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Original Article

A novel TAL1/miR-149* axis accelerates tumor growth of human T cell acute lymphoblastic leukemia

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Abstract: *Background*: Herein, we aimed to investigate the roles of TAL1 and miR-149* in T cell acute lymphoblastic leukemia (T-ALL). *Methods*: The biological characteristics, including cell proliferation, cell apoptosis, and cell cycle, were analyzed in Molt4 cells. *Results*: ChIP results revealed that miR-149* expression in Jurkat cells transfected with overexpression TAL1 plasmid was higher than that in Jurkat cells alone, while miR-149* expression in Molt-4 cells transfected with knockdown TAL1 plasmid was lower than that in Molt-4 cells alone, suggesting that TAL1 might direct target miR-149*. This was further confirmed by a luciferase activity report assay. Finally, biological functions, such as cell proliferation, cell cycle, and apoptosis of TAL1 and miR-149* were measured by MTT and flow cytometry, respectively. It was uncovered that enhanced TAL1 and miR-149* expression promoted cell proliferation, induced cell cycle arrest in G0/G1 phase, and inhibited apoptosis in Molt-4 cells. In contrast, decreased TAL1 and miR-149* expression suppressed cell proliferation, abolished cell cycle arrest in G0/G1 phase, and accelerated apoptosis in Molt-4 cells. *Conclusion*: Thus, these data indicate that TAL1 directly regulates miR-149* expression and TAL1/miR-149* link is implicated in the pathogenesis of T-ALL.

Keywords: T cell acute lymphoblastic leukemia (T-ALL), TAL1, microRNA-149* (miR-149*)

Introduction

Acute lymphoblastic leukemia (ALL), mostly diagnosed in children and adults, is an aggressive hematologic malignancy that arises from hematopoietic precursors of the lymphoid lineage [1]. T cell ALL (T-ALL), accounting for 15% of pediatric and 25% of adult ALL cases, is the ALL transformed from developing thymocytes and resulting from cooperative genetic lesions which generally could affect proliferation, survival, and differentiation of precursor T cells [2, 3]. Despite recent advances in the treatment for T-ALL, therapeutic outcomes of patients with resistance or relapsed disease are still unsatisfactory [4, 5]. Accumulating studies have been indicated that stepwise alterations in cellular mechanisms contributed to T-ALL pathogenesis and progression included the aberrant expression of intact master developmental regulatory transcription factors (TFs) [6]. Among a variety of TFs, it was found that TAL1 presented an ectopically expression level in nearly up to 60% of T-ALL patients [2]. Moreover, clinical statistical data revealed that TAL1-positive T-ALL patients commonly displayed a particularly poor prognosis as compared to TAL1-negative T-ALL patients [7, 8]. In physiologic conditions, TAL1, encoded a class II basic helix-loop-helix (bHLH) TF, not only lies at the apex of the hierarchy of TFs involved in hematopoietic specification, but it is also required for terminal maturation of select blood lineages [9]. Nevertheless, the regulatory mechanism of TAL1 in pathogenesis of T-ALL has not yet been fully elucidated.

MicroRNAs (miRNAs), a family of short (approximately 19~24 nt in length), non-coding RNAs, could negatively the expression of target genes by base pairing with target mRNAs in the 3' untranslated region (3'-UTR), ultimately leading to mRNA cleavage or translational repression [10]. In the recent years, emerging evidence has demonstrated that miRNAs play multiple roles in the occurrence, development, and progression of T-ALL [11]. For example, miR-204 inhibited cell proliferation, migration, and invasion of T-ALL cell lines by down-regulating SOX4 [12]. miR-101 regulated T-ALL progres-

sion and chemotherapeutic sensitivity by targeting Notch1 [13]. Therefore, in-depth explorations in miRNAs might provide a potential biomarker for T-ALL diagnosis and a novel therapeutic target for T-ALL intervention [14].

