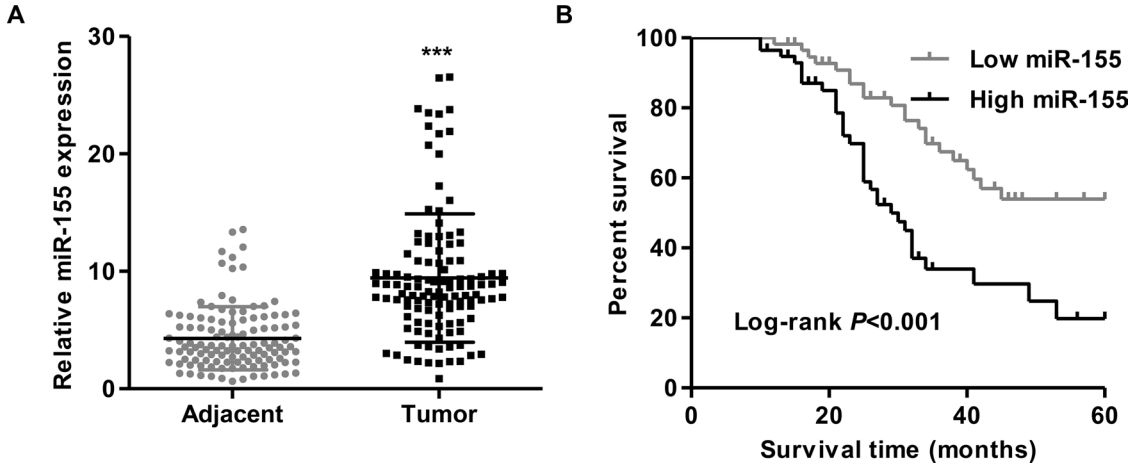

Figure 1. Correlation between miR-155 expression and survival time of patients with lymphoma. (A) Expression level of miR-155 detected by real-time PCR in 118 lymphoma tissues and adjacent tissues. (B) In 118 lymphoma patients, survival time of lower miR-155 expression level patients was notably longer than that of higher miR-155 expression level patients. ***$p<0.001$ compared with adjacent tissues.

expression of cyclin B1, cyclin D1, and CDK4 in OCI-Ly3 and Rose cells, while upregulation of miR-155 increased expression levels of these proteins. Meanwhile, increases in Bax/Bcl-2 ratio and caspase 3 expression were also found in OCI-Ly3 and Rose cells infected with anti-miR-155, while miR-155 mimics decreased Bax/Bcl-2 ratio and caspase 3 expression (Fig. 5C and D). Taken together, these results suggest that inhibition of miR-155 induced cell cycle arrest and apoptosis by regulating these protein expressions.

Table 2. Correlation of the Expression of miR-155 With Clinicopathologic Features

Clinicopathological Parameter	Low miR-155 Group, No. of Patients	High miR-155 Group, No. of Patients	*p* Value
Age			0.251
<55	33	26	
≥55	28	31	
Sex			0.261
Female	29	22	
Male	30	37	
Performance status			<0.001
<2	45	33	
≥2	3	29	
Unknown	8	–	
Extranodal involvement			0.642
Yes	33	29	
No	25	31	
Tumor type			<0.001
Non-Hodgkin's B-cell lymphoma	10	29	
DLBCL	24	55	
Stages			0.590
I	22	18	
II	12	16	
III	12	23	
IV	6	9	
International prognostic index risk			0.004
Low	31	19	
High	14	31	
Unknown	23	–	

