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MicroRNA-370-3p inhibits cell proliferation and induces chronic myelogenous leukemia cell apoptosis by suppressing PDLIM1/Wnt/β-catenin signaling

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Growing evidence has suggested that microRNA-370-3p (miR-370-3p) is downregulated and acts as a suppressor in several cancers. However, the role of miR-370-3p in chronic myeloid leukemia (CML) remains unknown. Here, the expression level and molecular mechanism of miR-370-3p in CML were investigated. Firstly, the expression of miR-370-3p has markedly decreased in the peripheral blood mononuclear cells (PBMCs) of patients with CML and in cell lines. Moreover, miR-370-3p in CML cells upregulated and downregulated proliferation and apoptosis, respectively. Notably, miR-370-3p directly targeted the 3'-untranslated region of PDZ and LIM domain protein 1 (PDLIM1). A negative correlation was observed between the levels of miR-370-3p and PDLIM1 in the PBMCs of patients with CML and healthy volunteers. PDLIM1 was shown to have an oncogenic role in CML cells by promoting proliferation and suppressing apoptosis. Finally, the miR-370-3p-PDLIM1-Wnt/β-catenin signaling axis was indicated to play an important role in CML progression.

Key words: miR-370-3p, CML, PDLIM1, proliferation, apoptosis, Wnt/β-catenin signaling

Chronic myeloid leukemia (CML) is a malignant clonal disease that occurs due to a disorder in the hematopoietic stem cells and is characterized by the production of large numbers of immature white blood cells [1]. The Philadelphia (Ph) chromosome is a genetic, molecular and biological feature of CML; moreover, the newly fusion gene *BCR–ABL*, which shows inherent tyrosine kinase activity, is considered crucial in the pathogenesis of CML [2]. Although the 5-year survival rate of patients with CML significantly increased owing to the use of imatinib, which is a small-molecule inhibitor of *BCR–ABL*, patients with imatinib resistance continue to have an extremely poor prognosis [3–6]. Therefore, exploring the molecular mechanisms underlying CML pathogenesis may provide insights for discovering alternative therapeutic strategies or diagnostic biomarkers.

MicroRNAs (miRNAs) are a group of small noncoding RNAs with a length of 18–25 nucleotides that can suppress target gene expression by binding to the 3'-untranslated region (3'UTR) of target mRNAs [7–9]. miRNAs can act as prognostic biomarkers or play crucial roles in regulating multiple biological processes in several human diseases including cancers [10–13]. Although many studies have conducted to study the role of miRNAs, the biological functions of numerous miRNAs remain unclear. A recent report has shown that miR-370-3p functions as a tumor suppressor by inhibiting cell growth, cycle and migration and by inducing apoptosis in or invading several cancers, such as gliomas [14], ovarian cancer [15], bladder cancer [16], colorectal cancer [17] and thyroid cancer [18]. However, the role of miR-370-3p in CML remains unclear.

In this study, we showed that miR-370-3p was downregulated in the PBMCs of patients with CML and CML cell lines. Furthermore, miR-370-3p acted as a tumor suppressor by significantly inhibiting cell proliferation and promoting apoptosis in CML cells. Importantly, we found that miR-370-3p inhibited Wnt/β-catenin signaling by downregulating PDLIM1. These results indicate that miR-370-3p is a novel diagnostic biomarker or a potential therapeutic target in CML.

Materials and methods

Blood samples. Blood samples from 50 patients with CML and 50 healthy volunteers were collected at the Department of Clinical Laboratory, Zhuji People's Hospital of Zhejiang Province, between January 2014 and March 2019. The ethics committee of the Zhuji People's Hospital of Zhejiang Province approved the study. Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy volunteers, and CML cells were isolated from patients using Ficoll

Plus™ 1.077 (Solarbio, Beijing Solarbio Technology Co. Ltd, China) according to the manufacturer's instructions.

Cell culture and transfection. CML cell lines (K562, KCL-22, and KU812) and HEK 293T cells were obtained from the American Type Culture Collection. K562 and KCL-22 cells were cultured in the Roswell Park Memorial Institute-1640 (RPMI-1640; Sigma-Aldrich, USA) medium along with 10% (v/v) fetal bovine serum (Gibco, Life Technologies, USA) and 100 mg/ml penicillin/ streptomycin as supplements.

CML cells were transiently transfected with miR-370-3p mimics, miR-370-3p inhibitor and negative control (GENEWIZ, Suzhou, China) and wild type (wt) and mutant type (mut) PDLIM1 3'UTR luciferase reporter vector (pmir/luc-PDLIM1 3'UTR and pmir/luc-PDLIM1 3'UTR mut, respectively) using the Lipofectamine 2000 reagent (Thermo Fisher Scientific).

RNA isolation and real-time quantitative polymerase chain reaction (RT-qPCR). Total RNA from PBMCs and CML cells was extracted using the TRIzol Reagent (Thermo Fisher Scientific, USA). The miRNA-specific TaqMan MiRNA Assay Kit was used to quantify miR-370-3p (ID 002275, Thermo Fisher Scientific, USA) using U6 snRNA (ID 001973, Thermo Fisher Scientific, USA) as an internal control. For quantifying mRNA levels, RT-qPCR was performed using the SYBR Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, USA) and was normalized by using glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data were analyzed using the $2^{-\Delta\Delta Ct}$ method.

