

RETRACTED ARTICLE: miR-302 cluster inhibits angiogenesis and growth of K562 leukemia cells by targeting VEGFA

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Background: miR-302 cluster has been reported as a tumor suppressor in many human cancers; yet, its function in chronic myeloid leukemia (CML) tumorigenesis remains largely unclear. The study was aimed to explore the functional roles of miR-302 cluster in CML progression.

Materials and methods: Quantitative reverse transcriptase PCR and Western blot were performed to evaluate miR-302 cluster and vascular endothelial growth factor A (VEGFA) expression levels. Cell Counting Kit-8 assay, colony formation assay and human umbilical vein endothelial cell line capillary tube formation were used to determine the influence of miR-302 cluster on the growth and angiogenesis of K562 cells, respectively. Luciferase reporter assay was employed to confirm the direct target interaction between miR-302 cluster and VEGFA.

Results: This study demonstrated that miR-302 cluster was frequently downregulated in CML samples and cell lines and high level of miR-302 cluster was significantly associated with good prognosis of CML patients. Compared with miRNA negative control, miR-302 cluster mimics obviously suppressed cell growth, colony formation and angiogenesis. Further studies revealed that VEGFA was a direct target gene of miR-302 cluster. Moreover, overexpression of VEGFA dramatically abated the inhibition of miR-302 cluster on cell growth and angiogenesis.

Conclusion: The present study, for the first time, identified miR-302 cluster as a tumor suppressor, and overexpression of miR-302 cluster inhibited growth and angiogenesis in K562 cells. miR-302 cluster may be a potential therapeutic target in CML to develop the adjuvant antiangiogenic therapy based on VEGFA.

Keywords: chronic myeloid leukemia, angiogenesis, miR-302, VEGFA

Introduction

Chronic myeloid leukemia (CML), a clonal hematopoietic stem cell disorder, is caused by the constitutively active BCR-ABL tyrosine kinase resulting from the t(9;22)(q34;q11) reciprocal translocation (the Philadelphia translocation).^{1,2} With the introduction of imatinib, a small-molecule BCR-ABL-specific tyrosine kinase inhibitor, the 5-year survival rate of CML patients has greatly improved.³⁻⁵ Unfortunately, the prognosis of some patients who are resistant to imatinib therapy still remains poor.^{6,7} Therefore, a better understanding of how CML initiates and progresses will be pivotal to the development of new therapeutic strategies.

miRNAs are small non-coding RNAs that regulate the expression of target genes at the post-transcriptional level.⁸ Recent studies have demonstrated that miRNAs are involved in a variety of biological processes such as cell proliferation, apoptosis and metastasis.^{9,10} They are often dysregulated and may serve as oncogenes or tumor suppressors in many cancers. miR-302 cluster was initially identified in human embryonic stem cells and human embryonic carcinoma cells and was involved in stemness maintenance.¹¹⁻¹³ Several studies have also indicated the potential roles of miR-302

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433



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cluster in human cancers. For example, miR-302 inhibited cell growth by targeting MTDH in hepatocellular carcinoma, suppressed cell tumorigenesis and metastasis by regulating EphA2 in gastric cancer, repressed epithelial–mesenchymal transition and metastasis by reducing AP-4 in colorectal cancer and restrained cell migration and invasion by controlling CXCR4 in breast cancer.^{14–17} However, little is known about the roles of miR-302 cluster in CML.

Angiogenesis has been associated with the growth, dissemination and metastasis of solid tumors.^{18,19} In CML, increased vascularity has been observed, which can provide sufficient blood supply to feed growing leukemic cells.²⁰ Vascular endothelial growth factor A (VEGFA) is an important tumor angiogenic factor involved.²¹ It was reported that antisense VEGF cDNA transfection inhibited cell growth, reduced VEGF secretion and the microvessel density in CML.²² These studies indicate the pivotal role of VEGFA-mediated angiogenesis in CML progression. Recent studies have reported several miRNAs could directly target VEGFA and regulate angiogenesis and metastasis, such as miR-134, miR-140-5p, miR-199a-3p and miR-503.^{23–26} These findings suggest therapies based on miRNA-VEGFA may be a potent and promise strategy for cancer treatment.

In the present study, we examined miR-302 cluster expression in CML patients and cell lines and investigated the functional roles of miR-302 cluster in angiogenesis and the underlying mechanisms.

Materials and methods

Patient samples

Bone marrow mononuclear cells were collected from 70 CML patients and 20 healthy age-matched controls at the Affiliated Hospital of Xuzhou Medical University (Xuzhou, China) between January 2014 and March 2016. The diagnosis of CML was based on clinical features, hematological characteristics and the presence of Philadelphia chromosome. The characteristics of the CML patients included in the study

are summarized in Table 1. Bone marrow mononuclear cells were isolated by Ficoll Histopaque density gradient method. The study was approved by the Ethical Committee of the Affiliated Hospital of Xuzhou Medical University, and informed consent was obtained in accordance with the Declaration of Helsinki.

