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Circ_DLEU2 knockdown represses cell proliferation, migration and invasion, and induces cell apoptosis through the miR-582-5p/COX2 pathway in acute myeloid leukemia

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Running title: The role of circ_DLEU2/miR-582-5p/COX2 in AML malignant progression

Abstract

Background: Acute myeloid leukemia (AML) is a malignant hematological neoplasm in adults. Researchers indicate that circular RNAs (circRNAs) play paramount roles in the pathological process of AML. In this study, the role of circ_DLEU2 (circ_0000488) in AML is revealed.

Methods: The expression of circ_DLEU2, microRNA-582-5p (miR-582-5p) and cyclooxygenase 2 (COX2) was determined by quantitative real-time PCR. Protein expression was detected by western blot. Cell proliferation was investigated by cell cycle, 5-Ethynyl-29-deoxyuridine and 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di-phenyltetrazoliumromide (MTT) assays. Cell apoptosis was elucidated by apoptosis analysis assay. The targeting relationship between miR-582-5p and circ_DLEU2 or COX2 was predicted by the starbase online database, and identified by a dual-luciferase reporter assay.

Results: Circ_DLEU2 and COX2 expression were substantially up-regulated, while
miR-582-5p was down-regulated in AML marrow samples and cells compared with control groups. Circ_DLEU2 knockdown suppressed cell proliferation, whereas it induced cell arrest at G0/G1 phase and cell apoptosis in AML; however, these effects were attenuated by miR-582-5p inhibitor. Additionally, circ_DLEU2 was associated with miR-582-5p, and miR-582-5p bound to COX2 in AML cells. Also, we found that circ_DLEU2 regulated COX2 expression by interacting with miR-582-5p.

**Conclusion:** Circ_DLEU2 silencing hindered AML malignant progression via downregulating COX2 through sponging miR-582-5p. Our finding provides a theoretical basis for studying circRNA-directed therapy of AML.

**Keywords:** AML, circ_DLEU2, miR-582-5p, COX2

1. Introduction

As a representative of myeloid malignancies, acute myeloid leukemia (AML) displays complicated biological and clinical heterogeneity in sufferers, especially in adults (Wen et al., 2018). AML is characterized by heterogeneity in biology and in the clinic in different patients (Sill et al., 2011; Li et al., 2017a). Up to now, the major therapeutic method for AML is still chemotherapy. However, serious ill effects of chemotherapy and syndromes such as infection and bleeding also bring a heavy burden to human life. Therefore, in-depth exploration of the pathogenesis of AML is necessary to seek a therapeutic target promoting AML treatment.

Circular RNA (circRNA) is a stable non-coding transcript and has a closed continuous loop that is typically non-polyadenylated (Uchida et al., 2008). One special function of circRNA is to modulate the coactions between microRNAs (miRNAs) and genes (Rybak-Wolf et al., 2015). CircRNA is involved in cancer progression and serves a key role in its pathogenesis (Zhong et al., 2017; Kristensen et al., 2018). For example, Chen et al. explained that circ_100395 suppressed cell proliferation through miR-1228
in lung carcinoma (Chen et al., 2018). Tian and his partners explained that low circ_0003159 expression was related to tumor metastasis in gastric cancer (Tian et al., 2018). Recently, an altered amount of circRNA expression was reported in AML (Li et al., 2017b; Zhou et al., 2019). For instance, Zhang et al. unveiled that circ_000370 contributed to cell viability and repressed cell apoptosis via interacting with miR-1299 in AML (Zhang et al., 2020). Circ_0121582 also hindered the progression of leukemia (Chen et al., 2020). However, the function of circ_DLEU2 (circ_000488) in AML development has not been fully studied.

MiRNA is a small RNA with approximately 21 nucleotides without protein-coding capacity (Gardiner et al., 2015). MiRNA commonly functions in cancer progression by associating with a non-coding region of the target gene (Femminella et al., 2015). Multiple studies present that the dysregulation of miRNA is linked with the pathobiology and clinical manifestations of cancers (Reddy, 2015). MiR-582-5p, a cancer-related miRNA, has been disclosed to inhibit the progression of prostate cancer (Huang et al., 2019), hepatocellular carcinoma (Zhang et al., 2015) and gastric cancer (Jin et al., 2017). Nevertheless, there are few reports revealing miR-582-5p function in the AML process. Cyclooxygenase 2 (COX2), a membrane-bound and rate-limiting enzyme, exerts a pleiotropic and multifaceted role in cancer progression (Hashemi Goradel et al., 2019). COX2 expression can be mediated via mitosis, inflammation and the stimulation of carcinogenic factors (Lara-Pezzi et al., 2002). It has been discovered that COX2 is highly expressed in human leukaemia (Bernard et al., 2008). Importantly, COX2 inhibitor can be used to treat cancers and alleviate the burden of hematologic malignancies (Ramon et al., 2013; Ranger, 2014).