Cell counting kit-8 (CCK-8) assay. The CCK-8 (Sigma-Aldrich, USA) assay was used to measure the activity of CML cells. Briefly, K562 and KCL-22 cells were transfected with the indicated miRNAs or vectors and cultured for 24 h. Subsequently, K562 and KCL-22 cells were seeded in 96-well culture plates at a density of 3×10^3 cells/well. 100 µl RPMI-1640 medium (serum-free) containing 10% CCK-8 reagent (v/v) was added to each well at 48, 72, and 96 h after transfection. The cells were incubated for 1 h at 37 °C under dark conditions. The Quant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Winooski, USA) was used to measure the absorbance at 450 nm.

Flow cytometry analysis. Cells were washed and resuspended in binding buffer. The apoptosis assay was analyzed using Annexin V-fluorescein isothiocyanate as previously reported [19, 20].

Dual-luciferase reporter assay. 3'UTRpmir/luc-PDLIM1 3'UTR and 3'UTRpmir/luc-PDLIM1 3'UTR mut were constructed using GENERAL BIOSYSTEMS (Anhui) Co., Ltd (Chuzhou, China). These reporter vectors contained a firefly luciferase gene upstream of PDLIM1-3'UTR or PDLIM1-3'UTR mut. The miR-370-3p mimics or miR-370-3p inhibitor and their negative control (NC mimics or NC inhibitor) were co-transfected with pmir/luc-PDLIM1 3'UTR or pmir/luc-PDLIM1 3'UTR mut and a pRL Renilla luciferase control reporter vector (pRL-CMV, Promega Corporation, Beijing) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, USA) according to the manufacturer's instructions. The relative firefly/Renilla luciferase activity was measured using a dual-luciferase reporter assay kit (Promega Corporation, Beijing) at 48 h after transfection.

TOP/FOP flash reporter assays. TOP/FOP flash reporter assays were conducted according to the previously reported method [21].

Western blot. The RIPA lysis buffer (Sigma-Aldrich, USA) was used for the extraction of the proteins from cells; the concentration of proteins was quantified using the BCA Protein Assay Kit (Sigma-Aldrich, USA). Protein samples were first separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto 0.45 nm polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, USA). Subsequently, relevant primary antibodies were added to the PVDF membranes at 4 °C and incubated overnight. Subsequently, horseradish peroxidase-labeled secondary antibodies were added to the PVDF membranes at room temperature for 2 h. Finally, these proteins were measured using an enhanced chemiluminescence kit (Millipore, USA).

Statistical analysis. The results were presented as means ± standard deviation. Statistical significance was determined using the Student's t-test and one-way analysis of variance. Values of $p<0.05$ were considered to be statistically significant.

Results

Downregulation of miR-370-3p in the PBMCs of patients with CML. First, the expression levels of miR-370-3p in K562 cells, in PBMCs of healthy volunteers and patients with CML were assessed using RT-qPCR. As shown in Figure 1A, the expression of miR-370-3p markedly decreased in PBMCs from CML patients compared with healthy volunteers. Further, the expression levels of miR-370-3p were evaluated in the PBMCs of healthy volunteers and K562, KCL-22 and KU812 cells. Results showed that miR-370-3p was downregulated in CML cells compared to PBMCs (Figure 1B).

miR-370-3p inhibited the proliferation and promoted apoptosis of CML cells. To further confirm the role of miR-370-3p in CML, miR-370-3p mimics, miR-370-3p inhibitor and their blank controls were transiently transfected into K562 and KCL-22 cells. Verifying the levels of miR-370-3p in these cells using RT-qPCR revealed that the expression of miR-370-3p markedly increased after transfection with miR-370-3p mimics, whereas the expression of endogenous miR-370-3p markedly reduced after transfection with miR-370-3p inhibitor in K562 and KCL-22 cells (Figure 2A). We also observed that the proliferation of miR-370-3p-transfected cells was markedly inhibited in K562 and KCL-22 cells, whereas the transfection with the miR-370-3p inhibitor increased the proliferation of these cells based on the results of the CCK-8 assay (Figure 2B).

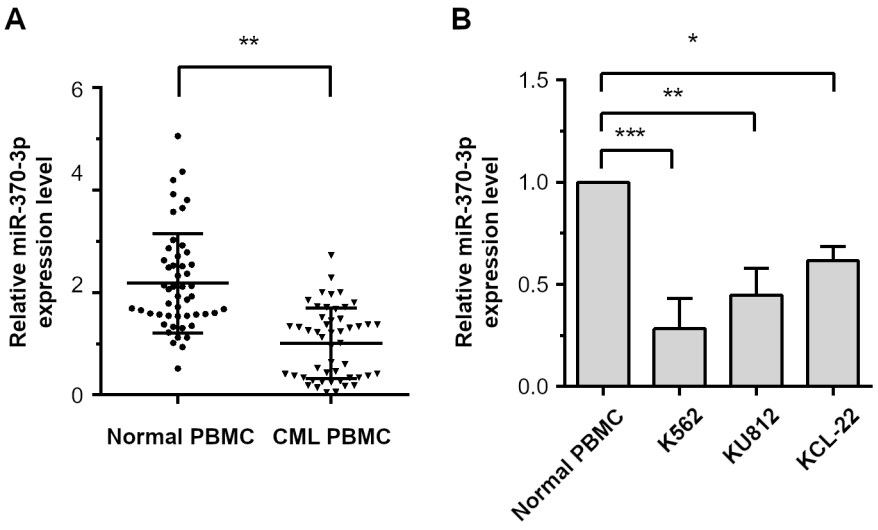

Figure 1. Downregulation of miR-370-3p in the PBMCs of patients with CML and CML cell lines. A) RT-qPCR assay shows the expression of miR-370-3p in the PBMCs of healthy volunteers and patients with CML. B) The expression of miR-370-3p in 293T and CML cells.

