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## Long Noncoding RNA MEG3 Inhibits Cell Proliferation and Metastasis in Chronic Myeloid Leukemia via Targeting miR-184

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Maternally expressed gene 3 (MEG3), a long noncoding RNA, has been reported to be associated with the pathogenesis of multiple malignancies. However, little is known regarding the role of MEG3 in leukemia. In this study, we found that the expression of MEG3 was decreased in leukemia patients and cell lines and has potential to be considered as a biomarker for leukemia. In addition, overexpression of MEG3 inhibited cell proliferation and invasion in vitro and in vivo. Moreover, a potential bonding site between miR-184 and MEG3 was predicted, and low expression of miR-184 was found in leukemia patients and cell lines. In vitro loss and gain of function showed that overexpression of MEG3 significantly decreased the expression of miR-184, and MEG3 knockdown markedly increased it. Furthermore, our results showed that MEG3 interacted with miR-184 and subsequently mitigated the proliferation and invasion of leukemia cells by downregulating related proteins. In conclusion, our study has identified a novel pathway through which MEG3 acts as a tumor suppressor in leukemia at the level of miRNAs and provided a molecular basis for potential applications of MEG3 in the prognosis and treatment of leukemia.

**Key words:** Long noncoding RNAs (lncRNAs); Maternally expressed gene 3 (MEG3); miR-184; Leukemia

### INTRODUCTION

Chronic myeloid leukemia (CML) is a major myeloproliferative neoplasm, which accounts for around 15% of newly diagnosed leukemia in adults<sup>1</sup>. Currently, CML treatment is based on the use of the tyrosine kinase inhibitors (TKIs), such as imatinib, which inhibit the tyrosine kinase activity of the breakpoint cluster region (BCR)-Abelson murine leukemia (ABL) protein<sup>2-4</sup>. However, TKIs can effectively target proliferating mature cells, but do not eradicate leukemic stem cells<sup>5</sup>. Patients can develop resistance to TKIs, which limits the long-term benefits of the drug<sup>6</sup>. Thus, a deeper understanding of the molecular and genetic mechanisms that induce the initiation and progression of CML is crucial.

Long noncoding RNAs (lncRNAs) are a type of RNAs that are longer than 200 nucleotides and are implicated in multiple biological processes. Recently, lncRNAs were recognized to be diagnostic and prognostic biomarkers for malignant tumors. Maternally expressed gene 3 (MEG3) is one of the most important lncRNAs, which is located on chromosome 14q32 and expressed in many normal tissues<sup>7</sup>. Loss of MEG3 has been shown in various human cancers, including bladder, bone marrow, breast, and liver cancers, and exogenous expression of MEG3 significantly

suppresses the proliferation of human cancer cells<sup>8,9</sup>. However, the biological significance of MEG3 in leukemia is not well explored.

MicroRNAs (miRNAs) are a conserved family of small noncoding RNA molecules that posttranscriptionally regulate gene expression. It was estimated that about 60% of genes can be regulated by miRNAs. miR-184 is a recently identified cancer-related miRNA. It was reported to be highly expressed in many cancers, such as hepatocellular carcinoma, glioblastoma, and pancreatic ductal adenocarcinoma<sup>10-12</sup>. In addition, the upregulation of miR-184 may facilitate cell proliferation and invasion<sup>10-12</sup>. However, the exact expression of miR-184 and the molecular mechanism underlying its role in leukemia have not been reported.

In this study, we examined the expression of MEG3 and miR-184 in leukemia patients, cell lines, and mouse models in an attempt to provide a new basis for the development of the mechanism of leukemia.

### MATERIALS AND METHODS

#### *Patients and Samples*

Peripheral blood samples were obtained from 57 patients with acute myeloid leukemia (AML) (diagnosed from January 2015 to December 2016 at the Department of

Hematology in the First Affiliated Hospital of Xinxiang Medical University) and 57 healthy volunteers. Written informed consent was signed by all participants. This study was approved by the ethics committee of the First Affiliated Hospital of Xinxiang Medical University.

#### Cell Culture

THP-1, HL-60, CCL-240, and CRL-1582 cells (ATCC, Manassas, VA, USA) were grown in RPMI-1640 medium complemented with 10% FBS (v/v; Life Technologies, Grand Island, NY, USA). All cells were cultured at 37°C in a 5% CO<sub>2</sub> incubator.

#### Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from cells or tissues using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Equal amounts of RNA were reverse transcribed to cDNA with the SuperScript Reverse Transcriptase Kit (Thermo Fisher Scientific, Waltham, MA, USA). Then the total cDNA was amplified and analyzed by SYBR Green PCR Master Mix (Thermo Fisher Scientific) in a Fast Real-Time PCR 7500 System (Applied Biosystems, Foster City, CA, USA). The following primers were used<sup>12,13</sup>: MEG3, 5'-CTGCCC ATCTACACCTCACG-3' (forward) and 5'-CTCTCCG CCGTCTGCGCTAGGGGCT-3' (reverse); miR-184, 5'-TACGACTATGTGACCTGCCTG-3' (forward) and 5'-TGGTTCAACT CTCCT TTCCA-3' (reverse); GAPDH, 5'-GGCCTTCCGTGTTCTTAC-3' (forward) and 5'-TG TCATCATATCTGGCAGGTT-3' (reverse). The original Ct values were adjusted to GAPDH.

#### Construction of the Recombinant Lentiviral Vector

The MEG3 gene was designed and synthesized by PCR according to the GenBank of human MEG3 (NR\_002766) gene sequence<sup>14</sup>. The LV-MG3 was constructed essentially as previously described<sup>15</sup>.