Cell culture and transfection

Three CML cell lines (K562, KCL-22 and KU812) were purchased from ATCC and grown in Roswell Park Memorial Institute-1640 (Thermo Fisher Scientific, Waltham, MA, USA) medium. Human umbilical vein endothelial cell lines (HUVECs) were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). K562 cells were transiently transfected with 50 nmol/L miR-302 cluster mimics and miRNA negative control (GenePharma, Shanghai, China), 50 nmol/L wild type (WT) and mutant type (Mut) of VEGFA reporter vector, 50 nmol/L pcDNA3.1-VEGFA and pcDNA3.1 (Promega Corporation, Madison, WI, USA) using Lipofectamine 2000 reagent (Thermo Fisher Scientific).

Cell Counting Kit-8 (CCK-8) assay

After 24 hours of transfection, K562 cells (3×10^3 cells/well) were seeded in 96-well culture plate. At 24, 48, 72 and 96 hours, 100 μ L of serum-free Roswell Park Memorial Institute-1640 containing 10% CCK-8 reagent (v/v) was added to each well, and cells were cultured for 1 hour at 37°C. The number of viable cells was assessed by measurement of absorbance at 450 nm using an Enzyme Immunoassay Analyzer (Bio-Rad Laboratories Inc., Hercules, CA, USA).

Colony formation assay

After 24 hours of transfection, 150 cells were plated in 6-well plates and grown for 2 weeks. Cells were fixed with acetic acid:methanol (1:4) and stained with dilute crystal violet (1:30). The number of visible colonies was counted manually. All samples were assayed in triplicate.

Table 1 Clinical features of the CML patients included in the study

| Characteristics | CML in chronic phase (n=32) | CML in accelerated phase (n=17) | CML in blast phase (n=21) |
|--|-----------------------------|---------------------------------|---------------------------|
| Age (years), median (range) | 48.8 (10–70) | 45.5 (9–65) | 47.3 (18–69) |
| Male/female, (n/n) | 18/14 | 9/8 | 10/11 |
| White blood cells, $\times 10^9$ /median (range) | 213.4 (30.2–517) | 243.5 (47.4–396) | 63.5 (27.4–224) |
| Hemoglobin level (g/L), median (range) | 94 (72–123) | 82 (60–107) | 63 (53–80) |
| Platelet count, 10^9 /median (range) | 614 (102–821) | 287 (49–748) | 47 (18–72) |

Abbreviation: CML, chronic myeloid leukemia.

RNA isolation and quantitative reverse transcriptase PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific). The expression of miR-302 cluster was evaluated using Taqman miRNA assays (Thermo Fisher Scientific). The mRNA level of VEGFA was determined using SYBR Green PCR master mix (Thermo Fisher Scientific). U6 or β -actin was used as an endogenous control. All samples were normalized to internal controls, and fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method.

Luciferase reporter assay

The 3'UTR of VEGFA containing the putative or mutated miR-302 cluster binding site was synthesized and cloned into the pGL3 vector (Promega Corporation). pGL3-VEGFA-WT or pGL3-VEGFA-Mut was co-transfected with miR-302 cluster mimics or miRNA negative control, respectively. After 48 hours of transfection, luciferase activity was measured using Dual Luciferase Assay (Promega Corporation). Experiments were performed in triplicate.

Western blot

Cells were lysed in RIPA lysis buffer (Cell Signaling) with protease inhibitor. Western blot analysis was performed using rabbit monoclonal anti-VEGFA (#ab52917; Abcam) and mouse monoclonal β -actin (#ab8226; Abcam). β -Actin was used as an internal control.

HUVEC capillary tube formation assay

After 24 hours of transfection, K562 cells were incubated with serum-free medium for 2 days. The medium was then collected as conditioned medium. HUVECs at a density of 5×10^3 per well were grown with conditioned medium in a 24-well plate precoated with 200 μ L Matrigel (BD Biosciences, San Jose, CA, USA) and then incubated at 37°C for 6 hours. The formation of capillary-like structures was captured under phase microscope. The number of connected tubes was counted.

Prediction of target genes

Potential target genes that interacted with miR-302 cluster were analyzed with TargetScan 7.2 and starBase v3.0 software (Table S1).

Statistical analysis

Data are expressed as the mean \pm SD of at least triplicate experiments. Statistical analyses were performed with

Student's *t*-test (two-tailed) using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA). *P*-value < 0.05 was defined as statistically significant.

Results

Downregulated miR-302 cluster expression is associated with poor overall survival of CML patients

To investigate the functional roles of miR-302 cluster in the development and progression of CML, we first determined its expression levels in CML patient samples and cell lines. As shown in Figure 1A–D, the expression levels of miR-302a, miR-302b, miR-302c and miR-302d in CML patients were significantly downregulated 2.41-fold, 3.90-fold, 3.16-fold and 2.22-fold, respectively, compared with normal samples. Consistent with CML samples, decreased miR-302 cluster was also observed in CML cell lines, especially in K562 cells (Figure 1E). We also assessed the relationship between miR-302 cluster and overall survival of CML patients and found that high expression level of miR-302 cluster was significantly correlated with good prognosis ($P=0.038$, $P=0.032$, $P=0.033$ and $P=0.045$, respectively; Figure 1F–I). These results suggest that miR-302 cluster may play key roles in CML tumorigenesis.