Herein, the influences of circ_DLEU2 on AML cell malignancy were unveiled. Additionally, with the help of bioinformatics tools, a circ_DLEU2/miR-582-5p/COX2 regulatory axis was assembled to explain the mechanism of circ_DLEU2 in regulating AML processes.
2. Materials and methods

2.1. Clinical samples

Human marrow samples were obtained from 41 AML subjects from the Children’s Hospital of Chongqing Medical University and 41 healthy contributors following written informed consents. The age distribution in the healthy control groups was similar to that in the AML patient group, with 16 cases over the age of 41 and 25 cases under the age of 41. The clinical information of AML sufferers is listed in Table 1. Marrow samples were kept at -80˚C in a freezer. The Ethics Committee of Children’s Hospital of Chongqing Medical University endorsed this study.

2.2. Cell acquirement and culture

Ciboer Biosciences (Nanjing, China) provided AML cell lines (HL-60 and THP-1). Otwo Biotech (Shenzhen, China) provided human bone marrow stromal cell line HS-5. Cells were cultivated in Roswell Park Memorial Institute-1640 (RPMI-1640; Biosun, Shanghai, China) containing 10% fetal bovine serum (FBS; Thermo Fisher, Waltham, MA, USA) and 1% streptomycin/penicillin (Thermo Fisher) at 37˚C in an incubator with 5% CO₂.

2.3. Cell transfection

The small hairpin RNA against circ_DLEU2 (sh-circ_DLEU2, 5’-AACACAGTTTTAGATGGAGTT-3’), miR-582-5p mimic (miR-582-5p, 5’-UUACAGGUUGUCAACCAGUACU-3’), miR-582-5p inhibitor (anti-miR-582-5p, 5’-AGUAACUGGUUGAACAACUGUAA-3’), the overexpression plasmid of COX2 (COX2) and control groups (sh-NC, miR-NC, anti-miR-NC and pcDNA) were synthesized and built by GENEWIZ (Suzhou, China). Cell transfection was performed using FuGENE6 (Roche, Basel, Switzerland).
2.4. Quantitative real-time PCR (qRT-PCR)

RNA was isolated with RNAiso Plus (TaKaRa, Dalian, China). Then, cDNA was amplified with a commercial cDNA synthesis kit (TaKaRa). For quantifying the levels of circ_DLEU2, miR-582-5p and COX2, SYBR Green Master Mix (Roche) was employed. Data were assessed with the 2^ΔΔCt method. The sequences of sense and antisense primers are listed as follows. Circ_DLEU2 5'-TAGAATGACTGCGTAAAG-3’ and 5’-CCTAAAACAAGTAAGGAGCTATAAG-3’, DLEU2 5’-GCCGCCGGTTACTTATCTCC-3’ and 5’-CCAGGGAGGATGTAGCTGT-3’, miR-582-5p 5’-GCGGTTACAGTTGTTCAACC-3’ and 5’-CTCAACTGGGTGTCGTGGA-3’, COX2 5’-CCCTTCTGCGGCCACCTTT-3’ and 5’-TTCTGTACTGCAGGTTGGAAC-3’, U6 5’-CTCGCTTCGGCAGCACA-3’ and 5’-AACGCTTCACGAATTTGCAAT-3’, GAPDH 5’-AATGGGCAGCCGTTAGAAA-3’ and 5’-GCCCGCAATACGACCAATTC-3’. U6 and GAPDH were employed as reference substances.

2.5. RNase R and actinomycin D treatment assays

The isolated RNA was incubated with RNase R (3 U/µg RNA; Tiangen, Beijing, China) for 30 min at 37°C. Then, Qiagen RNA purification reagents (Valencia, CA, USA) were performed as per the guidebook. For the actinomycin D treatment assay, cells were incubated with 1 mg/L actinomycin D (Amresco, Solon, OH, USA) for 0, 8, 16 and 24 h. Finally, RNA was isolated and qRT-PCR was conducted to analyze gene expression. DLEU2 was chosen as a control.