The status of apoptosis in these cells, determined using flow cytometry, revealed that the upregulation of miR-370-3p increased cell apoptosis and that the blockage of endogenous miR-370-3p in CML cells decreased apoptosis (Figures 2C and 2D). Taken together, these results demonstrated that miR-370-3p played a suppressive role in CML cells by suppressing cell proliferation and enhancing cell apoptosis.

miR-370-3p was directly bound to the 3'UTR of PDLIM1. Based on the results of bioinformatics prediction using TargetScan7.2, PDLIM1 was chosen as a candidate target of miR-370-3p, which was predicted to bind to the 3'UTR of PDLIM1 at the 26–32 nts position (Figure 3A). The direct interaction between miR-370-3p and the 3'UTR of PDLIM1 confirmed using luciferase report assays. The results revealed that transfection with miR-370-3p mimics significantly inhibited the luciferase activity of PDLIM1 3'UTR, whereas the downregulation of miR-370-3p showed a reverse outcome (Figure 3B). However, neither overexpression nor blocking of miR-370-3p had a significant effect on the luciferase activity when co-transfected with the mut PDLIM1 3'UTR vector (Figure 3C). Additionally, we found that the endogenous mRNA level of PDLIM1 was obviously increased when miR-370-3p mimics was transfected into K562 and KCL-22 cells, and the endogenous mRNA level of PDLIM1 showed an opposite trend when miR-370-3p inhibitor was transfected (Figure 3D). Moreover, compared with healthy volunteers, in patients with CML, the mRNA level of PDLIM1 in PBMCs markedly increased (Figure 3E). Moreover, we found a negative correlation between the levels of miR-370-3p and PDLIM1 in the PBMCs of patients with CML and healthy volunteers (Figure 3F). Collectively, these results indicated that PDLIM1 was the direct target of miR-370-3p, which could inhibit the expression of PDLIM1 at the post-transcriptional level.

PDLIM1 promoted the proliferation and suppressed apoptosis of CML cells. To further confirm the role of PDLIM1 in CML, we first analyzed the efficacy of PDLIM1-overexpressing or -silencing plasmids in K562 and KCL-22 cells using RT-qPCR. Results demonstrated that the mRNA levels of PDLIM1 were markedly increased following transfection with the PDLIM1-overexpressing plasmid and that transfection with the PDLIM1-knockdown plasmid significantly decreased the mRNA level of endogenous PDLIM1 in K562 and KCL-22 cells (Figure 4A). Transfection with PDLIM1 overexpressing plasmid could markedly inhibit the proliferation of K562 and KCL-22 cells, whereas transfection with PDLIM1 knockdown plasmid significantly increased the proliferation of these cells (Figure 4B). The overexpression of PDLIM1 obviously decreased cell apoptosis rate, whereas the silencing of PDLIM1 enhanced the apoptosis rate in CML cells (Figures 4C and 4D). Altogether, these results suggest that PDLIM1 has an oncogenic role in CML cells by promoting cell proliferation and suppressing cell apoptosis.

Overexpression of PDLIM1 abated the role of miR-370-3p in CML. To further verify that PDLIM1 was an exact functional target of miR-370-3p, we investigated the effect of overexpressing PDLIM1 on CML cells transfected with miR-370-3p mimics. miR-370-3p mimics decreased the proliferation of CML cells, and this effect was partially rescued by the overexpression of PDLIM1 (Figure 5A). Similarly, the overexpression of PDLIM1 was found to partly rescue apoptosis induced by miR-370-3p in CML cells (Figures 5B and 5C). The above results suggested that cell