Overexpression of miR-302 cluster inhibits cell growth, colony formation and angiogenesis

To further explore the function of miR-302 cluster in CML, miR-302 cluster mimics or miRNA negative control was transfected into K562 cells. The transfection efficiency was first evaluated using qRT-PCR. The expression level of miR-302 cluster were significantly upregulated (123.14 ± 19.36)-fold, (105.60 ± 11.87)-fold, (117.92 ± 15.24)-fold and (134.28 ± 16.73)-fold compared with negative control (Table 2). Overexpression of miR-302 cluster obviously inhibited cell viability after 72 hours of transfection (Figure 2A). The number of colonies formed was significantly reduced in K562 cells transfected with miR-302 cluster mimics compared with negative control (Figure 2B). HUVEC capillary tube formation assay results showed that miR-302 cluster mimics-transfected K562 cells presented incomplete and fluffy tubular structures with less tube length, but miRNA negative control-transfected cells formed elongated and robust tubular structure (Figures 2C and S1). These findings indicate that miR-302 cluster functions as a tumor suppressor in CML carcinogenicity.

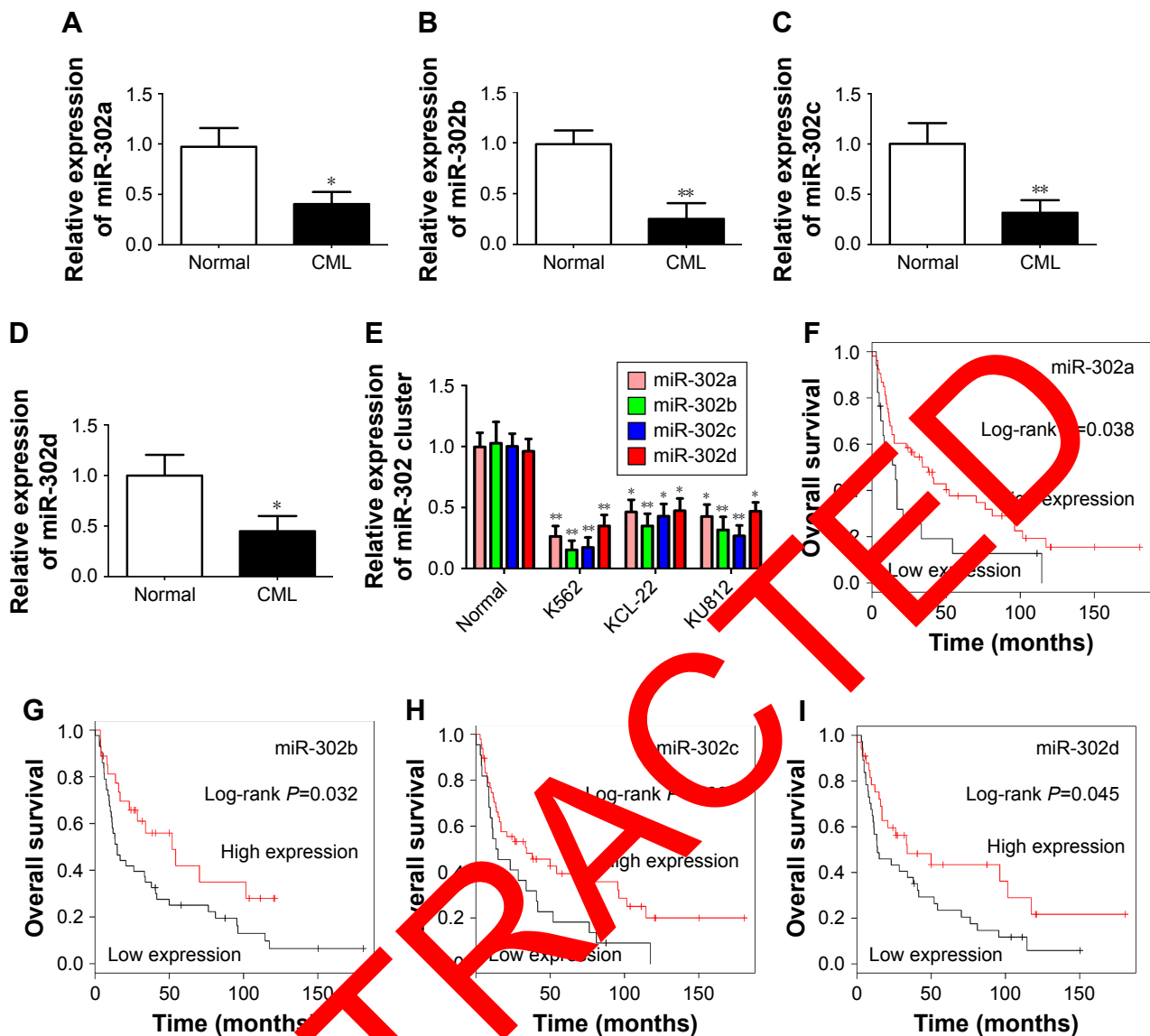


Figure 1 Downregulated miR-302 cluster expression is associated with poor overall survival of CML patients.