2.6. Cell cycle assay

HL-60 and THP-1 cells were digested and collected. Cold phosphate buffer solution (PBS; Solarbio, Beijing, China) was used to wash cells. Cells were immobilized with 70% ethanol (Millipore, Bradford, MA, USA) overnight at 4°C. Afterward, cells were co-cultured with RNase A (Beyotime, Shanghai, China), and dyed using propidium
iodide (PI; Beyotime). Cell cycle process was confirmed by flow cytometer (Thermo Fisher).

2.7. 5-Ethynyl-29-deoxyuridine (EdU) assay
The cells treated with test compounds were cultured in 12-well plates for 48 h. The cells were then digested and seeded in 96-well microplates added with EdU-labeled medium. After that, EdU assay was performed using an EdU staining kit (Ribobio, Guangzhou, China) as per the guidebook. Cell proliferation was confirmed by a fluorescence microscope (Olympus, Tokyo, Japan).

2.8. 3-(4,5)-dimethylthiaziaiazol (-z-y1)-3,5-di-phenytetrazoliumromide (MTT) assay
After the desired treatment, HL-60 and THP-1 cells (5×10^3 cells each hole) were plated in 96-well plates and cultured for 24, 48 and 72 h, respectively. Then, 10 µL MTT reagent (Beyotime) was placed into each well. After the formazan was resolved by dimethyl sulfoxide, a microplate reader (Thermo Fisher) was used to assess samples with the absorbance at 570 nm.

2.9. Western blot assay
Protein extraction kit (Phygene, Fuzhou, China) was used to isolate protein from AML marrow samples and cells on ice. Loading buffer (Thermo Fisher) was mixed with the lysates. The protein sample was loaded onto 12% SDS-PAGE (Beyotime). Then, all proteins were transferred onto nitrocellulose membranes (Millipore). After blocking membranes with 5% nonfat milk (Solarbio) at 4°C for 4 h, these membranes were bred with primary antibodies overnight, and reacted with the secondary antibodies labeled with horseradish peroxidase (1:2000; CST, Boston, MA, USA) at 37°C for 2 h. Protein bands were visualized with ECL chemiluminescent detection equipment (Thermo Fisher). Primary antibodies were anti-Ki67 (Affinity, Nanjing, China), anti-B-
cell lymphoma-2 (anti-Bcl-2; 1:1000; Affinity), anti-BCL2-associated x protein (anti-Bax; 1:1500; Affinity), anti-COX2 (1:2000; Affinity) and anti-GAPDH (1:10000; Affinity). GAPDH was selected as a control.

2.10. Apoptosis analysis

Annexin V-FITC apoptosis detection kit (Solarbio) was conducted to detect cell apoptosis. Briefly, cells were harvested after various treatments and precipitated by centrifuging at 100 rpm for 12 min. The cells were suspended in binding buffer (Solarbio). After that, Annexin V-FITC (Solarbio) and PI (Solarbio) were employed to incubate the cells, respectively. Results were assessed by flow cytometry (Thermo Fisher).

2.11. Dual-luciferase reporter assay

The binding sites between miR-582-5p and circ_DLEU2 or COX2 were predicted through the starbase online database. The wild-type (WT) sequences of circ_DLEU2 and COX2 3’UTR containing the targeting sites of miR-582-5p were used to build wild-type plasmids including circ_DLEU2-WT and COX2-WT. Mutant (MUT) circ_DLEU2 or COX2 3’UTR without the putative miR-582-5p-binding sites were employed to construct circ_DLEU2-MUT and COX2-MUT. The plasmids were transfected into HL-60 and THP-1 cells with miR-582-5p mimic or miR-NC. Forty-eight hours later, luciferase activity was detected using a dual-luciferase assay kit (Solarbio) with Renilla Luciferase activity as a reference.

2.12. Statistical analysis

All data from three independent duplicate tests were analyzed with SPSS 21.0 software. Results are presented as means ± standard deviations. Significant differences were assessed by two-tailed Student’s t-tests, Wilcoxon rank-sum test or one-way analysis of variance. The linear relationship between circ_DLEU2 and miR-582-5p or
COX2 was unveiled using Spearman correlation analysis. *P* value < 0.05 indicated statistical significance.