Currently, it has been reported that TAL1 could direct target miRNAs, which in turn involved in pathogenesis of T-ALL [15]. For instance, TAL1-mediated up-regulation of miR-223 promoted the malignant phenotype in T-ALL through repression of the FBXW7 tumor suppressor [16]. Thus, to identify a TAL1-dependent miRNA may be a new strategy for T-ALL treatment in the future. It was discovered that miR-149* was highly expressed in T-ALL cell lines and T-ALL patients' bone marrow samples. Moreover, miR-149*, served as an oncogenic molecule, enhanced cell proliferation, and suppressed apoptosis via modulating JunB in T-ALL [17]. Additionally, we also previously found that TAL1 might be an upstream regulator of miR-149*. Hence, in the present study we attempted to disclose the TAL1/miR-149* link and the effects of TAL1 and miR-149* on T-ALL.

Materials and methods

Cell culture and transfection

Cell lines, including human T lymphocyte line Kit225, human T cell lymphoblast-like cell line Jurkat, T-ALL cell line Molt-4, human Burkitt's lymphoma cell line Raja, and human acute monocytic leukemia cell line THP-1, purchased from American Type Culture Collection (ATCC), were maintained in Roswell Park Memorial Institute (RPMI)-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 2 mM L-glutamine and antibiotics (100 U/mL penicillin G and 100 mg/mL streptomycin) at 37°C in a 95% air and 5% CO_2 incubator and passaged between every 2 and 3 days. Additionally, 293T cells, similarly obtained from ATCC, were cultivated in Dulbecco's modified eagle medium (DMEM; Corning, USA) with the addition of 10% FBS, 2% L-glutamine and 1% penicillin/streptomycin in a 37°C humidified atmosphere with 5% CO_2.

TAL1 over-expression and knockdown plasmids, as well as miR-149* mimic and miR-149* inhibitor, were designed and synthesized from Sangon Biotech, Shanghai, China. Logarithmically growing Jurkat and Molt-4 cells (2×10^5 cells/well) were plated in 6-well sterile plastic culture plates and transfected with TAL1 overexpression and knockdown plasmids, respectively, with FuGENE® HD Transfection Reagent (Promega, USA) according to the manufacturer's instructions. After 48 h of transfection, cells were collected for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and Western blotting (WB) analysis. Moreover, Jurkat cells transfected with TAL1 overexpression plasmid and miR-149* mimic, and Molt-4 cells transfected with TAL1 knockdown plasmid and miR-149* inhibitor similarly using FuGENE® HD Transfection Reagent, were harvested for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cell cycle and apoptosis examination.

qRT-PCR analysis

The above mentioned 5 cell lines, plus 2 transfected strains cells, were collected and total RNA were extracted using Trizol Reagent (TIANGEN, China) in accordance with the manufacturer's recommendations. The isolated RNA was cleared of contaminating genomic DNA by DNase treatment (Thermo, USA) and then reverse transcribed into complementary DNA (cDNA) with an M-MLV reverse transcriptase Kit (Promega, USA) following the manufacturer's guidelines. The amplifications of PCR were performed with commercially available a SYBR Green qPCR SuperMix Kit (Invitrogen, USA) on ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, USA). PCR primers used were as follows: TAL1 forward, 5'-GTGGAGATCCTATTCAGAGGG-3', reverse, 5'-GCGACGCCGTTCAGCAGGAC-3'; 18S rRNA forward, 5'-CCTGGATACCGCAGCTAGGA-3', reverse, 5'-GCGGCGCAATACGAATGCCCC-3'. The relative expression of TAL1 normalized to the 18S rRNA internal control was determined by the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Whole cell lysates were prepared using ice-cold radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime, China) containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 100 mM phenylmethanesulfonyl fluoride (PMSF) and 10 μl protease inhibitor cocktail. The supernatant was harvested after centrifuging at 12000 rpm

for 10 min at 4°C and the concentrations of protein were quantified by a BCA protein assay kit (Beyotime, China) following the manufacturer's protocols. An equal amount of denatured protein (20 μg) was added per lane, separated by SDS-polyacrylamide gels for electrophoresis (SDS-PAGE) on an 8~10% gel and transferred onto polyvinylidene difluoride (PVDF) membranes. Following blocking with 5 % skim milk in Tris-buffered saline with 0.1% Tween-20 (TBST) for 2 h at room temperature with a gentle shaking, PVDF membranes were sequentially incubated with the desired primary antibodies at 4°C overnight at the following dilutions: TAL1 (1:1500; Abcam, USA) and GAPDH (1:2000, as an endogenous control; Abmart, USA). The next day, after 3 extensive washes, the membranes were probed with the appropriate secondary antibodies: horseradish peroxidase (HRP)-conjugated goat-anti-rabbit antibody (1:12000 dilution; BOSTER, China) and HRP-conjugated goat-anti mouse antibody (1:12000 dilution; BOSTER, China).