Notes: qRT-PCR was performed to determine miR-302 cluster expression in 70 CML patients and 20 healthy age-matched controls. (A) miR-302a expression. (B) miR-302b expression. (C) miR-302c expression. (D) miR-302d expression. (E) miR-302 cluster expression in three CML cell lines and normal control cells detected by qRT-PCR. Overall survival Kaplan–Meier curves of miR-302 were plotted in 70 CML patients. (F) Prognostic value of miR-302a. (G) Prognostic value of miR-302b. (H) Prognostic value of miR-302c. (I) Prognostic value of miR-302d. (F–I) The threshold between the high and low groups was “median”. * $P < 0.05$, ** $P < 0.01$.

Abbreviations: CML, chronic myeloid leukemia; qRT-PCR, quantitative reverse transcriptase PCR.

VEGFA is target gene of miR-302 cluster

Several studies have demonstrated that VEGFA is a well-known driver of tumor angiogenesis in many human cancers. To investigate whether miR-302 cluster exerts its function

through regulating VEGFA, we searched for its putative binding miRNAs using TargetScan and starBase. Both online tools suggested VEGFA is a candidate target gene for miR-302 cluster (Figure 3A). Luciferase reporter assays revealed that miR-302 cluster mimics significantly decreased luciferase activity co-transfected with WT 3'UTR of VEGFA compared with miRNA negative control, but the activity of luciferase transfected with Mut 3'UTR vector was not significantly changed (Figure 3B). To directly determine the effect of miR-302 cluster on VEGFA expression, we performed qRT-PCR and Western blot analysis. As shown in Figure 3C and D, miR-302 cluster mimics obviously reduced

Table 2 Fold change of miR-302 cluster in K562 cells transfected with miRNA mimics compared with negative control

| miRNA | Fold change |
|----------|--------------|
| miR-302a | 123.14±19.36 |
| miR-302b | 105.60±11.87 |
| miR-302c | 117.92±15.24 |
| miR-302d | 134.28±16.73 |

Note: Data presented as mean ± SD.