3. Results

3.1. Circ_DLEU2 expression was significantly up-regulated in AML marrow samples and cells

In order to reveal the properties of circ_DLEU2 in AML progression, circ_DLEU2 expression was firstly determined. Results showed that circ_DLEU2 expression was significantly up-regulated in AML marrow samples and HL-60 and THP-1 cells when compared with normal marrow samples and HS-5 cells, respectively (Figure 1A and B). Subsequently, the RNase R treatment assay presented that circ_DLEU2 expression had no obvious change after RNase R treatment, whereas linear DLEU2 expression was significantly down-regulated in HL-60 and THP-1 cells (Figure 1C and D). Actinomycin D treatment assay also unveiled that there was no striking variation in circ_DLEU2 expression after actinomycin D pretreatment in HL-60 and THP-1 cells, but linear DLEU2 expression was prominently repressed by actinomycin D (Figure 1E and F). All data showed that circ_DLEU2 was a circular RNA and was highly expressed in AML marrow samples and cells.

3.2. Circ_DLEU2 knockdown repressed cell proliferation, whereas it induced cell apoptosis and cell arrest at G0/G1 phase in AML

The function of circ_DLEU2 in AML progression was further studied. Circ_DLEU2 expression level was determined in both the HL-60 and THP-1 cells transfected with sh-circ_DLEU2 or sh-NC. Results showed that circ_DLEU2 expression was substantially down-regulated after circ_DLEU2 knockdown in HL-60 and THP-1 cells relative to control groups, whereas DLEU2 expression was not obviously changed by circ_DLEU2 silencing (Figure 2A and B). Subsequently, the cell cycle assay showed that circ_DLEU2 knockdown promoted cell arrest at G0/G1 phase in HL-60 and THP-
1 cells (Figure 2C and D). As shown in Figure 2E-H, the proliferation of HL-60 and THP-1 cells was inhibited by circ_DLEU2 depletion. In support, western blot analysis presented that circ_DLEU2 knockdown reduced the protein expression of proliferation-related Ki67 (Figure 2I). On the contrary, the apoptosis of HL-60 and THP-1 cells was induced by circ_DLEU2 knockdown (Figure 3A). Additionally, the protein expression of apoptosis-linked Bcl-2 and Bax was detected in circ_DLEU2-reduced HL-60 and THP-1 cells. Comparatively, circ_DLEU2 absence decreased Bcl-2 expression, and increased Bax expression (Figure 3B and C). The above data indicated that circ_DLEU2 might be an oncogene in AML development.

### 3.3. Circ_DLEU2 acted as a sponge of miR-582-5p

Given the effects of circ_DLEU2 knockdown on the AML process, we continued to explore the underlying regulatory mechanism. Starbase online database showed that circ_DLEU2 contained the binding sites of miR-582-5p (Figure 4A). Dual-luciferase reporter assay illustrated that luciferase activity was significantly repressed after circ_DLEU2-WT and miR-582-5p co-transfection in HL-60 and THP-1 cells, whereas that was rarely changed in the circ_DLEU2-MUT+miR-582-5p group (Figure 4B and C). In addition, miR-582-5p expression was obviously up-regulated after circ_DLEU2 knockdown in HL-60 and THP-1 cells (Figure 4D). QRT-PCR results displayed that miR-582-5p was lowly expressed in HL-60 cells, THP-1 cells and AML marrow samples as compared to control groups (Figure 4E and F). Furthermore, circ_DLEU2 expression was negatively related to miR-582-5p expression in AML marrow samples (Figure 4G). These findings suggested that circ_DLEU2 was associated with miR-582-5p and might regulate AML progression by sponging miR-582-5p.
3.4. Circ_DLEU2 knockdown repressed AML malignant progression by sponging miR-582-5p