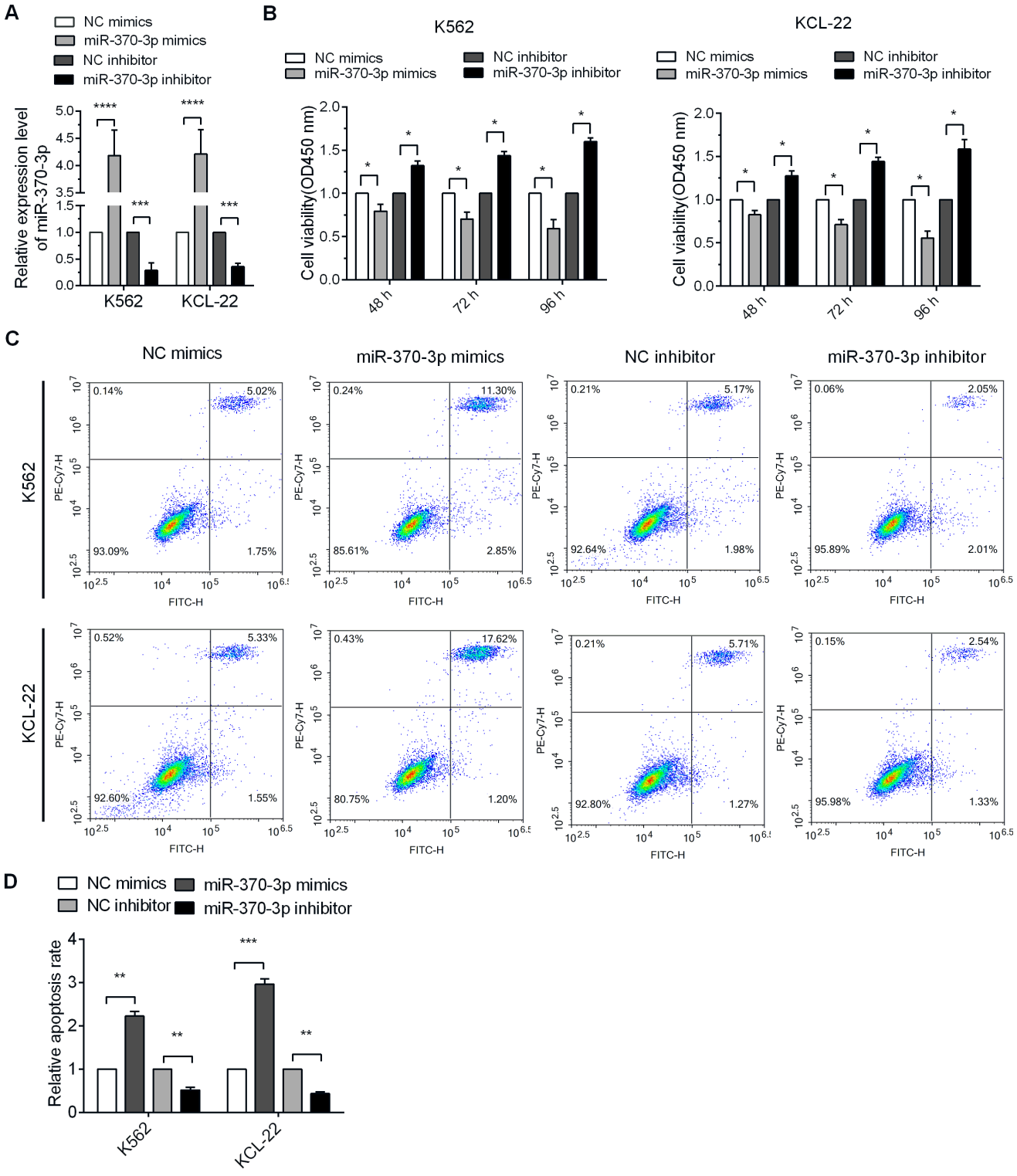

Figure 2. miR-370-3p inhibits the proliferation and promotes the apoptosis of CML cells. A) The expression level of miR-370-3p was detected using RT-qPCR after transfection with miR-370-3p and its inhibitor. B) The proliferative ability of miR-370-3p and its inhibitor on CML cells using CCK-8 assay. C) Flow cytometry analysis was used to detect the apoptosis of CML cells after transfection with miR-370-3p and its inhibitor. D) Quantitative analysis of the relative apoptosis rates from Figure 2C. Experiments were repeated in triplicates. *p<0.05; ***p<0.001; ****p<0.0001.