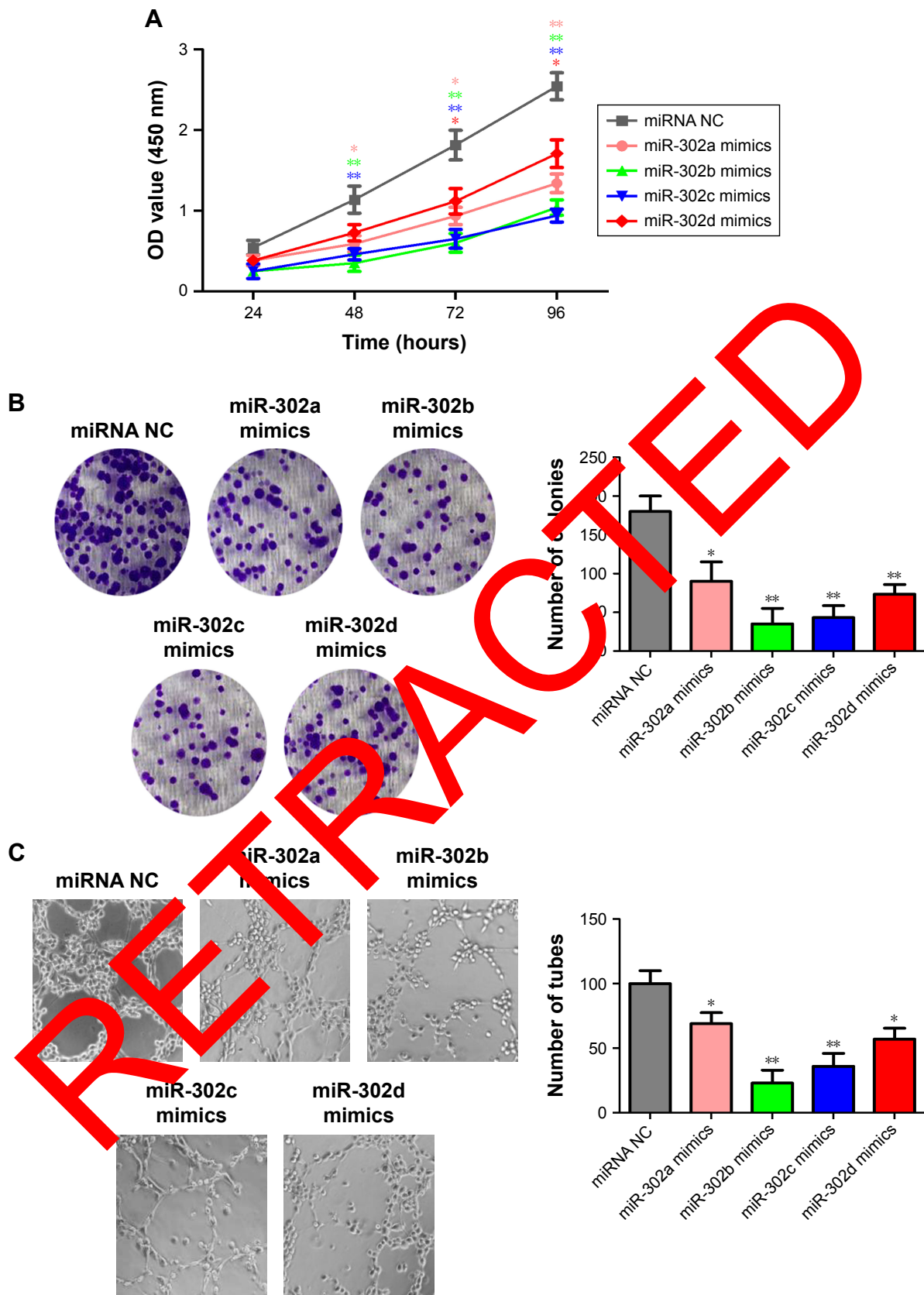


Figure 2 Overexpression of miR-302 cluster inhibits cell growth, colony formation and angiogenesis.

Notes: (A) Effect of miR-302 cluster on cell viability was assessed using CCK-8 assay. (B) Representative images of colony formation assay of K562 cells transfected with miR-302 mimics and miRNA NC. (C) Conditioned medium from miR-302 cluster mimic-transfected K562 cells significantly inhibited capillary tube formation. * $P < 0.05$, ** $P < 0.01$.

Abbreviations: CCK-8, Cell Counting Kit-8; NC, negative control.

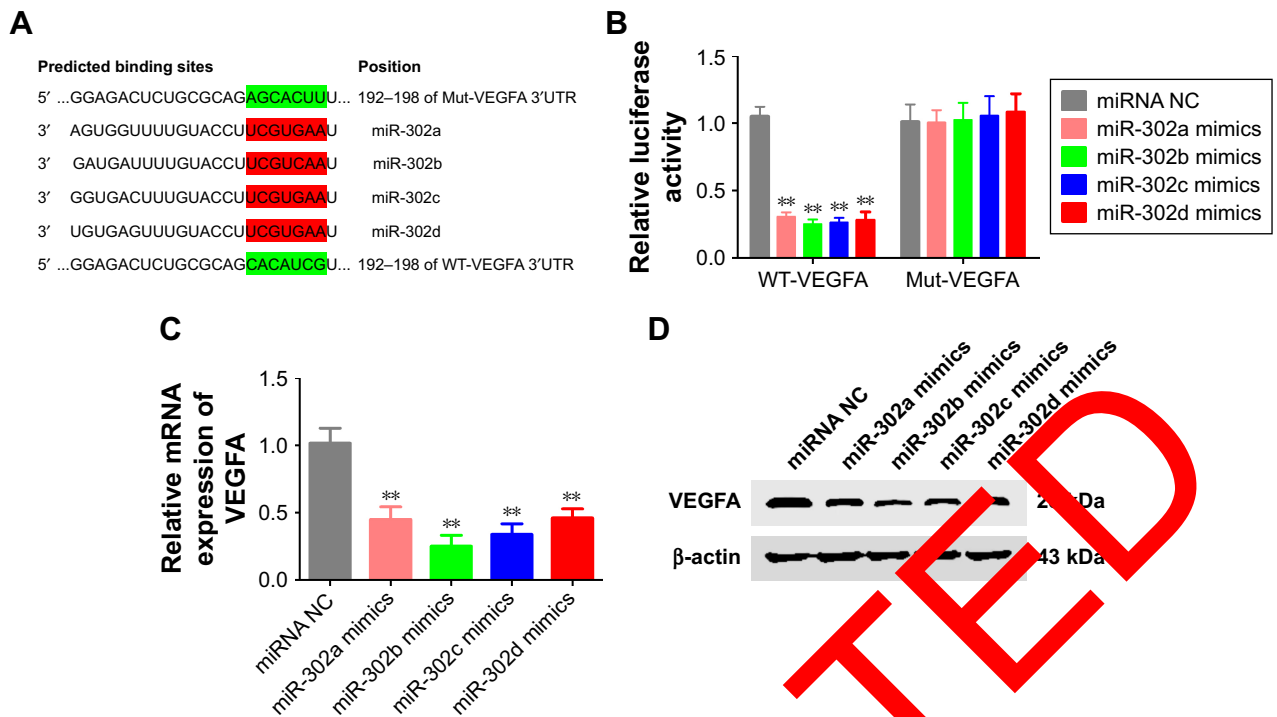


Figure 3 miR-302 cluster directly targets VEGFA.