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were conducted according to protocols of the EZ-ChIPTM Chromatin Immunoprecipitation kit (Millipore, USA). Briefly, 1×10^6 Jurkat and Molt-4 cells were cross-linked with 1% (v/v) formaldehyde (Sigma, USA) for 10 min at 37°C and terminated by the addition of 125 mM glycine for 5 min. Subsequently, crossed-linked cells were washed extensively with PBS, lysed in 1.2 ml lysis buffer for 30 min on ice (50 mM HEPES-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, pH 8.0, 1% Triton X-100, 0.1% Sodium Deoxycholate, 1% SDS, protease inhibitors) and sonicated by a bioruptor sonification device (Diagenode, Belgium) resulting in fragments with an average of 200~400 bp length. Thereafter, chromatin extracts were diluted 5-fold with dilution buffer, pre-cleared with Protein-A/G-Sepharose beads, and immunoprecipitated with specific anti-TAL1 antibody or IgG (as a control) on Protein-A/G-Sepharose beads at 4°C on a rotating wheel. One sample was spared for input control. The precipitates were washed sequentially with buffers, including low salt wash buffer, high salt wash buffer, LiCl wash buffer, and TE buffer, on a rotating wheel at 4°C and then antibody-DNA complexes were eluted using elution buffer (1% SDS, 100 mM $NaHCO_3$) at room temperature for 15 min with shaking. The eluates were heated for 5~6 hours at 65°C to dissociate the cross-linking. Finally, the ChIP-DNA was extracted and purified by phenol: chloroform extraction and ethanol precipitation, followed by a traditional PCR detection. Primer pairs used for ChIP-PCR were as follows: miR-149* promoter forward, 5'-CCAGCGGCATCCATACGAGC-3', reverse, 5'-AGGACATCCTCTGTCTCCTTGGTC-3'. The PCR products were run in 3% agarose gel electrophoresis and visualized with ethidium bromide staining.

miR-149 promoter luciferase activity reporter assay*

The predicted promoter binding sites and mutant sites of miR-149* interacted with TAL1 were amplified and cloned in the luciferase vector pGL3 (Promega, USA) to form two constructs of miR-149* and mutant-miR-149*. Jurkat and Molt-4 cells (1×10^5/well of a six-well plate) were transiently transfected with miR-149*, mutant-miR-149*, and pGL3 vectors together with NC plasmid, TAL1 mimic, TAL1 inhibitor the by use of the FuGENE® HD Transfection Reagent. Forty-eight hours later, cells were ultimately lysed and analyzed by a Dual luciferase assay (Promega, USA) in accordance with the manufacturer's protocols and data expressed as ratios of renilla to firefly luminescence in the experimental cell extracts normalized to control cell extracts.

Cell proliferation examination

Cell proliferation was measured by a MTT kit (Promega, USA) according to the manufacturer's guidelines. The treated Molt-4 cells were digested by 0.25% trypsin (Sigma, USA) and seeded in 96-well plates at a density of 1×10^4 per well. Subsequently, at indicated incubation time (0 day, 1 day, 2 day, 3 day, and 4 day), 50 μl of MTT working solution (5 mg/ml) was added to each well for 4 h incubation at 37°C. The reaction was stopped with the addition of solubilization buffer (i.e., 10% dimethylsulfoxide (DMSO) in 0.01 M HCl). After continuous shaking at room temperature for 15 min, the absorbance of the culture medium was read at a wavelength of 490 nm using a scanning microplate spectrophotometer (Thermo Fisher, USA).