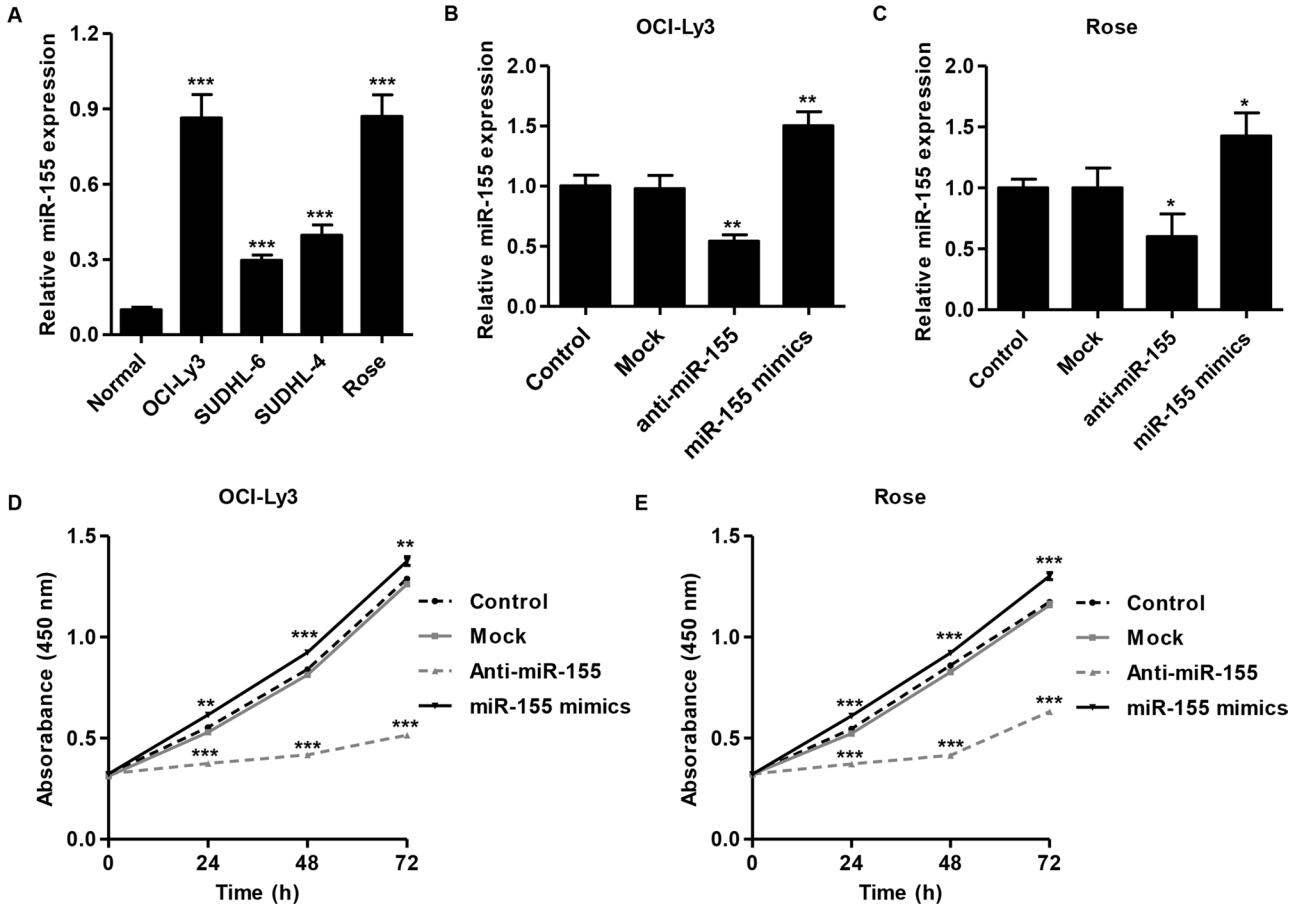

Figure 2. miR-155 downregulation inhibits cell proliferation. (A) Expressions of miR-155 in five DLBCL cell lines detected by real-time PCR. (B, C) Expressions of miR-155 in OCI-Ly3 and Rose cells detected by real-time PCR. OCI-Ly3 and Rose cells were infected with negative control miRNA (Mock), anti-miR-155, or miR-155 mimics, and 0, 24, 48, and 72 h later, cells were collected. (D, E) Cell proliferation was detected by CCK-8 assay. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with normal B cells or mock.

miR-155 Downregulation Suppresses Inflammatory Factor Secretions in Lymphoma Cell Lines

Next we measured TNF-α, IL-1β, IL-6, and IL-10 secretions in response to anti-miR-155 or miR-155 mimics. After infection of OCI-Ly3 and Rose cells with anti-miR-155, TNF-α, IL-1β, IL-6, and IL-10 secretions were significantly decreased, respectively (Fig. 6A and B). However, infection with miR-155 mimics increased TNF-α, IL-1β, IL-6, and IL-10 secretions in OCI-Ly3 and Rose cells. These results indicate that downregulation of miR-155 possesses an anti-inflammatory effect in lymphoma cell lines.