Notes: (A) Base pairing between miR-302 cluster and the putative target site in the 3'UTR of VEGFA predicted by TargetScan. (B) K562 cells were co-transfected with WT or Mut 3'UTR of VEGFA and miR-302 cluster mimics or miRNA NC. After 48 hours of transfection, luciferase activity was measured using dual luciferase assay. (C) miR-302 cluster mimics suppressed VEGFA mRNA expression in K562 cells, which was determined by qRT-PCR. (D) miR-302 cluster mimics suppressed VEGFA protein expression in K562 cells, which was determined by Western blot. ** $P < 0.01$.

Abbreviations: Mut, mutant; NC, negative control; qRT-PCR, quantitative reverse transcriptase PCR; VEGFA, vascular endothelial growth factor A; WT, wild type.

VEGFA expression in both mRNA and protein levels. Taken together, these results indicate that VEGFA is a direct target gene of miR-302 cluster.

Relationship between miR-302 cluster and VEGFA in CML samples

To further verify the regulatory relationship between miR-302 cluster and VEGFA, we assessed the mRNA level of VEGFA in CML samples using qRT-PCR. The mRNA level of VEGFA in CML samples was upregulated 3.21-fold compared with normal samples (Figure 4A). As shown in Figure 4B–E, Pearson correlation analysis revealed that miR-302 cluster was significantly associated with VEGFA mRNA level in CML patients ($P < 0.0001$, $R = -0.5713$; $P < 0.0001$, $R = -0.6605$; $P < 0.0001$, $R = -0.6707$ and $P < 0.0001$, $R = -0.7733$; respectively).

Overexpression of VEGFA abates the inhibition of miR-302 cluster on cell growth and angiogenesis

To confirm the regulatory roles of VEGFA in miR-302 cluster-mediated inhibition of cell growth and angiogenesis,

pcDNA 3.1-VEGFA vector was transfected into K562 cells. The mRNA and protein levels of VEGFA were confirmed using qRT-PCR and Western blot. The results showed both mRNA and protein levels of VEGFA were significantly upregulated (Figure 5A and B). CCK-8 assay results demonstrated overexpression of VEGFA notably increased cell viability compared with that of co-transfected with VEGFA and miR-302 cluster or transfected with pcDNA 3.1 vector alone (Figure 5C). In addition, pcDNA3.1-VEGFA transfection dramatically increased the number of colonies compared with negative control (Figure 5D). Similarly, more elongated and robust tubular structure was observed in VEGFA-transfected cells (Figure 5E). These results validate that miR-302 cluster regulates cell growth and angiogenesis at least partially through targeting VEGFA.

Discussion

miR-302 cluster has been identified as a tumor suppressor in many human cancers; yet, its function in CML tumorigenesis remains largely unclear.^{14–17} In this study, we first determined miR-302 cluster expression levels in CML samples and cell lines and found that miR-302a, miR-302b, miR-302c and