Flow cytometry

Cell cycle and apoptosis were examined with a Cell Cycle and Apoptosis Analysis Kit (Beyotime, China) following the manufacturer's instruc-

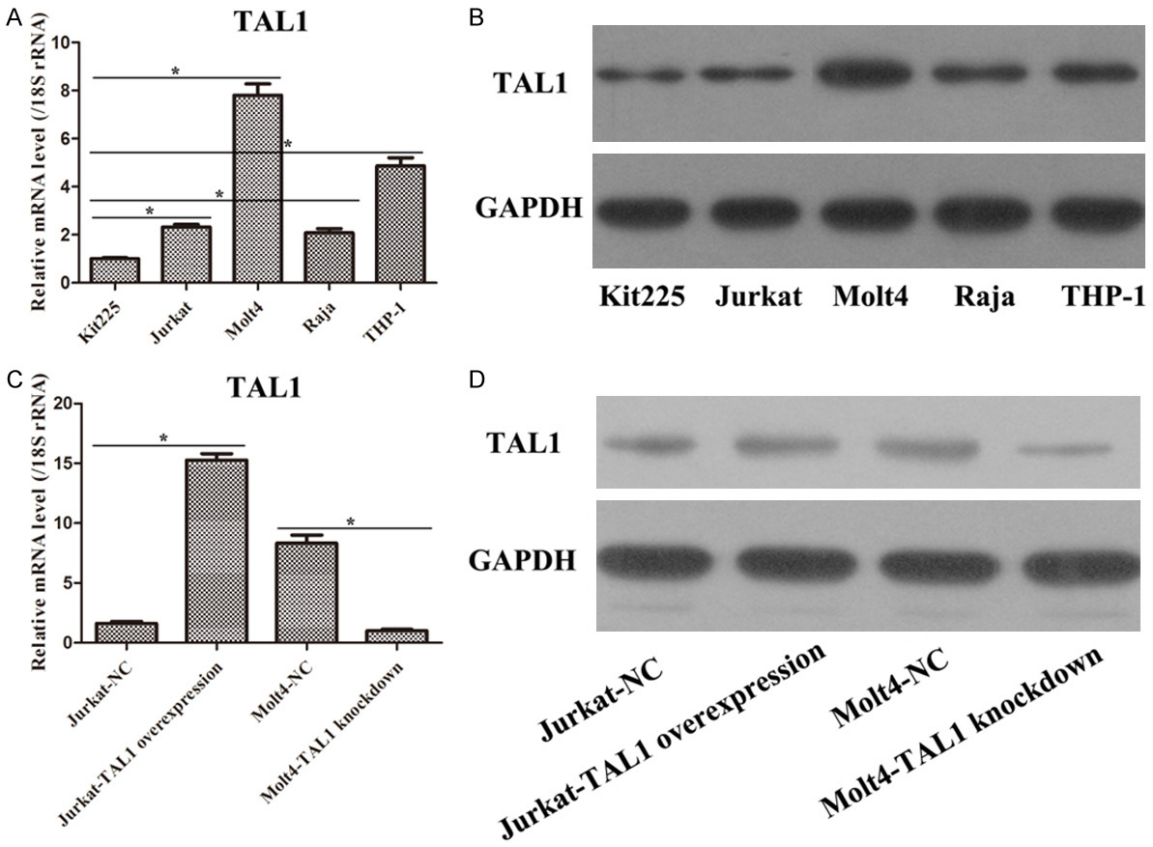

Figure 1. TAL1 expression at the mRNA and protein levels. A. TAL1 mRNA expression was determined by qRT-PCR in five cell lines. B. TAL1 protein expression was measured by WB in five cell lines. C. TAL1 mRNA expression was detected by qRT-PCR in Jurkat and Molt-4 cells transfected with TAL1 overexpression and knockdown plasmids, respectively. D. TAL1 protein expression was tested by WB in Jurkat and Molt-4 cells transfected with TAL1 overexpression and knockdown plasmids, respectively.