TGFBR2 Is a Target of miR-155 in Lymphoma Cell Lines

To confirm TGFBR2 as a target and is regulated by miR-155 in lymphoma cell lines, klotho 3′-UTR was cloned and inserted into a luciferase reporter vector. The luciferase assay showed that miR-155 significantly suppressed luciferase activity containing the klotho 3′-UTR (Fig. 7A). Western blot assay showed that TGFBR2 was notably increased after infection of anti-miR-155 into OCI-Ly3 and Rose cells (Fig. 7B and C). Consistent with these results, a significant decrease in TGFBR2 expression was observed upon upregulation of miR-155. These results suggest that TGFBR2 may be a direct target of miR-155 in lymphoma cell lines.

miR-155 Downregulation Inhibits Tumor Growth of Lymphoma In Vivo

To further demonstrate its function, we tested the effect of miR-155 on tumorigenicity in mouse models. When injected with OCI-Ly3 cells stably expressing miR-155 inhibitors (anti-miR-155) in nude mice, miR-155 downregulated tumors grew slower in mice, whereas mock tumors grew fast in mice (Fig. 8A and B). Consistently, the tumor weights were markedly decreased in anti-miR-155-expressing tumors compared with the mock (Fig. 8C). These data indicated that miR-155 downregulation suppresses tumor growth in vivo.