In order to unveil whether circ_DLEU2 regulated AML development via binding to miR-582-5p, the effects between circ_DLEU2 knockdown and miR-582-5p inhibitor on AML cell malignancy were investigated. The results incipiently showed that circ_DLEU2 silencing up-regulated miR-582-5p expression, whereas the effect was attenuated by miR-582-5p inhibitor (Figure 5A). Subsequently, the cell cycle assay displayed that circ_DLEU2 depletion induced cell arrest at G0/G1 phase in HL-60 and THP-1 cells, which was restored by miR-582-5p inhibitor (Figure 5B and C). It was found using EdU and MTT assays that circ_DLEU2 knockdown inhibited the proliferation of HL-60 and THP-1 cells; however, this impact was hindered by miR-582-5p inhibitor (Figure 5D-F). In support, circ_DLEU2 knockdown decreased Ki67 protein expression, whereas miR-582-5p depletion impaired this inhibitory effect (Figure 5G). Apoptosis analysis assay demonstrated that the promoting effect of circ_DLEU2 silencing on cell apoptosis was reversed by miR-582-5p inhibitor in HL-60 and THP-1 cells (Figure 6A). MiR-582-5p inhibitor also blocked the inhibitory effects of circ_DLEU2 knockdown on the protein expression of Bcl-2, and the stimulatory impact of circ_DLEU2 silencing on Bax protein level in HL-60 and THP-1 cells (Figure 6B and C). Therefore, these results meant that circ_DLEU2 regulated AML progression through sponging miR-582-5p.

3.5. MiR-582-5p was associated with COX2 in AML cells

The gene associated with miR-582-5p was explored further. Starbase online database showed that COX2, Rho associated coiled-coil containing protein kinase 1 (ROCK1), vascular endothelial growth factor A (VEGFA) and MCL1 contained the binding sites of miR-582-5p, and further analysis showed that miR-582-5p mimic could obviously reduce the expression of COX2, ROCK1, VEGFA in both HL-60 and THP-1 cells, especially the expression of COX2 (Figure 7A and B). Based on the above results,
COX2 was employed as a target gene of miR-582-5p. The binding sites between COX2 and miR-582-5p are shown in Figure 7C. Dual-luciferase reporter assay demonstrated that the luciferase activity of COX2-WT and miR-582-5p group was significantly suppressed in HL-60 and THP-1 cells, whereas the luciferase activity had no apparent change after COX2-MUT and miR-582-5p co-transfection (Figure 7D and E). In addition, miR-582-5p expression was significantly up-regulated by miR-582-5p mimic and down-regulated after miR-582-5p inhibitor transfection (Figure 7F). And the mRNA and protein expression of COX2 were significantly down-regulated by miR-582-5p mimic, and up-regulated by miR-582-5p inhibitor in HL-60 and THP-1 cells (Figure 7G and H). Subsequently, data showed that the mRNA and protein expression of COX2 were substantially up-regulated in HL-60 and THP cells compared with HS-5 cells (Figure 7I and J). The results also demonstrated that COX2 expression was prominently increased at mRNA and protein levels in AML samples in contrast to normal control (Figure 7K and N). Furthermore, COX2 expression was negatively related to miR-582-5p expression and positively related to circ_DLEU2 expression (Figure 7L and M). All data demonstrated that miR-582-5p bound to COX2.

3.6. MiR-582-5p inhibited AML cell processes by binding to COX2

This study continued to explore whether miR-582-5p regulated AML progression via interacting with COX2. Results firstly showed that the mRNA and protein expression of COX2 were down-regulated by miR-582-5p mimic, whereas COX2 overexpression attenuated these effects (Figure 8A and B). Subsequently, it was found that miR-582-5p mimic induced cell arrest at G0/G1 phase in HL-60 and THP-1 cells, whereas the effect was restored after COX2 overexpression (Figure 8C and D). EdU and MTT assays exhibited that COX2 overexpression hindered the inhibitory effect of miR-582-5p on cell proliferation (Figure 8E-G). Western blot demonstrated that miR-582-5p decreased Ki67 protein expression; however, COX2 overexpression abolished this effect (Figure 8H). Additionally, the apoptosis of HL-60 and THP-1 cells was induced
by miR-582-5p, which was blocked after COX2 overexpression (Figure 9A). In support, western blot exhibited that miR-582-5p led to decreased Bcl-2 expression and increased Bax level in HL-60 and THP-1 cells, but these effects were abolished after COX2 overexpression (Figure 9B and C). Collectively, all these findings implied that miR-582-5p modulated AML development through interacting with COX2.

3.7. Circ_DLEU2 knockdown down-regulated COX2 expression by sequestering miR-582-5p

The above evidence proved that circ_DLEU2 could sponge miR-582-5p, and miR-582-5p targeted COX2. Whether circ_DLEU2 could regulate COX2 expression by binding to miR-582-5p was further revealed. Results showed that the mRNA and protein expression of COX2 were repressed after circ_DLEU2 knockdown; however, miR-582-5p inhibitor attenuated these impacts (Figure 10A and B). Our data suggested that COX2 can be regulated by circ_DLEU2 through miR-582-5p as a bridge in HL-60 and THP-1 cells.