tions. For cell cycle analysis, the harvested cells were washed with phosphate-buffered saline (PBS) twice and fixed in 70% ethanol at -20°C overnight. Then, cells were stained with 500 μl propidium iodide (PI) (50 μg/ml) solution containing 100 μg/ml RNase A and 0.2% Triton X-100 away from light for 10 min at room temperature. DNA content and cell number were determined using a FACSCalibur cytometer (BD Biosciences, USA). Nevertheless, for apoptosis analysis, the collected cells were similarly washed with PBS twice and resuspended in Annexin V binding buffer. Next, 10 μl of freshly prepared Annexin V-PI mixed reagent was added into cell suspension. Following pipette up and down, the cells were incubated in the dark for 10 min at 37°C. Eventually, fluorescence was measured using a FACSCalibur flow cytometer within 30 min. The cell cycle distribution was calculated from 10000 cells using the ModFit 3.3 program, while the apoptosis cells were analyzed by CellQuest Pro software.

Statistical analysis

All statistical calculations were performed using SPSS 18.0 software (IBM SPSS, USA). All data represent at least three independent experiments and are expressed as the means ± standard deviation (SD). Statistical differences between groups were evaluated using two-tailed student-t test and one-way analysis of variance (ANOVA), as appropriate. Significance was set for $P < 0.05$.

Results

Comparisons of TAL1 expression among the five cell lines

qRT-PCR and WB were performed to measure TAL1 mRNA and protein expressions, respectively, in five cell lines. It was found that TAL1 expression in mRNA and protein levels in the four tumor cell lines was apparently increased as compared to Kit225 cells. Moreover, in the