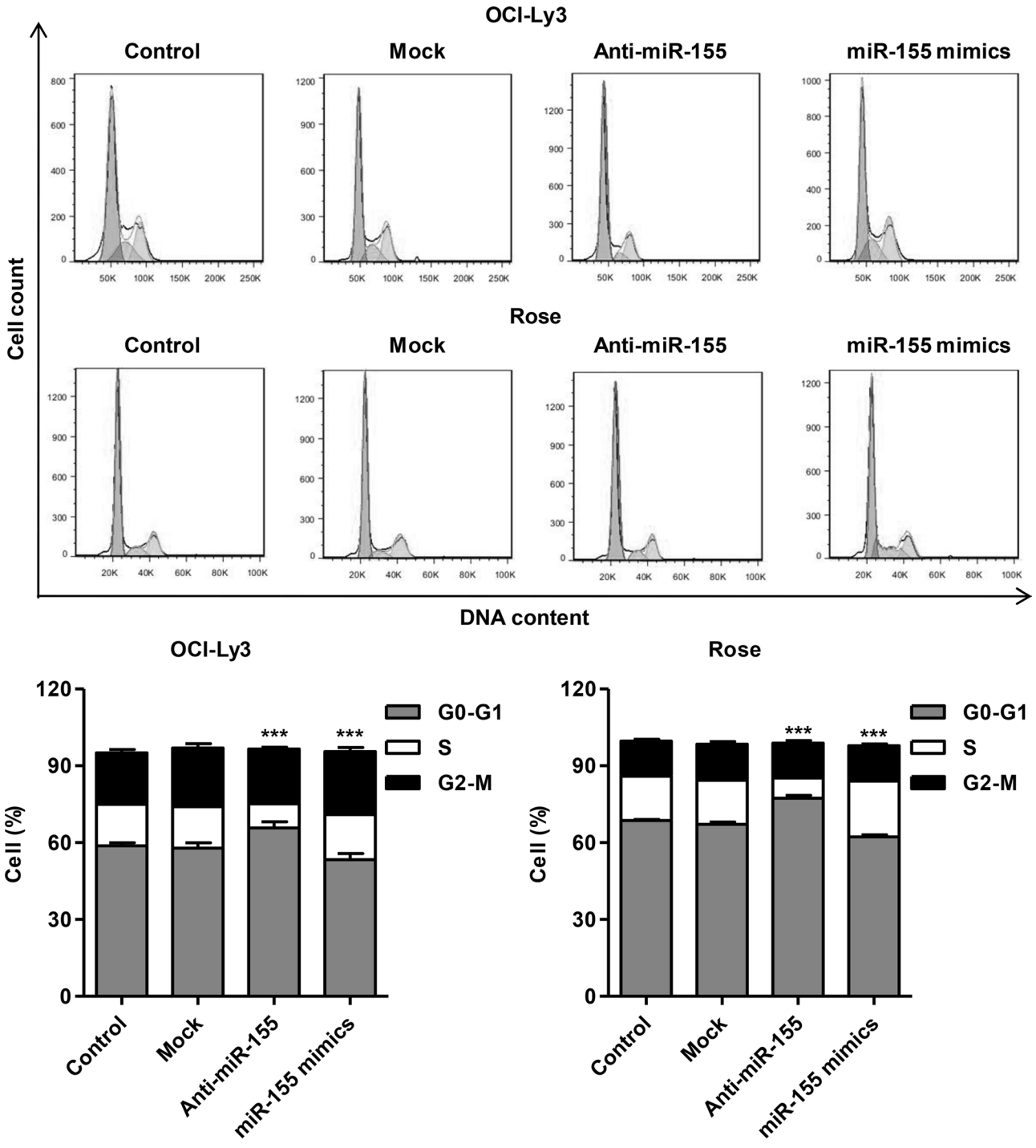

Figure 3. miR-155 downregulation arrests cell cycle at the G_0/G_1 phase. OCI-Ly3 and Rose cells were infected with negative control miRNA (Mock), anti-miR-155, or miR-155 mimics, and 48 h later cells were collected. Cell cycle profile was analyzed using flow cytometry and PI staining. ***$p < 0.001$ compared with mock.