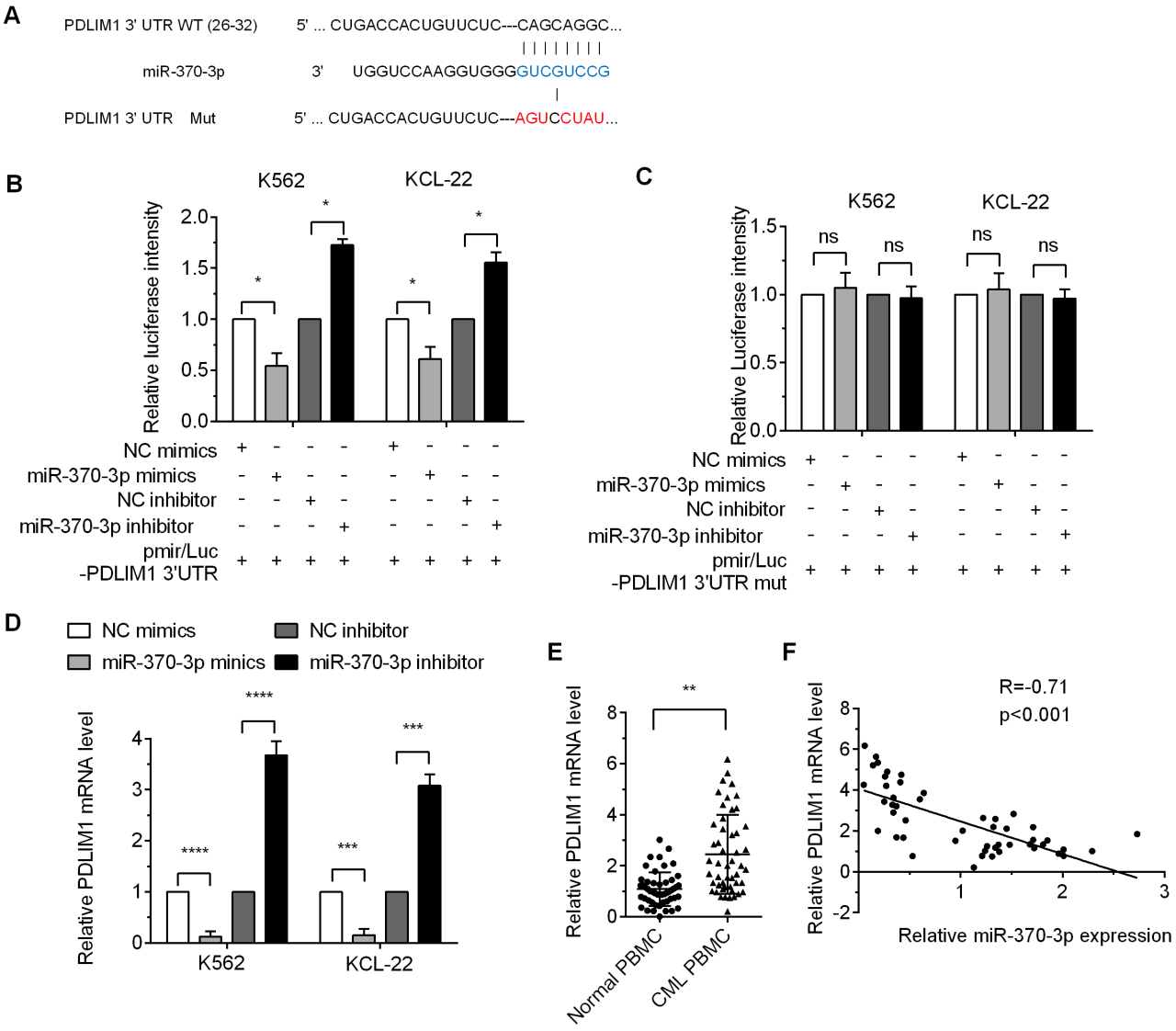

Figure 3. PDLIM1 is the direct target of miR-370-3p. A) Bioinformatics prediction indicated that PDLIM1 was the potential target of miR-370-3p, and the mutational PDLIM1 3'UTR sequence was shown. B and C) Luciferase report system was used to verify the direct interaction between miR-370-3p and the 3'UTR of PDLIM1. D) RT-qPCR detected the mRNA level of PDLIM1 after transfection with miR-370-3p and its inhibitor. E) The mRNA level of PDLIM1 in the PBMCs of patients with CML and healthy volunteers was detected using RT-qPCR. F) Negative correlation between miR-370-3p and PDLIM1 in the PBMCs of patients with CML and healthy volunteers. Experiments were repeated in triplicates. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns, not significant.

proliferation and apoptosis were regulated by miR-370-3p, and these functions induced, by miR-370-3p, partially relied on the expression of PDLIM1 in CML cells.

miR-370-3p inhibited Wnt/β-catenin signaling by targeting PDLIM1. Wingless (Wnt)-β-catenin signaling plays a key role in cancer progression, and β-catenin is involved in the activation of Wnt/β-catenin signaling [22–25]. To further investigate whether miR-370-3p affected the Wnt/β-catenin signaling pathway, we first confirmed the activation of this signaling pathway by using pTOP-flash/pFOP-flash reporter vectors. The results showed that transfection with miR-370-3p mimics markedly inhibited the pTOP-flash/pFOP-flash ratio in K562 and KCL-22 cells, while transfection with miR-370-3p inhibitor significantly increased the pTOP-flash/pFOP-flash ratio in CML cells (Figure 6A), indicating the suppressive activation of Wnt/β-catenin signaling by miR-370-3p. In addition, we observed that the overexpression of PDLIM1 partly rescued pTOP-flash/pFOP-flash ratio induced by miR-370-3p in CML cells (Figure 6B), indicating that miR-370-3p inhibited Wnt/β-catenin signaling, which at