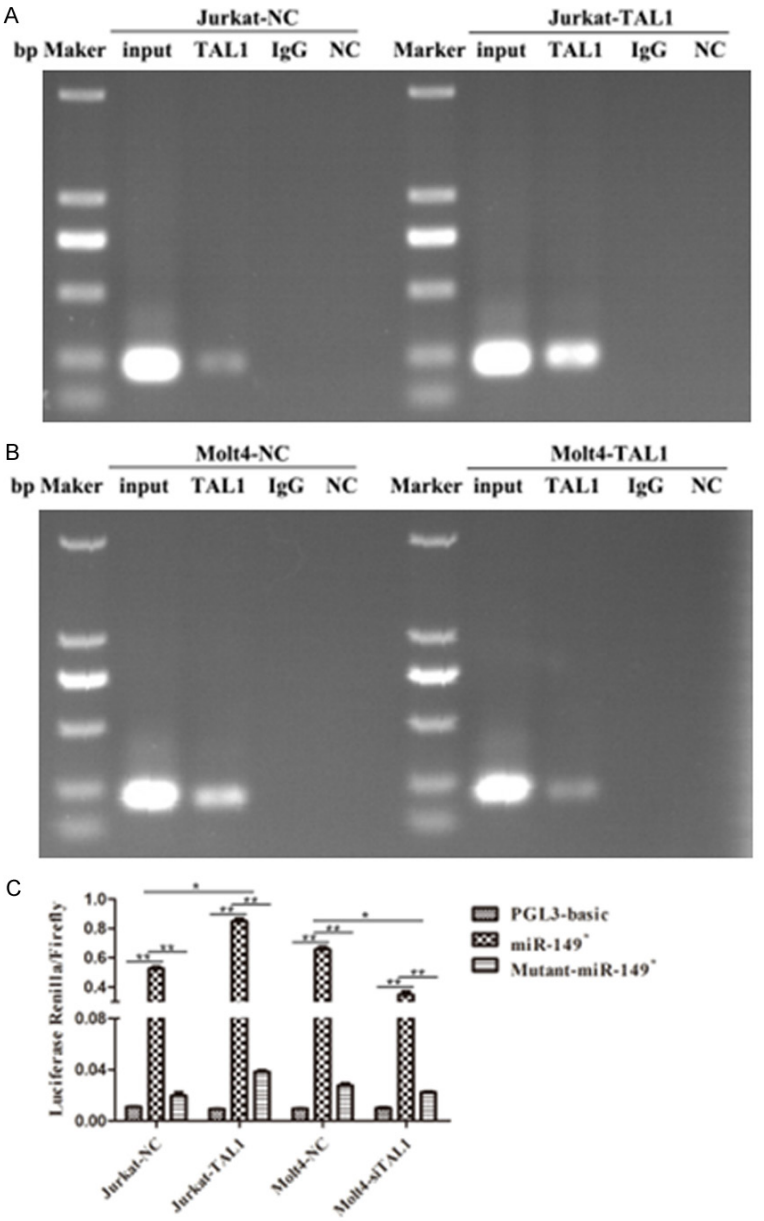

Figure 2. TAL1 directly regulates miR-149* expression through interacting with the promoter of miR-149*. A. ChIP with anti-TAL1 followed by PCR with miR-149* promoter primers in TAL1-overexpressed Jurkat cells. B. ChIP with anti-TAL1 followed by PCR with miR-149* promoter primers in TAL1-knockdown Molt-4 cells. C. miR-149* promoter luciferase activity assay in TAL1-overexpressed Jurkat cells and TAL1-knockdown Molt-4 cells. *$P < 0.05$ or **$P < 0.01$.

four tumor cell lines, the highest TAL1 expression in mRNA and protein levels were detected in Molt4 cells, while the lowest TAL1 expression in mRNA and protein levels were tested in Jurkat cells (**Figure 1A** and **1B**). Therefore, Molt-4 and Jurkat cells were selected to apply in the subsequent experiments. Next, TAL1 overexpression and knockdown plasmids were transfected into Jurkat and Molt-4 cells, respectively. Moreover, it was verified that the mRNA and protein expression levels of TAL1 in Jurkat cells with TAL1 overexpression treatment were increased, while the mRNA and protein expression levels of TAL1 in Molt-4 cells with TAL1 knockdown treatment were decreased (**Figure 1C** and **1D**).

*TAL1 directly binds with the promoter of miR-149**

To elucidate whether TAL1 altered the expression of miR-149* in T-ALL, we first predicted the binding sites between TAL1 and miR-149* by a TransmiR online software. It was revealed that TAL1 could bind the promoter domain of miR-149*. Then, we carried out ChIP using a TAL1 specific antibody, followed by PCR with miR-149* promoter primers in Jurkat and Molt-4 cells subjected to TAL1 and si-TAL1 transfection, respectively. Our data show that enhanced expression of TAL1 in Jurkat cells increased the miR-149* expression as compared to Jurkat-NC group (**Figure 2A**), whereas silencing of TAL1 in Molt-4 cells apparently decreased miR-149* expression in compared with Molt-4-NC group (**Figure 2B**). Thus, these data suggested that TAL1 might regulate miR-149* expression via interacting with the promoter domain of miR-149*, which was further demonstrated by a miR-149* promoter luciferase activity report assay (**Figure 2C**).

The effects of TAL1 and miR-149 on cell proliferation, cell cycle, and apoptosis in Molt-4 cells*

Based on the above results, we adopted different treatments methods to evaluate the cell