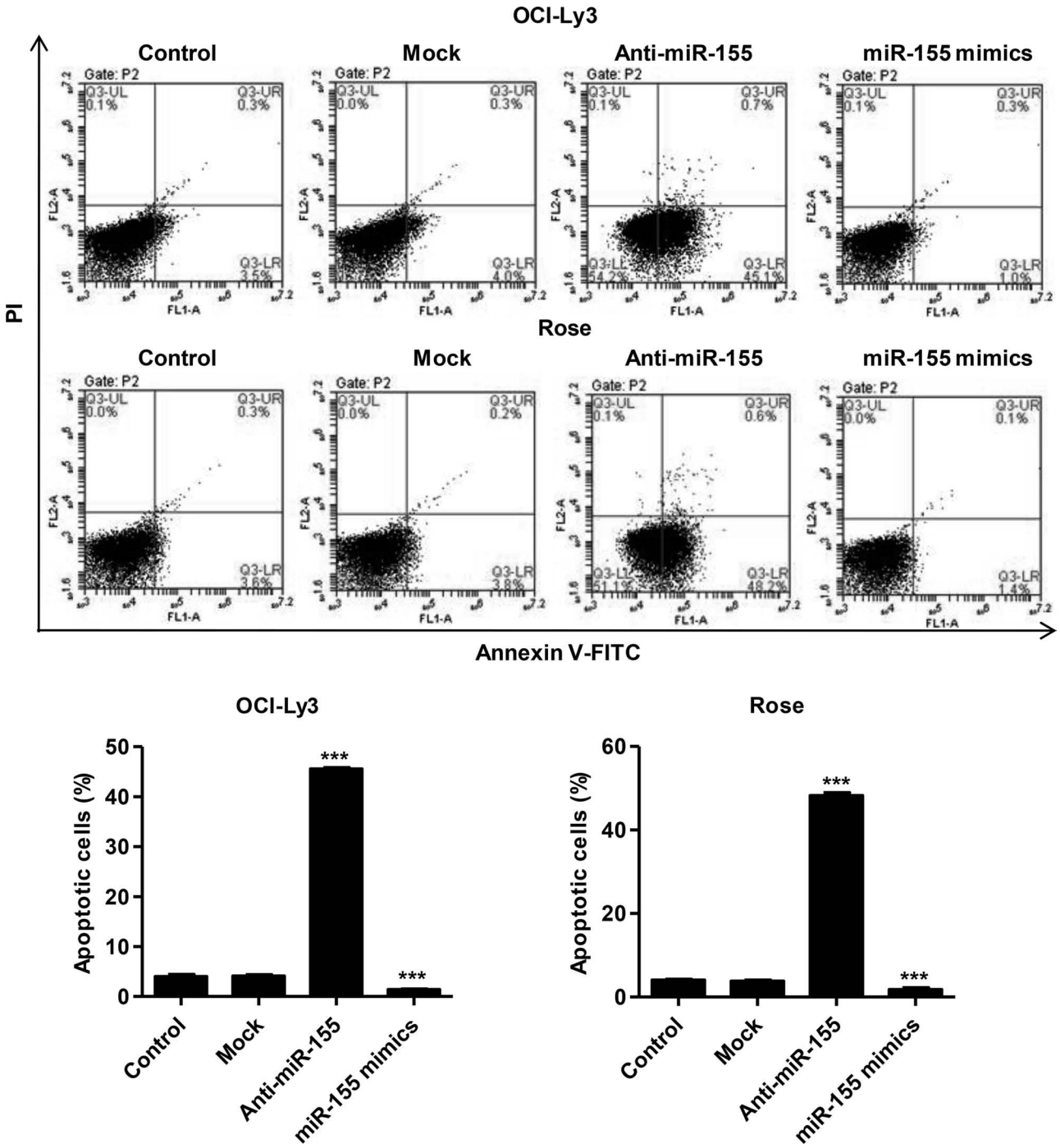

Figure 4. miR-155 downregulation induces cell apoptosis. OCI-Ly3 and Rose cells were infected with negative control miRNA (Mock), anti-miR-155, or miR-155 mimics, and 48 h later cells were collected. Cell apoptosis was analyzed using flow cytometry and annexin V-FITC/PI staining. ***$p<0.001$ compared with mock.

DISCUSSION

miR-155 has emerged as an important regulator with impact on a wide range of cancers, including lymphoma (13), breast cancer (16), pancreatic cancer (25), and non-small cell lung cancer (26). miR-155 has been extensively investigated in immunology and lymphoma; however, it is only evident that miR-155 expression is increased in lymphoma, and to date the detailed function of miR-155 remains elusive. In the present study, miR-155 inhibitor and miR-155 mimics were infected into the OCI-Ly3 and