4. Discussion

AML, a malignant myeloid cancer, accounts for almost 80% of all adult leukemia (Döhner et al., 2015). Existing studies have shown the importance of circRNA in various diseases, including cancers (Zhu et al., 2017; Shang et al., 2019). For example, circ_0026344 suppressed cell growth through binding to miR-21 and miR-31 in colorectal cancer (Yuan et al., 2018). Li et al. explained that circ_0004277 could serve as a biomarker of AML with the help of circRNA mapping and bioinformatics (Li et al., 2017b). In another example, it was found that circ_0012152 and circ_0001857 were able to distinguish the Acute Lymphoblastic Leukemia from AML (Guo et al., 2020). In this study, we found that circ_DLEU2 promoted AML progression by the miR-582-5p/COX2 pathway.

Previous data have indicated that circ_DLEU2 expression was increased in AML
marrow samples and cells, and circ_DLEU2 overexpression repressed the AML process by facilitating cell proliferative ability and hindered cell apoptosis (Wu et al., 2018). In this research, circ_DLEU2 expression was detected and data exhibited that circ_DLEU2 was substantially increased in AML marrow samples and cells. Subsequent data showed that circ_DLEU2 knockdown repressed cell proliferation, but expedited cell apoptosis. Our evidence suggested that circ_DLEU2 was also a tumor promoter in AML progression. In addition, circ_DLEU2 was found to sponge miR-582-5p.

Research has revealed that miR-582-5p serves a tumor-repressing role in cancer development (Liang et al., 2021; Xue et al., 2021). In particular, considerable data also indicated miR-582-5p participated in radiation-induced AML, and its upregulation was closely associated with the reduced expression of Sfpi1 in radiation-induced AML (O'Brien et al., 2020). Also, previous data documented that miR-582-5p expression was downregulated in AML cells, and its introduction inhibited AML development (Wang et al., 2020). Herein, we also reported the low expression of miR-582-5p in AML cells, and its ectopic expression suppressed cell proliferation, but increased cell-apoptotic rate. These findings were consistent with the above data. Beyond that, we also manifested that miR-582-5p facilitated G0/G1 phase cell arrest in AML, and regulated AML processes by binding to circ_DLEU2. In addition, we searched for the target genes of miR-582-5p. Starbase online database showed that COX2, ROCK1, VEGFA and MCL1 contained the binding sites of miR-582-5p, and subsequent analysis showed that miR-582-5p introduction obviously reduced the expression of COX2, ROCK1, VEGFA and COX2, especially COX2, in both HL-60 and THP-1 cells. Thus, COX2 was chosen as a potential target of miR-582-5p in AML cells.

COX2 has been found to participate in the regulation of the apoptosis repressor through the interaction between leukemia and stroma mediated by caspase recruitment domain (ARC) (Carter et al., 2019). Hua et al. revealed that COX2 repression attenuated the stimulatory effect of vascular endothelial growth factor on cell
proliferation in AML (Hua et al., 2014), which suggested that COX2 acted as a
promoter during the proliferation of AML cells. In another report, we found that COX2
inhibitor inhibited the proliferative ability of cells and elevated cell apoptosis in acute
leukemia (Lu et al., 2016). From our findings, COX2 was overexpressed in AML
marrow samples and cells. COX2 overexpression restored the inhibitory impact of miR-
582-5p mimic on the proliferative ability of cells, as well as the stimulatory influence
of that on the apoptotic rate of cells in AML, implying that COX2 functioned as a
positive regulator in AML progression. Different from the above evidence, we also
provided evidence that COX2 repressed G0/G1 phase cell arrest in AML.