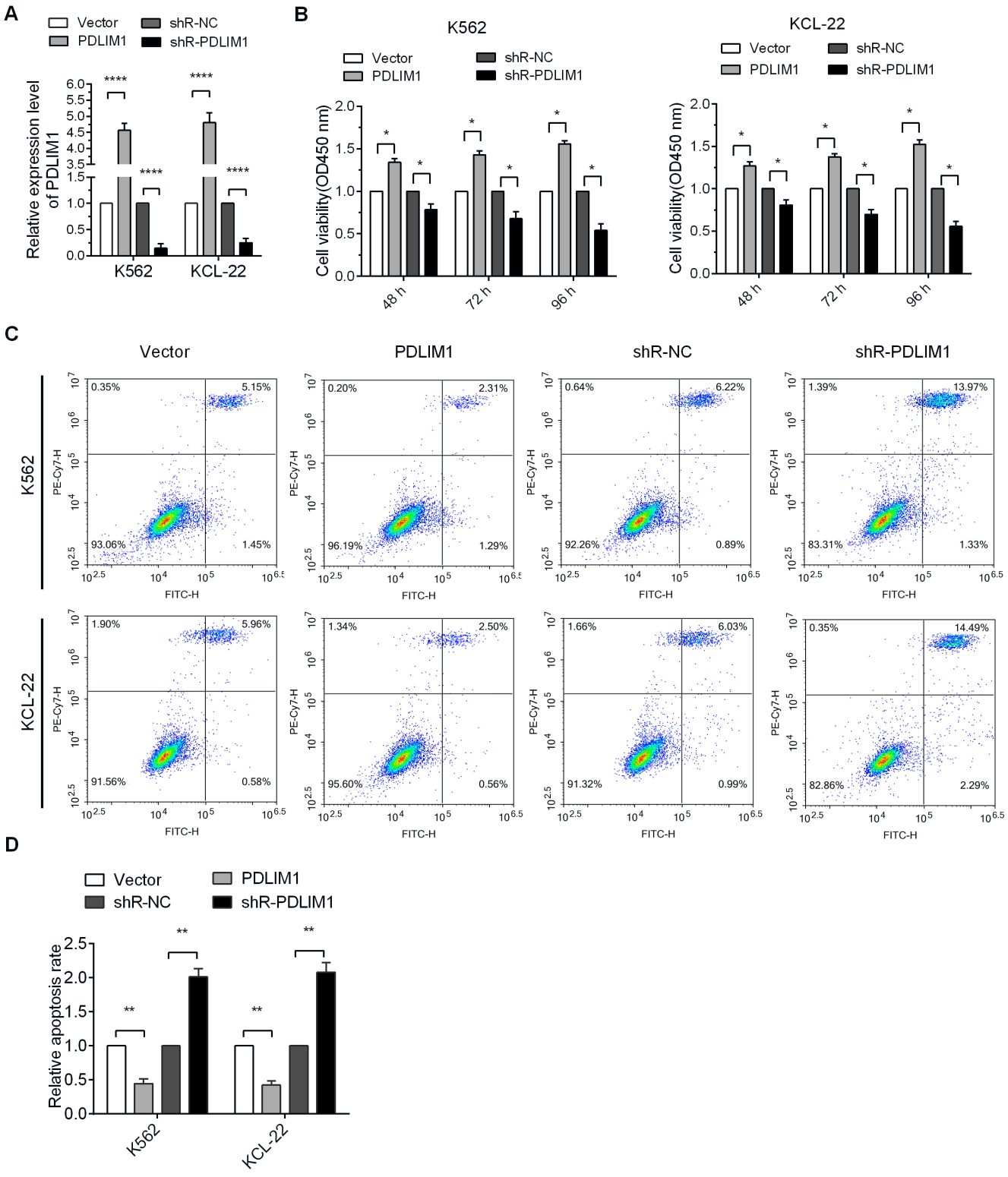

Figure 4. PDLIM1 promotes the proliferation and inhibits apoptosis of CML cells. A) The mRNA level of PDLIM1 was detected using RT-qPCR after transfection with PDLIM1-overexpressing or -silencing plasmids. B) The proliferative ability of PDLIM1 on CML cells was measured by using CCK-8 assay. C) Flow cytometry analysis was used to detect the apoptosis of CML cells after transfection with PDLIM1-overexpressing or -silencing plasmids. D) Quantitative analysis of the relative apoptosis rates from Figure 4C. Experiments were repeated in triplicates. *p<0.05; ****p<0.0001.