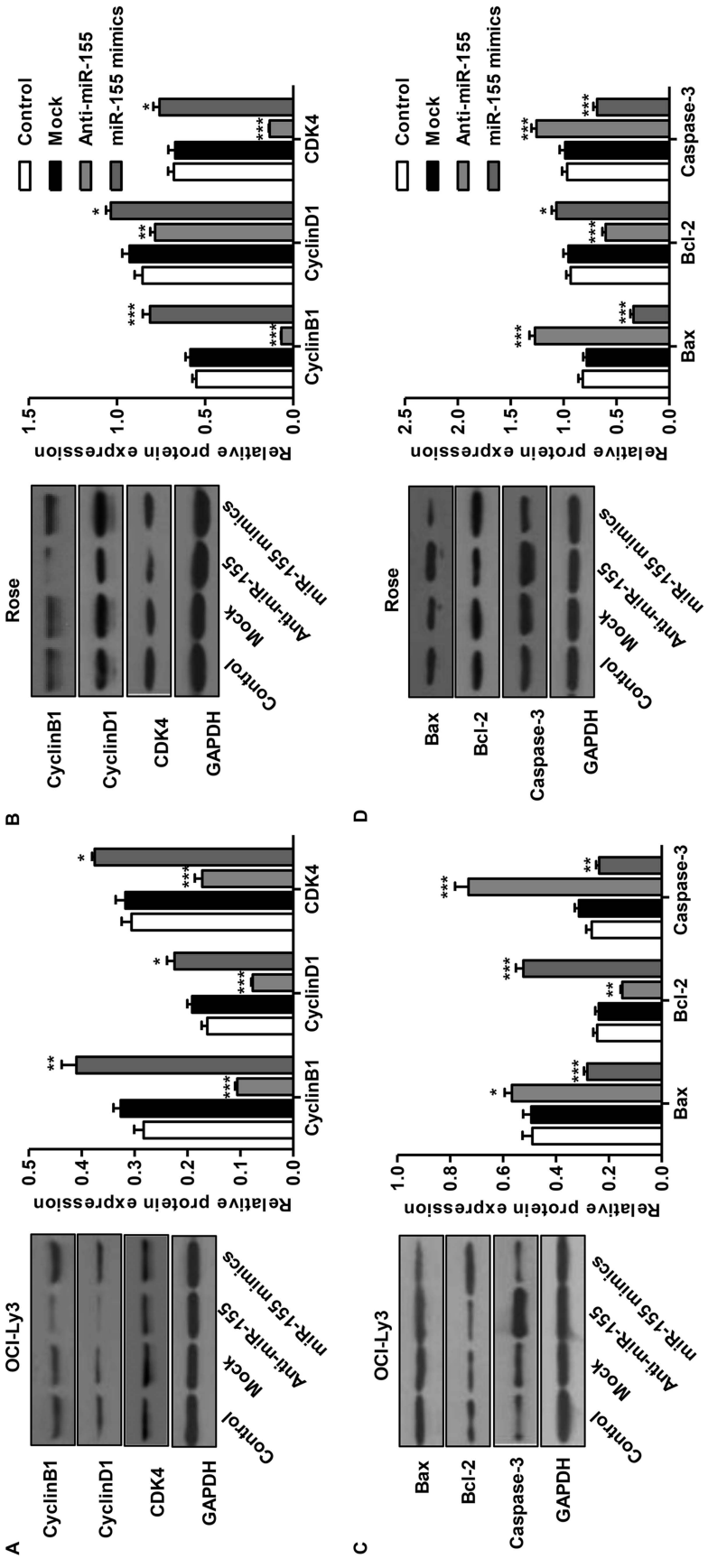

Figure 5. miR-155 regulates proteins correlated with cell cycle and apoptosis. OCI-Ly3 and Rose cells were infected with negative control miRNA (Mock), anti-miR-155, or miR-155 mimics, and 48 h later cells were collected. (A, B) Expression of three cell cycle regulatory factors cyclin B1, cyclin D1, and CDK4 was evaluated by Western blot. (C, D) Expression of three apoptosis regulatory factors Bax, Bcl-2, and caspase 3 was evaluated by Western blot. $*p<0.05$, $**p<0.01$, $***p<0.001$ compared with mock.

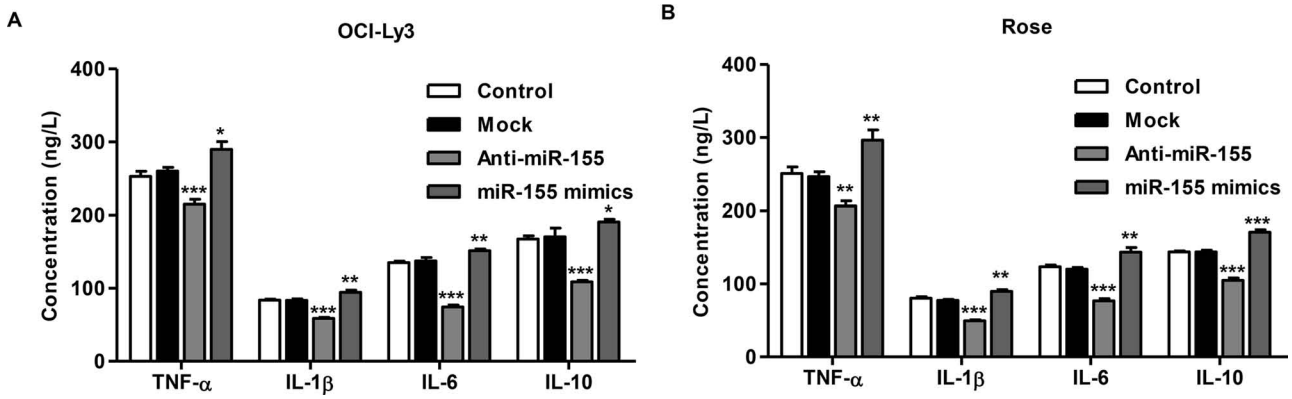

Figure 6. miR-155 downregulation inhibits inflammatory factor secretions. OCI-Ly3 and Rose cells were infected with negative control miRNA (Mock), anti-miR-155, or miR-155 mimics, and 48 h later cells were collected. (A, B) TNF-α, IL-1β, IL-6, and IL-10 secretions in OCI-Ly3 and Rose cells were measured by ELISA. *$p<0.05$, **$p<0.01$, ***$p<0.001$ compared with mock.

Rose DLBCL cells, respectively, to investigate the biological role of miR-155 and its potential functional target in DLBCL.