In summary, circ_DLEU2 and COX2 were highly expressed, and miR-582-5p was
lowly expressed in AML marrow samples and cells. Circ_DLEU2 depletion inhibited
cell proliferative ability, whereas it contributed to cell apoptosis in AML. In addition,
circ_DLEU2 regulated COX2 by interacting with miR-582-5p in AML cells. The novel
mechanism suggests that targeting the circ_DLEU2/miR-582-5p/COX2 pathway may
be effective for the therapy of AML. The biomarkers of cancers are the
clinicopathological factors used in the prediction or therapeutic intervention of cancers
partly due to their wide availability in routine clinical practice (Li et al., 2021). A
previous study has shown that TP53 mutation status has a predictive value in primary
AML (Ishizawa et al., 2018). Ten-Eleven Translocation 2 (TET2) has the potential to
monitor disease surveillance of AML patients (Zhang et al., 2018). Our studies indicate
that the altered expression of circ_DLEU2 in AML marrow samples manifests the
potential of circ_DLEU2 as a predictive marker in AML.

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None

Disclosure of interest

The authors declare that they have no financial conflicts of interest.
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None.

Ethics approval and consent to participate

This study was approved by the Medical Ethics Committee of Children’s Hospital of Chongqing Medical University. The patients gave their written informed consent in accordance with the Declaration of Helsinki.

Author contributions

Shifang Dong collected the data, analyzed the data, and wrote the manuscript; Yanyu Jiang, Nan Mou, and Zhenxing Yang performed laboratory work for this study; Haiying Zhong Li were responsible for acquisition and analysis of data and statistical analysis; Lin Li coordinated the study and revised it critically for important intellectual content; All of the authors approved the final manuscript.

Data Availability Statement

Please contact the correspondence author for the data request.

References


Table 1. The clinical information of AML patients.

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**Figure legends:**

**Figure 1 Circ_DLEU2 was highly expressed in AML marrow samples and cells.** (A and B) Circ_DLEU2 expression was detected by qRT-PCR. (C-F) RNase R and Actinomycin D treatment assays revealed the stability of circ_DLEU2. *P<0.05.

**Figure 2 Circ_DLEU2 knockdown hindered HL-60 and THP-1 cell proliferation.** (A-I) HL-60 and THP-1 cells were transfected with sh-NC or sh-circ_DLEU2. (A and
B) The efficiency of circ_DLEU2 knockdown was determined by qRT-PCR in both HL-60 and THP-1 cells. (C-H) Cell proliferation was investigated by cell cycle, EdU and MTT assays. (I) Western blot was employed to investigate Ki67 protein expression. *P<0.05.

Figure 3 Circ_DLEU2 knockdown induced HL-60 and THP-1 cell apoptosis. (A-C) HL-60 and THP-1 cells were transfected with sh-NC or sh-circ_DLEU2. (A) Apoptosis analysis assay was carried out to uncover the apoptosis rate of HL-60 and THP-1 cells. (B and C) Western blot was conducted to check the protein expression of Bcl-2 and Bax. *P<0.05.

Figure 4 Circ_DLEU2 bound to miR-582-5p in HL-60 and THP-1 cells. (A) The binding sites between circ_DLEU2 and miR-582-5p were predicted by the starbase online database. (B and C) Luciferase activities were detected by dual-luciferase reporter assay in HL-60 and THP-1 cells. (D) The effect of circ_DLEU2 knockdown on miR-582-5p expression was revealed by qRT-PCR. (E and F) MiR-582-5p expression was determined by qRT-PCR. (G) Spearman correlation analysis was performed to unveil the linear relationship between circ_DLEU2 and miR-582-5p expression. *P<0.05.

Figure 5 Circ_DLEU2 silencing suppressed HL-60 and THP-1 cell proliferation via binding to miR-582-5p. (A-G) HL-60 and THP-1 cells were transfected with sh-NC, sh-circ_DLEU2, sh-circ_DLEU2+anti-miR-NC or sh-circ_DLEU2+anti-miR-582-5p. (A) MiR-582-5p expression was determined by qRT-PCR. (B and C) Cell cycle was studied by cell cycle assay. (D-F) Cell proliferation was disclosed by EdU and MTT assays. (G) Western blot was conducted to detect the protein expression of Ki67. *P<0.05.
Figure 6 Circ_DLEU2 silencing induced HL-60 and THP-1 cell apoptosis via binding to miR-582-5p. (A-C) HL-60 and THP-1 cells were transfected with sh-NC, sh-circ_DLEU2, sh-circ_DLEU2+anti-miR-NC or sh-circ_DLEU2+anti-miR-582-5p. (A) Apoptosis of HL-60 and THP-1 cells was uncovered via apoptosis analysis assay. (B and C) Western blot was conducted to detect the protein expression of Bel-2 and Bax. *P<0.05.