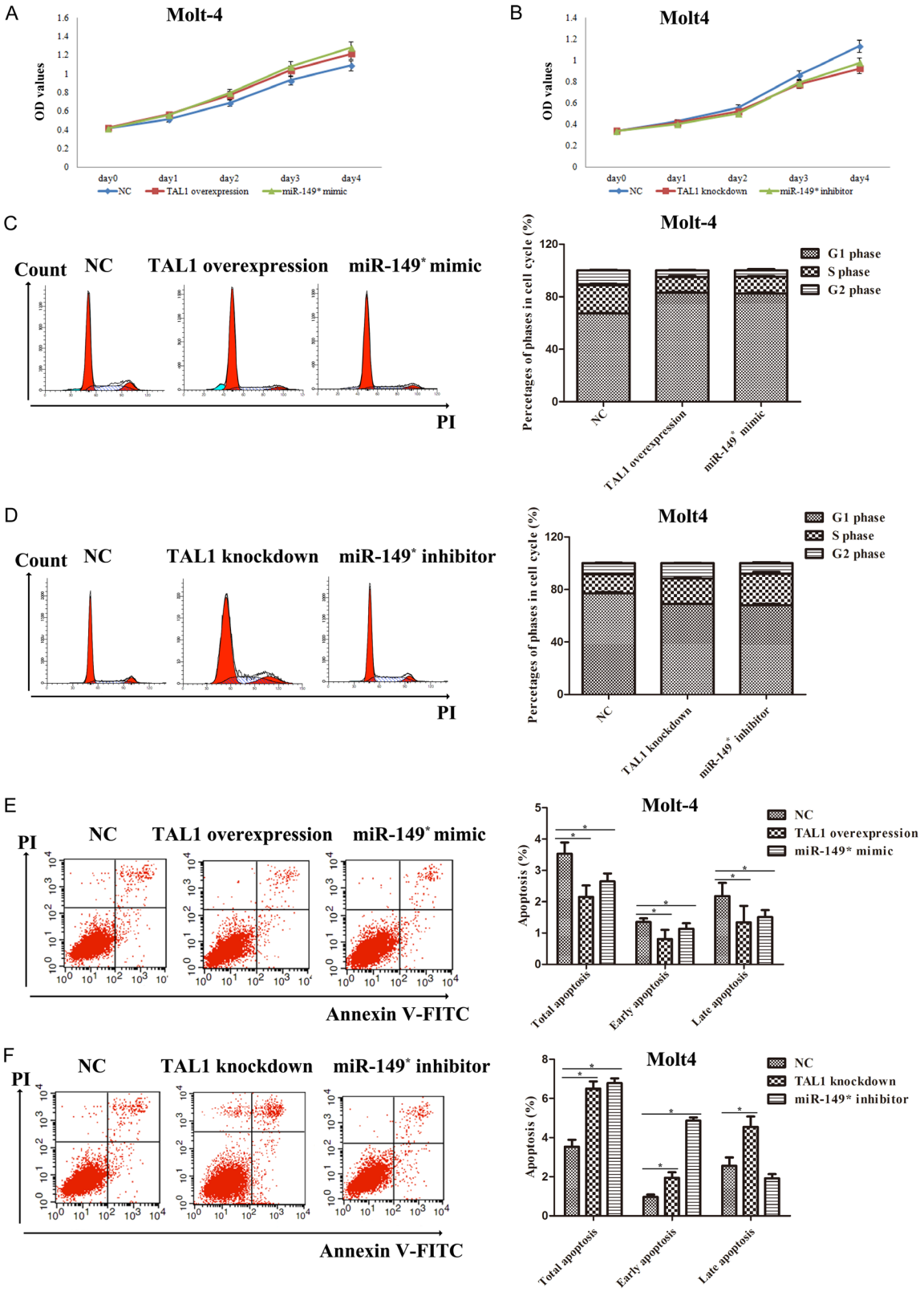

Figure 3. TAL1 and miR-149* impacts cell proliferation, cell cycle, and apoptosis in Molt-4 cells. A. Increased expression of TAL1 and miR-149* promoted Molt-4 cell proliferation, as determined by MTT assay. B. Decreased expression of TAL1 and miR-149* inhibited Molt-4 cell proliferation, as determined by MTT assay. C. Up-regulation of TAL1 and miR-149* caused Molt-4cell cycle arrest in G0/G1 phase examined by flow cytometry. D. Down-regulation of TAL1 and miR-149* reversed Molt-4 cell cycle arrest in G0/G1 phase examined by flow cytometry. E. Elevated TAL1 and miR-149* suppressed apoptosis measured by flow cytometry. F. Reduced TAL1 and miR-149* enhanced apoptosis measured by flow cytometry.

proliferation, cell cycle, and apoptosis in Molt-4 cells. As illustrated in **Figure 3**, in Molt-4 cells, it was found that restored TAL1 and miR-149* could promote cell proliferation, induced cell cycle arrest in G0/G1 phase, and inhibited apoptosis. On the contrary, in Molt-4 cells, it was uncovered that downregulation of TAL1 and miR-149* could suppress cell proliferation, abolish cell cycle arrest in G0/G1 phase, and accelerate apoptosis. Taken together, these findings implied that TAL1 and miR-149* might be involved in development of T-ALL by changing cell proliferation, cell cycle, and apoptosis.