The clinical significance of miR-155 in DLBCL showed that miR-155 was upregulated in lymphoma tissues and cell lines, and upregulation of miR-155 positively correlated with performance status, tumor type, and international prognostic index risk. However, we did not find any association between miR-155 expression levels and other clinical pathological features including patients' age, sex, extranodal involvement, and stages. Kaplan–Meier analysis showed that patients with high miR-155 expression had significantly poorer survival than those with low miR-155 expression. However, previous studies reported that levels of miR-155 were upregulated in patients with lymphoma, while associations between miR-155 levels and clinicopathological features of the lymphoma patients, including sex, stage, and prognosis, were found to have no significant correlations (27).

Regarding whether miR-155 may participate in tumor progression in DLBCL patients, we also measured the role of miR-155 in the DLBCL cell lines in vitro. Our study showed that inhibition of miR-155 significantly suppressed cell proliferation in OCI-Ly3 and Rose cells, which was consistent with the previous studies (19). Furthermore, lymphoblastoid SDLCL cells knocked down

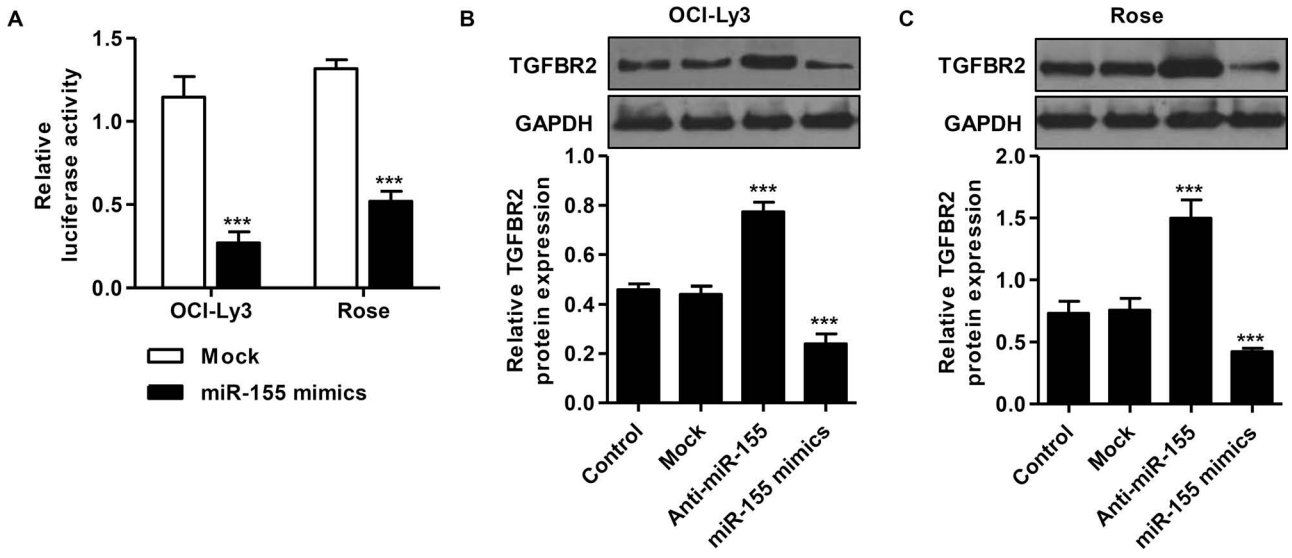

Figure 7. miR-155 regulates the expression of TGFBR2. (A) Luciferase assay. OCI-Ly3 and Rose cells were cotransfected with pGL3-TGFBR2 and mock. The firefly luciferase activity was normalized to Renilla luciferase activity. (B, C) TGFBR2 expression after infection of anti-miR-155 or miR-155 mimics in OCI-Ly3 and Rose cells analyzed by Western blot. ***$p<0.001$ compared with mock.