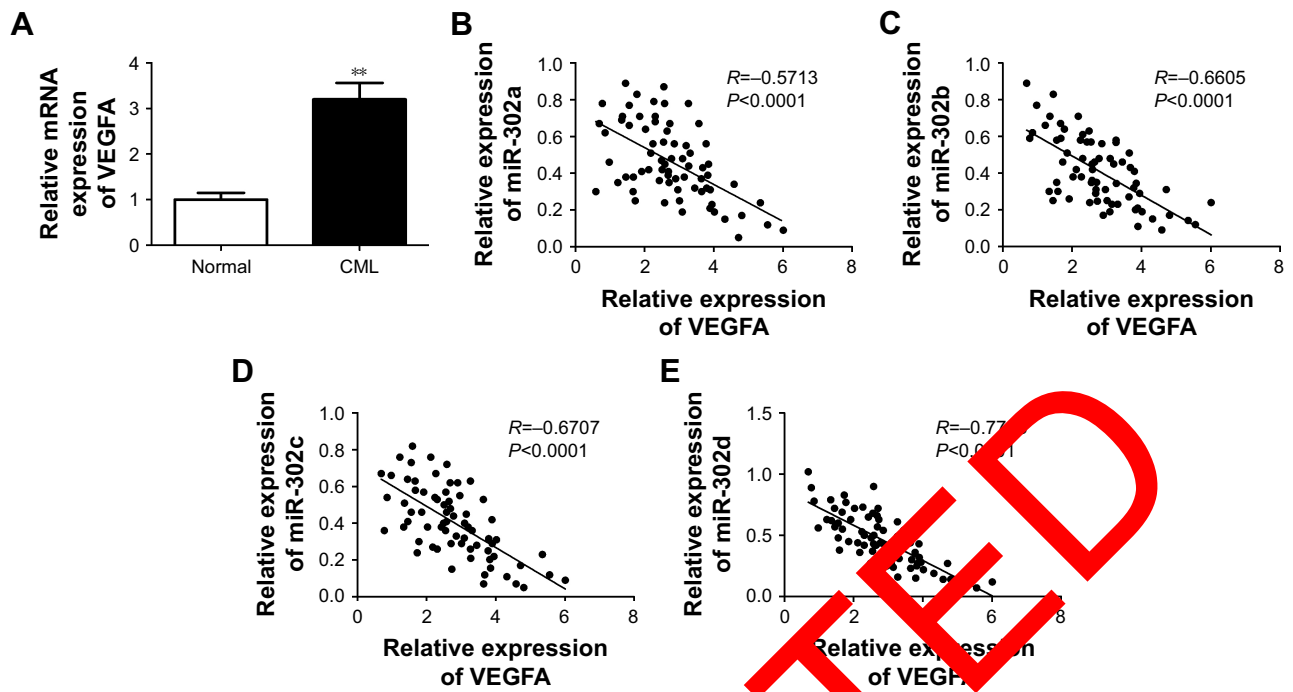


Figure 4 Expression level of miR-302 cluster is significantly negatively associated with VEGFA mRNA expression in CML patients. **Notes:** (A) VEGFA mRNA expression level in 70 CML patients and 20 healthy age-matched controls was determined using qRT-PCR. Pearson correlation analysis was performed to evaluate the relationship between miR-302 cluster and VEGFA in CML patients. (B) Pearson correlation analysis for miR-302a and VEGFA. (C) Pearson correlation analysis for miR-302b and VEGFA. (D) Pearson correlation analysis for miR-302c and VEGFA. (E) Pearson correlation analysis for miR-302d and VEGFA. ** $P < 0.01$. **Abbreviations:** CML, chronic myeloid leukemia; qRT-PCR, quantitative reverse transcriptase PCR; VEGFA, vascular endothelial growth factor A.

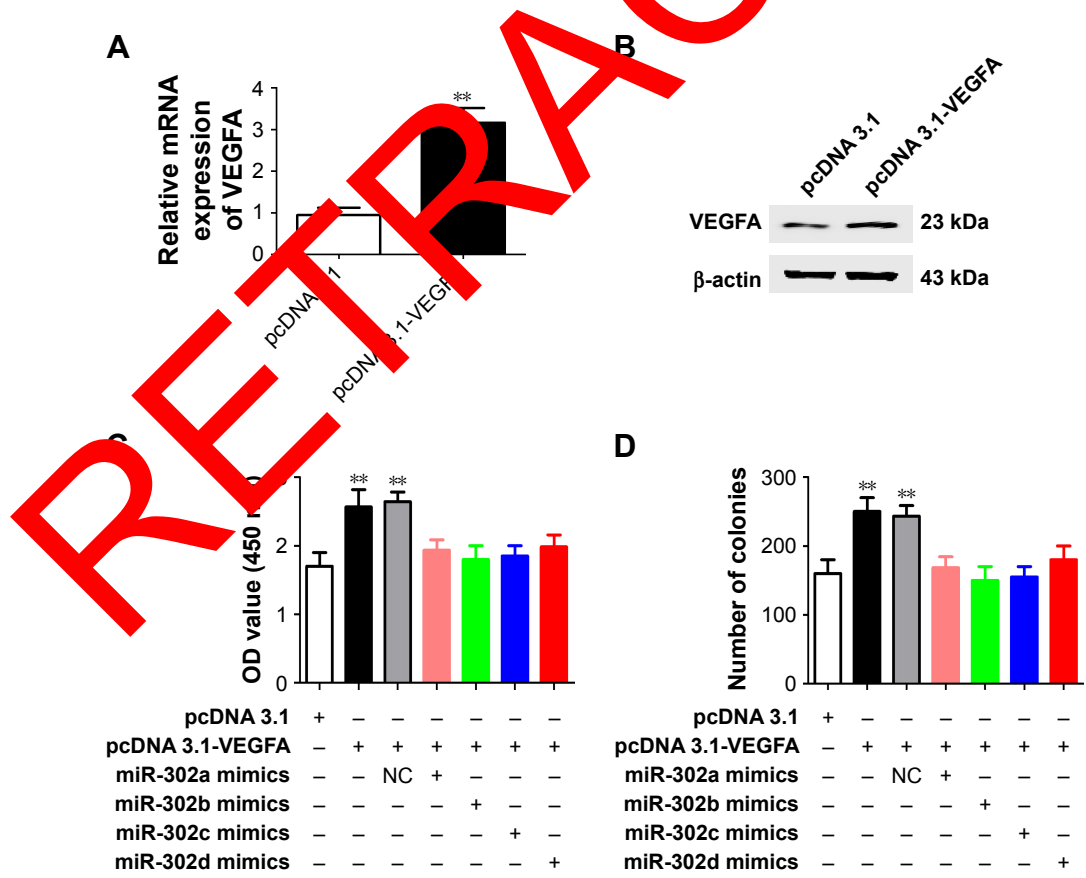


Figure 5 (Continued)