Discussion

T-ALL is a heterogeneous group of hematological tumors resulting from the oncogenic transformation of thymocytes at distinct stages of differentiation [3]. Moreover, it is still a significant health burden worldwide that commonly affects late childhood, adolescence, and young adulthood [18]. In spite of remarkable improvements in therapeutic strategies, the overall of survival rates in T-ALL patients have been limited owing to frequent relapse and remote metastasis [13]. The oncogenic transcription factor TAL1 is aberrantly expressed in more than 60% of cases of T-ALL and was first described in T-ALL over 20 years ago. However, the detailed mechanisms through which TAL1 is able to transform thymocytes have not been fully illustrated [19, 20]. In the current study, our data display that the expression levels of TAL1 in four tumor cell lines are higher than those in Kit225 cells, meanwhile the expression levels of TAL1 in Jurkat cells are the lowest, and those in Molt-4 cells are the highest among the four tumor cell lines. Thus, Jurkat cells were chosen to transfect with TAL1 overexpression plasmid, while Molt-4 cells were selected to transfect with TAL1 knockdown plasmid for the following study. Additionally, it is now known that TAL1 regulates the expression of numerous miRNA genes [15], which have been identified to be major post-transcriptional regulators implicated in the pathogenesis of T-ALL [14]. For example, TAL1 overexpression inhibited miR-146b-5p expression, which then promoted T cell developmental arrest during leukemogenesis [15]. miR-223 which was confirmed to be a direct target of TAL1 could function as a tumor suppressor in ALL [16]. Therefore, we also focused on the TAL1/miRNA target link. Previous studies demonstrated that miR-149* was apparently dysregulated in various primary human cancers, including T-ALL [17]. Furthermore, it was predicted that TAL1 could direct mediate miR-149* by binding the promoter of miR-149*. Next, the target interaction between TAL1 and miR-149* was validated by ChIP and luciferase report assays.

In order to further clarify the biological functions of TAL1 and miR-149*, we sequentially examined cell proliferation, cell cycle, and apoptosis. Cell proliferation, regulated by multiple molecular pathways, is one of the most important malignant characteristics in cancer, and abnormal pattern of cell proliferation is a dominant obstacle during the treatment of cancer [21]. For example, miR-543 suppressed breast cancer cell proliferation though directly repressing downstream factors of mitogen-activated protein kinase/extracellular signal-regulated kinase-2 (MAPK/ERK2) pathway, which ultimately leaded to tumor rapidly growth [22]. In our results, it was exhibited that enforced TAL1 and miR-149* in Molt-4 cells increased cell proliferation, but inhibited TAL1 and miR-149* in Molt-4 cells decreased cell proliferation, implying that TAL1 and miR-149* could accelerate tumor growth. In addition, cell growth is also strictly regulated by cell cycle and apoptosis, which not only were identified to be important processes in occurrence and development of cancer [23], but also could be mediated by miRNAs in the progression of cancer [24, 25]. For instance, miR-155 improved cell cycle progression and impeded apoptosis in hepatocellular carcinoma by targeting AT-rich interactive domain 2 (ARID2) [26]. In our data, it was observed that elevated TAL1 and miR-149* in Molt-4 cells up-regulated the proportion of cells in G1 phase and reduced apoptosis, whereas inhibition of TAL1 and miR-149* in Molt-4 cells down-regulated the percentage of cells in G1 phase and facilitated apoptosis, further indicating that TAL1 and miR-149* could promote tumor growth.

Conclusion

In conclusion, the present study reveals two important points: (1) It was validated that there was a target interaction between TAL1 and miR-149*; (2) TAL1 and miR-149* elevated cell proliferation, increased cell cycle arrest in G0/G1 phase, and hindered apoptosis. This novel TAL1/miR-149* axis may provide new insights into the mechanisms underlying tumor growth and inhibition of TAL1 and/or miR-149* may be

a potential therapeutic strategy for the treatment of T-ALL in the future.

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Disclosure of conflict of interest

None.

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