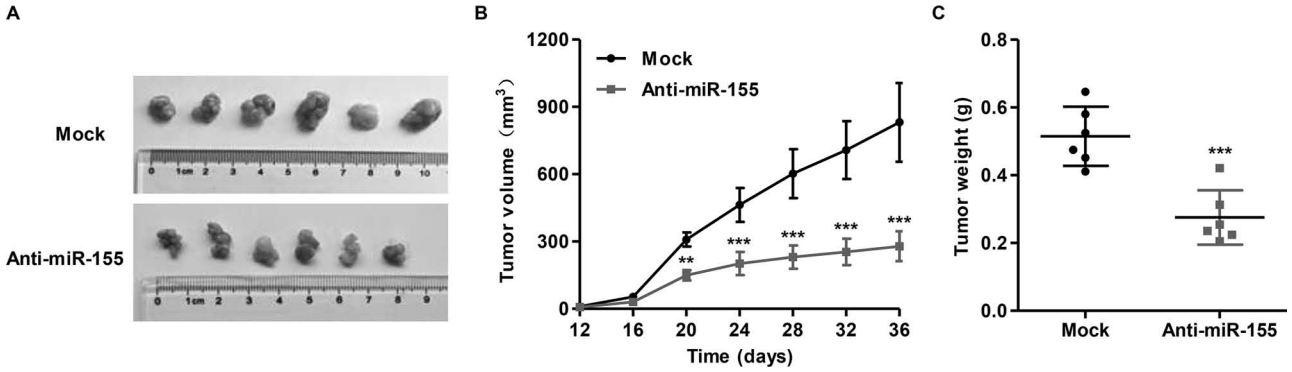

Figure 8. miR-155 downregulation in OCI-Ly3 cells reduced tumor growth in vivo. OCI-Ly3 cells infected with mock or anti-miR-155 were subcutaneously injected in nude mice. (A) Tumor diameter was evaluated for 36 days. (B) Tumor growth was significantly reduced in miR-155 downregulated tumors. (C) At day 36, mice were sacrificed, and tumors were weighted. $^{**}p<0.01$, $^{***}p<0.001$ compared with mock.

for miR-155 function arrested in G_0/G_1 and underwent increased apoptosis (28). In accordance with the above report, notably, inhibition of miR-155 led to cell cycle progression arrest in the G_0/G_1 phase in OCI-Ly3 and Rose cells. Cyclin B1 is required for the progression of the cell cycle through the G_2/M checkpoint, and profound loss of cyclin D1 and CDK4 resulted in cancer cells being unable to move to the S phase (29). The cell intrinsic apoptosis pathway is regulated by the Bcl-2 protein, which triggers the release of Bax and activation of caspases and causes mitochondrial outer membrane permeabilization and release of cytochrome *c* (30). In the present study, cell cycle-correlated proteins, including cyclin B1, cyclin D1, and CDK4, and apoptosis-correlated proteins, including Bax/Bcl-2 and caspase 3, were significantly increased in OCI-Ly3 and Rose cells after inhibition of miR-155. Further in vivo tumor formation studies in nude mice indicated that inhibition of miR-155 in OCI-Ly3 cells delayed the progress of tumor formation. These results suggested that miR-155 might be a potential therapy target for DLBCL.

miR-155, in addition to being upregulated in certain lymphomas, is necessary for normal immune function. It is known that TNF-α is involved in the modulation of lymphoma development and the balance between cell-mediated and humoral immunity. TNF-α plays a central role in inflammatory and immune responses, generating a cytokine cascade that includes the production of IL-1β, IL-6, and IL-10 (31). miR-155 deficiency was found to be associated with decreased TNF-α production, whereas forced expression of miR-155 leads to elevated TNF-α levels (32). Our data showed that inhibition of miR-155 was significantly increased in the secretions of TNF-α, IL-1β, IL-6, and IL-10 in OCI-Ly3 and Rose cells, suggesting that inflammatory miR-155 contributes to the development and progression of DLBCL.

Transforming growth factor-β (TGF-β) signaling plays a tumor-suppressive role in many cell types (33). Reduced expression of TGFBR2 is reported in several cancers including hepatocellular carcinoma (34), prostate (35), and pancreatic cancers (36). In the present study, we explored the mechanism by which miR-155 exerts influence on the development of DLBCL. Luciferase activity assay indicated that miR-155 could directly target the 3′-UTR of TGFBR2 mRNA. In addition, overexpression of miR-155 significantly downregulated the expression of TGFBR2 in OCI-Ly3 and Rose cells, suggesting that miR-155 functions as a novel oncogene in DLBCL and contributes to tumor progression of DLBCL.

CONCLUSION

In conclusion, our study shows that miR-155 plays an important role in cell proliferation, cell cycle, and apoptosis of DLBCL cell lines, and miR-155 may regulate biological progresses of DLBCL by targeting TGFBR2. These data suggest that miR-155 is a potent oncogene and a potential target for the treatment of DLBCL.

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