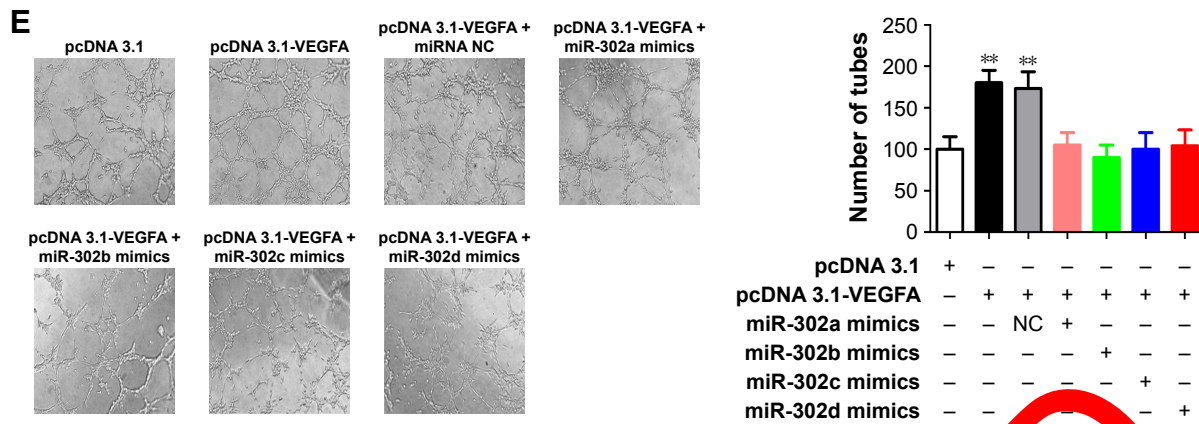


Figure 5 VEGFA is essential for miR-302 cluster-mediated cell growth and angiogenesis in CML.

Notes: (A) VEGFA mRNA level was determined by qRT-PCR in K562 cells transfected with pcDNA 3.1-VEGFA or pcDNA 3.1. (B) VEGFA protein level was determined by Western blot in K562 cells transfected with pcDNA 3.1-VEGFA or pcDNA 3.1. (C) Cell viability was assessed by CCK-8 assay after 72 hours of transfection. (D) pcDNA 3.1-VEGFA transfection increased colony-formation ability of K562 cells compared with that of cells co-transfected with pcDNA 3.1-VEGFA and miR-302 cluster mimics or transfected with pcDNA 3.1 vector alone. (E) Effect of miR-302 cluster on angiogenesis was evaluated by HUVEC capillary tube formation assay. * $P < 0.05$.

Abbreviations: CCK-8, Cell Counting Kit-8; CML, chronic myeloid leukemia; HUVEC, human umbilical vein endothelial cell line; NC, negative control; qRT-PCR, quantitative reverse transcriptase PCR; VEGFA, vascular endothelial growth factor A.

miR-302d were frequently downregulated. Meanwhile, high expression level of miR-302 cluster was significantly associated with good prognosis of CML patients. These findings suggest that miR-302 cluster may serve as a useful diagnostic marker and be involved in CML pathogenesis.

We observed that miR-302 cluster mimics could significantly suppress cell growth, colony formation and angiogenesis of K562 cells compared with miRNA negative control. In fact, miR-302 cluster was first known as a stem cell marker. Recent studies revealed that miR-302 cluster also played an important role in controlling tumor malignant phenotype as well as chemoresistance. In breast cancer, miR-302 was reported to inhibit cell metastasis and sensitize cells to cisplatin, adriamycin and mitomycin.^{27–30} In acute myeloid leukemia, Liu et al found that miR-302a was downregulated and overexpression of miR-302a sensitized leukemia cells to etoposide by targeting K562.³¹

To investigate the underlying mechanisms by which miR-302 cluster influences growth and angiogenesis of K562 cells, bioinformatics methods were used to predict the possible target genes. We found the 3'UTR of VEGFA contains binding sites of miR-302 cluster. qRT-PCR, Western blot and luciferase reporter assay results confirmed that VEGFA was a direct target of miR-302 cluster. These findings were consistent with a previous study in which Qin et al reported that miR-302a inhibited hepatocellular carcinoma cell proliferation and invasion through targeting VEGFA.³² We further revealed that there was a negative correlation between miR-302 cluster and VEGFA mRNA level in CML patients. Moreover, overexpression of VEGFA could relieve

the suppressive effect of miR-302 cluster on cell growth and angiogenesis. Taken together, these results indicate that miR-302 cluster inhibits cell growth and angiogenesis partly through regulating VEGFA.

VEGFA is frequently upregulated in many tumors and its expression level is correlated with tumor stage and progression. It is well known that VEGFA and its receptor VEGFR2 are the key regulators for tumor angiogenesis. A number of kinases, including ERKs, Src, PI3K/Akt, FAK, Rho GTPases and MAPK, have been identified as downstream effectors of VEGFA.³³ Further investigations are needed to explore the underlying mechanisms through which miR-302 cluster/VEGFA influences growth and angiogenesis in CML.

Conclusion

In summary, the present study revealed for the first time that frequently downregulated miR-302 cluster was associated with poor prognosis in CML patients. Our findings also demonstrated that miR-302 cluster inhibited growth and angiogenesis of K562 cells by targeting VEGFA. Thus, miR-302 cluster may be a potential prognostic and therapeutic target in CML. Understanding the underlying mechanisms of miR-302 cluster in CML may contribute to developing the adjuvant antiangiogenic therapy based on VEGFA target.

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Disclosure

The authors report no conflicts of interest in this work.

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