Figure 7 MiR-582-5p interacted with COX2. (A and B) The effects of miR-582-5p mimic on the expression of COX2, ROCK1, VEGFA and MCL1 were determined by qRT-PCR in HL-60 and THP-1 cells. (C) Starbase online database was performed to predict the binding sequence between miR-582-5p and COX2 3’UTR. (D and E) Dual-luciferase reporter assay was employed to detect luciferase activities. (F) The expression of miR-582-5p was determined by qRT-PCR. (G and H) COX2 expression was detected by qRT-PCR and western blot. (I and K) QRT-PCR was performed to check COX2 mRNA expression. (J and N) Western blot was conducted to detect the protein expression of COX2. (L and M) The linear relationship between COX2 and miR-582-5p or circ_DLEU2 expression was unveiled by Spearman correlation analysis. *P<0.05.

Figure 8 MiR-582-5p mimic suppressed HL-60 and THP-1 cell proliferation via targeting COX2. (A-H) HL-60 and THP-1 cells were transfected with miR-NC, miR-582-5p, miR-582-5p+pcDNA or miR-582-5p+COX2. (A and B) COX2 expression was determined by qRT-PCR and western blot. (C-G) Cell proliferation was studied via cell cycle, EdU and MTT assays. (H) Western blot was carried out to detect the protein expression of Ki67. *P<0.05.

Figure 9 MiR-582-5p mimic induced HL-60 and THP-1 cell apoptosis through COX2. (A) Cell apoptosis was evaluated by apoptosis analysis assay. (B and C)
Western blot was carried out to detect the protein expression of Bcl-2 and Bax. \( *P<0.05. \)

Figure 10 Circ_DLEU2 sponged miR-582-5p to modulate COX2 expression. (A and B) QRT-PCR and western blot were performed to detect COX2 expression. \( *P<0.05. \)
A

\[
\text{circ}_\text{DLEU2-WT} \quad \text{5}': \quad \text{UUGUUUAUUACCCCAUACUGUA} \quad \text{3}'
\]

\[
\text{miR-582-5p} \quad \text{3}': \quad \text{UCAUGGACACCUUGUUGCAAU} \quad \text{5}'
\]

\[
\text{circ}_\text{DLEU2-MUT} \quad \text{5}': \quad \text{UUGUUUAUUACCCCAUCAGUGC} \quad \text{3}'
\]

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B

C

E

F

G

\[ r = -0.6372 \quad P < 0.0001 \]
A

Relative miR-582-5p expression

HL-60 THP-1

B

Cell number (%)

G0/G1 S G2/M

HL-60 THP-1

C

EdU-positive cells (%)

HL-60 THP-1

D

sh-NC
sh-circ_DLEU2
sh-circ_DLEU2+anti-miR-NC
sh-circ_DLEU2+anti-miR-582-5p

E

OD value (570 nm)

Time (days)

HL-60 THP-1

F

OD value (570 nm)

Time (days)

HL-60 THP-1

G

Relative Ki67 protein expression

HL-60 THP-1

Ki67 GAPDH

HL-60 THP-1
HISTOLOGY AND HISTOPATHOLOGY

(A) Apoptotic rate (%)

(B) Relative protein expression

HL-60

THP-1

sh-NC

sh-circ_DLEU2

sh-circ_DLEU2+anti-miR-NC

sh-circ_DLEU2+anti-miR-582-5p

Bcl-2

Bax

GAPDH

HL-60

THP-1

sh-NC

sh-circ_DLEU2

sh-circ_DLEU2+anti-miR-NC

sh-circ_DLEU2+anti-miR-582-5p

Bcl-2

Bax

GAPDH
A

Relative COX2 mRNA expression

- sh-NC
- sh-circ_DLEU2
- sh-circ_DLEU2+anti-miR-NC
- sh-circ_DLEU2+anti-miR-582-5p

* * *

N

1.0

0.5

COX2

GAPDH

HL-60 THP-1

B

Relative COX2 protein expression

- sh-NC
- sh-circ_DLEU2
- sh-circ_DLEU2+anti-miR-NC
- sh-circ_DLEU2+anti-miR-582-5p

* * *

GAPDH

COX2

HL-60 THP-1

sh-NC
sh-circ_DLEU2
sh-circ_DLEU2+anti-miR-NC
sh-circ_DLEU2+anti-miR-582-5p