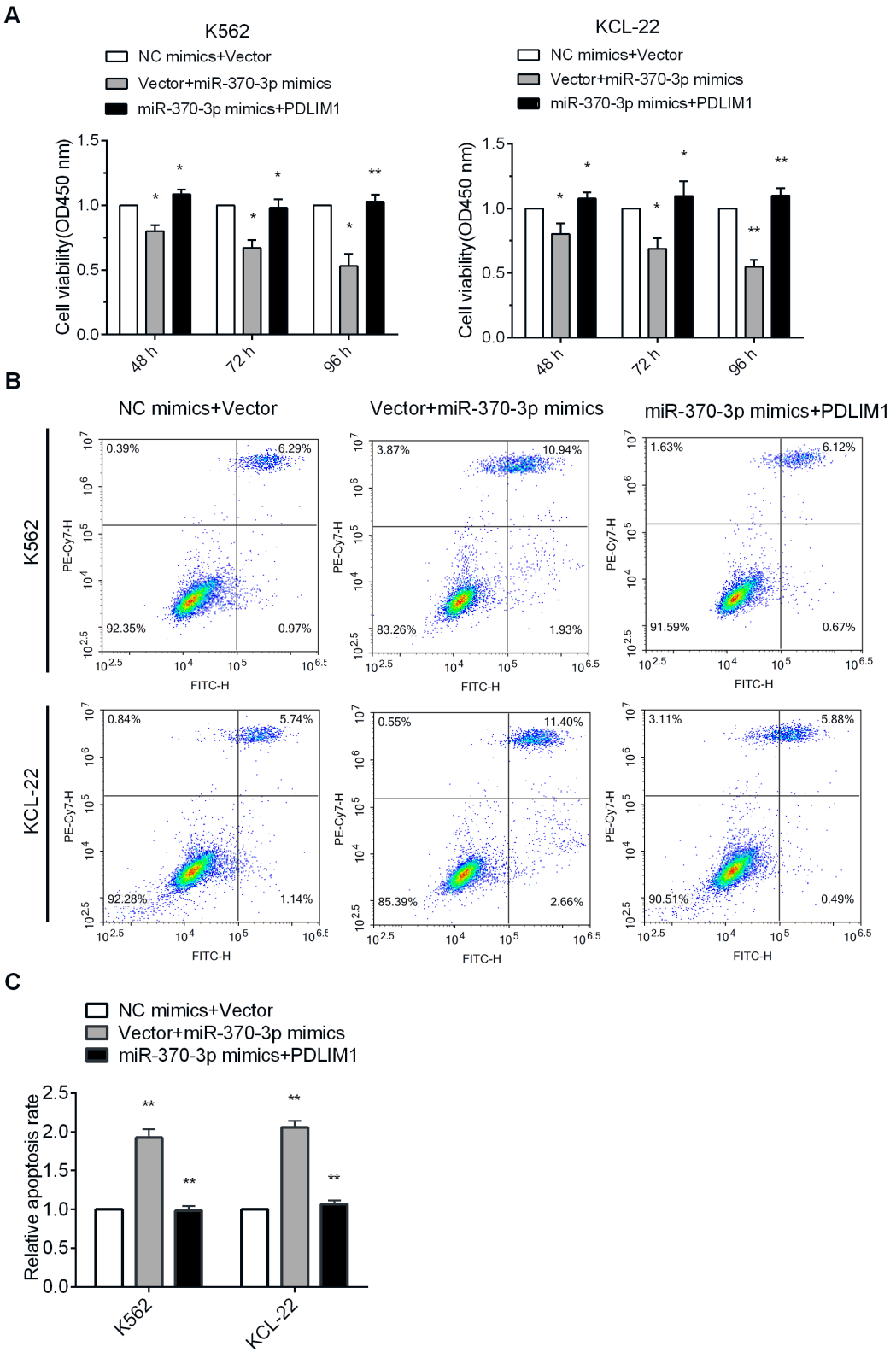

Figure 5. Overexpression of PDLIM1 partly rescues the tumor inhibition effect induced by miR-370-3p. The overexpression of PDLIM1 partly rescued cell proliferation (A) and apoptosis (B) induced by miR-370-3p in CML cells. C) Quantitative analysis of the relative apoptosis rates from Figure 5B. Experiments were repeated in triplicates. *p<0.05; **p<0.01; ***p<0.001.

least partially relied on the expression of PDLIM1 in CML cells. We found that the overexpression of miR-370-3p inhibited the expression of PDLIM1 and β-catenin, suggesting that Wnt/β-catenin signaling is inhibited in CML cells. Moreover, the overexpression of miR-370-3p could markedly downregulate the expression level of c-Myc, which was a downstream key molecular of Wnt/β-catenin signaling (Figures 6C and 6D). The blockage of miR-370-3p in CML cells had contradictory effects on Wnt/β-catenin signaling (Figures 6E and 6F). Therefore, miR-370-3p inhibited carcinogenesis by suppressing the suppression of Wnt/β-catenin signaling by targeting PDLIM1 (Figure 6G).

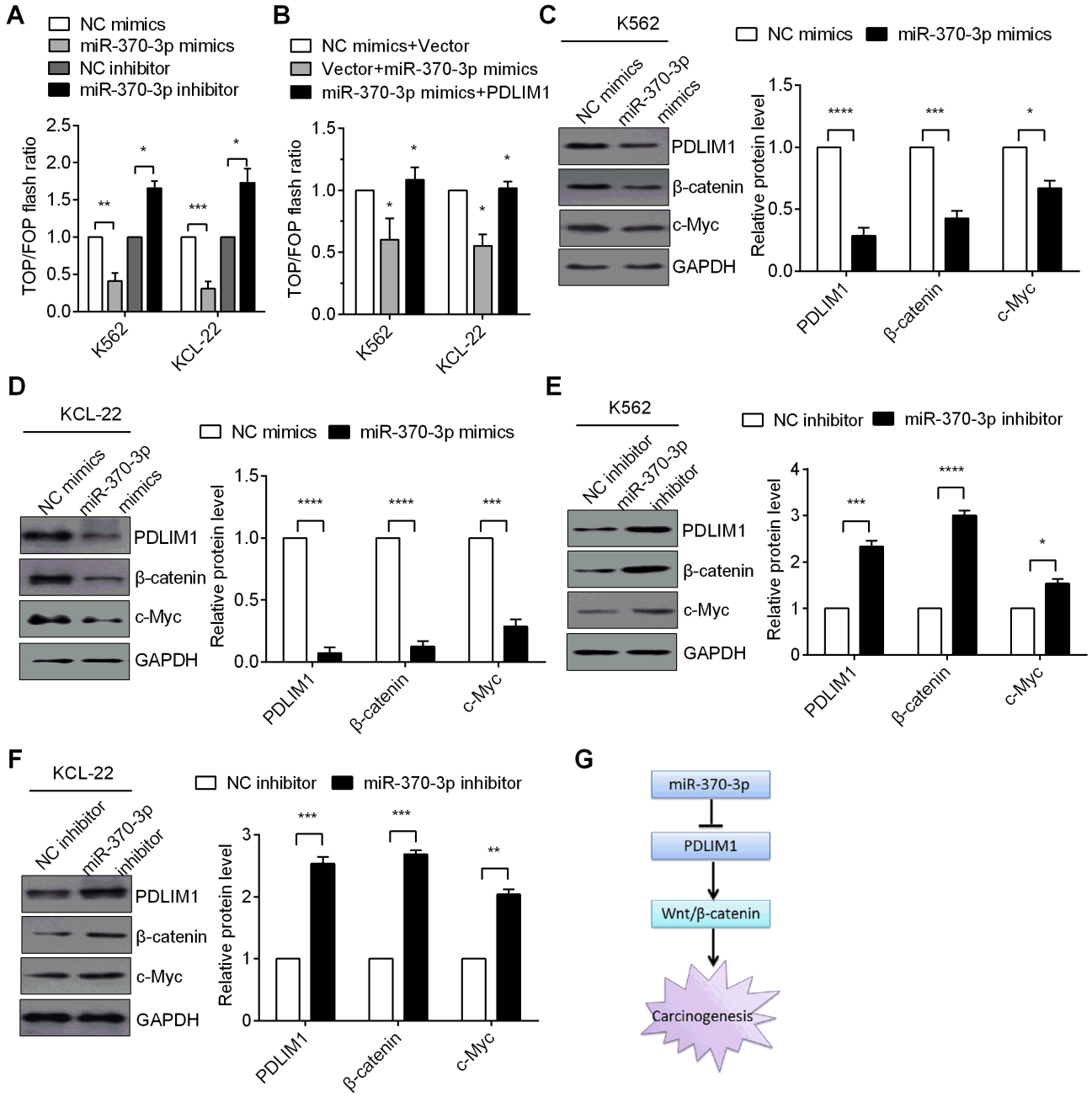

Figure 6. miR-370-3p inhibits Wnt/β-catenin signaling by downregulating PDLIM1. A and B) The pTOP-flash/pFOP-flash ratio was measured after the co-transfection of pTOP-flash/pFOP-flash reporter vectors with miR-370-3p mimics or inhibitors. C and D) Western blot was used to detect the expression levels of the PDLIM1, β-catenin and c-Myc in CML cells after transfection with PDLIM1 overexpressing plasmid. E and F) Western blot was used to detect the expression levels of PDLIM1, β-catenin and c-Myc in CML cells after transfection with PDLIM1-silencing plasmid. G) Proposed schematic diagram indicated that miR-370-3p downregulated the expression of PDLIM1 and thus inhibited carcinogenesis through Wnt/β-catenin signaling.

Discussion

Many studies have demonstrated that miRNAs play pivotal roles in numerous biological processes, such as cell growth, apoptosis, cell cycle, migration and invasion [9, 11, 26]. In this study, the role and mechanisms of miR-370-3p in the pathophysiology of CML were investigated. First, we observed that miR-370-3p was downregulated in the PBMCs of patients with CML and CML cell lines, indicating the dysregulation of miR-370-3p. Previous studies have demonstrated that miR-370-3p is frequently downregulated in cancers. Tian et al. observed that the expression of miR-370-3p was decreased in glioma tissues and was negatively correlated with the degree of glioma malignancy [14]. In addition, Yan et al. analyzed The Cancer Genome Atlas provisional databases and reported that miR-370-3p was downregulated in samples of urinary bladder cancer with lymph node invasion [16]. In the present study, we also found that miR-370-3p inhibited the proliferation and promoted the apoptosis of CML cells. These data suggested that miR-370-3p plays a suppressive role in CML cells. Of note, our findings were consistent with those previously reported for several other cancers, such as gliomas [14], ovarian cancer [15], bladder cancer [16], thyroid cancer [18], and pancreatic cancer [27].

To investigate the mechanism underlying the suppressive role of miR-370-3p in CML cells, we demonstrated that PDLIM1 was directly targeted by miR-370-3p, which could inhibit PDLIM1 expression at the post-transcriptional level. PDLIM1 was demonstrated to have an oncogenic role by enhancing cell proliferation and inhibiting cell apoptosis in CML cells. PDLIM1, also termed CLP36, has been demonstrated to be a key regulator in breast cancer by promoting cell migration and metastasis [28]. The function of PDLIM1 in tumor invasion has been demonstrated in breast cancer as well as in gliomas. Senger et al. observed that the knockdown of PDLIM1 markedly decreased the $p75^{NTR}$-mediated invasion in gliomas [29]. Considering tissue specificities, the role of PDLIM1 in different cancers may differ. For instance, Huang et al. demonstrated that the overexpression of PDLIM1 attenuated the epithelial-to-mesenchymal transition of colorectal cancer cells by stabilizing the E-cadherin/β-catenin complex [30]. However, our results were consistent with the fact that PDLIM1 is an oncogene in cancers.

Wnt/β-catenin signaling has been reported to play an important role in cancer progression [21–25]. A recent report has shown that miR-370-3p inhibits glioma cell growth by directly targeting β-catenin, which is a critical molecule of the Wnt/β-catenin signaling pathway [14]. Another report has suggested that miR-370-3p suppresses bladder cancer cell invasion by reducing the expression of Wnt7a, which activates canonical Wnt signaling in bladder cancer [16]. Here, we reported that miR-370-3p inhibited the carcinogenesis of CML through the suppression of Wnt/β-catenin signaling by targeting PDLIM1. Our results provide an alternative mechanism that miR-370-3p could inhibit Wnt/β-catenin signaling pathway by targeting PDLIM1 in CML cells.

In conclusion, we demonstrated that miR-370-3p was downregulated in the PBMCs of patients with CML and CML cell lines. The upregulation of miR-370-3p inhibited the proliferation but enhanced the apoptosis of CML cells by targeting PDLIM1 *in vitro*. Furthermore, we demonstrated that the miR-370-3p-PDLIM1-Wnt/β-catenin signaling axis might play an important role in CML progression. Targeting this signaling is an extremely promising strategy for CML treatment.

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