#### Cell Viability Analysis

HL-60 cells were cultured on a 96-well plate and transfected with LV-GFP or LV-MEG3 for various times. Cell viability was then measured by the CCK-8 kit (Beyotime Biotechnology, Shanghai, P.R. China) according to the manufacturer's instructions.

#### Western Blot

Total protein samples from cells and tissues were prepared with standard protocol. Western blot was performed essentially as previously described<sup>16</sup>.

#### Cell Invasion Analyses

The invasion capacity of the HL-60 cells was examined using Transwell invasion assay. Briefly, cells were

plated in the upper chamber in serum-free medium, and 20% FBS was added to the medium in the lower chamber. After incubating for 24 h, noninvading cells were removed from the top well with a cotton swab; the bottom cells were fixed in 95% ethanol and stained with hematoxylin. The cell numbers were determined by counting of the penetrating cells under a microscope at 200× magnification on 10 random fields in each well.

#### Bioinformatics Data Set

Prediction of the interaction between MEG3 and miR-184 was performed using DIANA TOOLS (<http://diana.imis.athena-innovation.gr/DianaTools>) as previously described<sup>17</sup>.

#### Luciferase Reporter Assays

The 3'-UTR of MEG3 mRNA containing miR-184 binding sites was PCR amplified and inserted to downstream of a *Renilla* luciferase reporter gene in the pGL3 vector. In addition, a mutant construct containing mutations within the binding sites was generated using the TaKaRa MutanBEST Kit (TaKaRa, Shiga, Japan) according to the manufacturer's instructions. HL-60 cells were cotransfected with miR-184 mimics and wild-type or mutant luciferase reporter plasmid by Lipofectamine 2000 reagent (Invitrogen). At 24 h after transfection, the luciferase activities were measured with a dual-luciferase reporter assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions.

#### Animal Work and Experimental Protocols

Fifty athymic BALB/c mice (Laboratory Animal Center of Xinxiang Medical University, Xinxiang, P.R. China) were used in the present study. Mice were housed under controlled conditions (25±2°C, 70% humidity, and 12-h light/dark periods) and fed a regular sterile chow diet and water ad libitum. All mice were randomly divided into two groups. One group was subcutaneously inoculated in the back with 5×10<sup>6</sup> HL-60 cells transfected with LV-MEG3, and another group was treated with scrambled HL-60 cells. Animals were monitored for signs of tumor growth. Tumor volumes were calculated at 6–30 days after injection. Upon termination, the tumor tissues were collected. All the animal experiments were performed according to relevant national and international guidelines and were approved by the Animal Experimental Ethical Committee.

#### Statistical Analysis

All results were presented as mean±SD. The statistical significance of the studies was analyzed using Student's *t*-test. The difference was considered statistically significant with a value of *p*<0.05.

## RESULTS

### *Expression of MEG3 Is Decreased in Leukemia Patients and Cell Lines*

To understand the biological significance of lncRNA MEG3 in leukemia, the mRNA levels of MEG3 were examined in serum from 57 patients. The results showed that the expressions of MEG3 in patients with leukemia were significantly higher than those in healthy controls ( $p < 0.01$ ) (Fig. 1A). The expressions of MEG3 were subsequently measured in THP-1 cells and three leukemia cell lines (HL-60, CCL-240, and CRL-1582). As shown in Figure 1B, the mRNA expression levels of MEG3 were significantly increased in leukemia cells compared with HL-60 cells ( $p < 0.01$ ). These results indicate that leukemia cells display a low expression of MEG3.

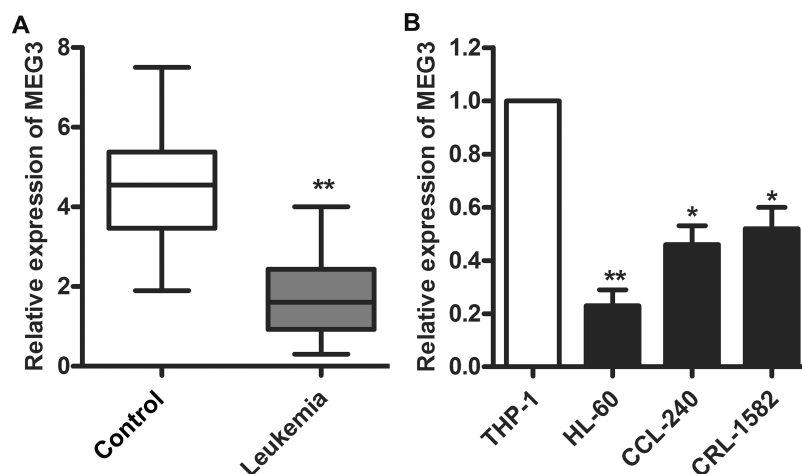
### *MEG3 Overexpression Suppresses Proliferation and Invasion of Leukemia Cells*

To investigate the effect of MEG3 on cell proliferation and invasion, HL-60 cells were transfected with LV-MEG3 to overexpress MEG3. According to the qRT-PCR analysis, the relative mRNA level of MEG3 was significantly increased after the transfection of LV-MEG3 ( $p < 0.01$ ) (Fig. 2A). This revealed that the LV-MEG3 used in the present study successfully overexpressed MEG3 in HL-60 cells. The cell viability analysis showed that MEG3 overexpression significantly decreased cell proliferation after 72 or 96 h posttransfection in HL-60 cells ( $p < 0.05$ ) (Fig. 2B). Then a Matrigel Transwell assay was carried out. As shown in Figure 2C and D, the capacity of cell invasion was obviously decreased in HL-60 cells after MEG3 overexpression ( $p < 0.01$ ). In addition, the expression levels of proliferation-associated

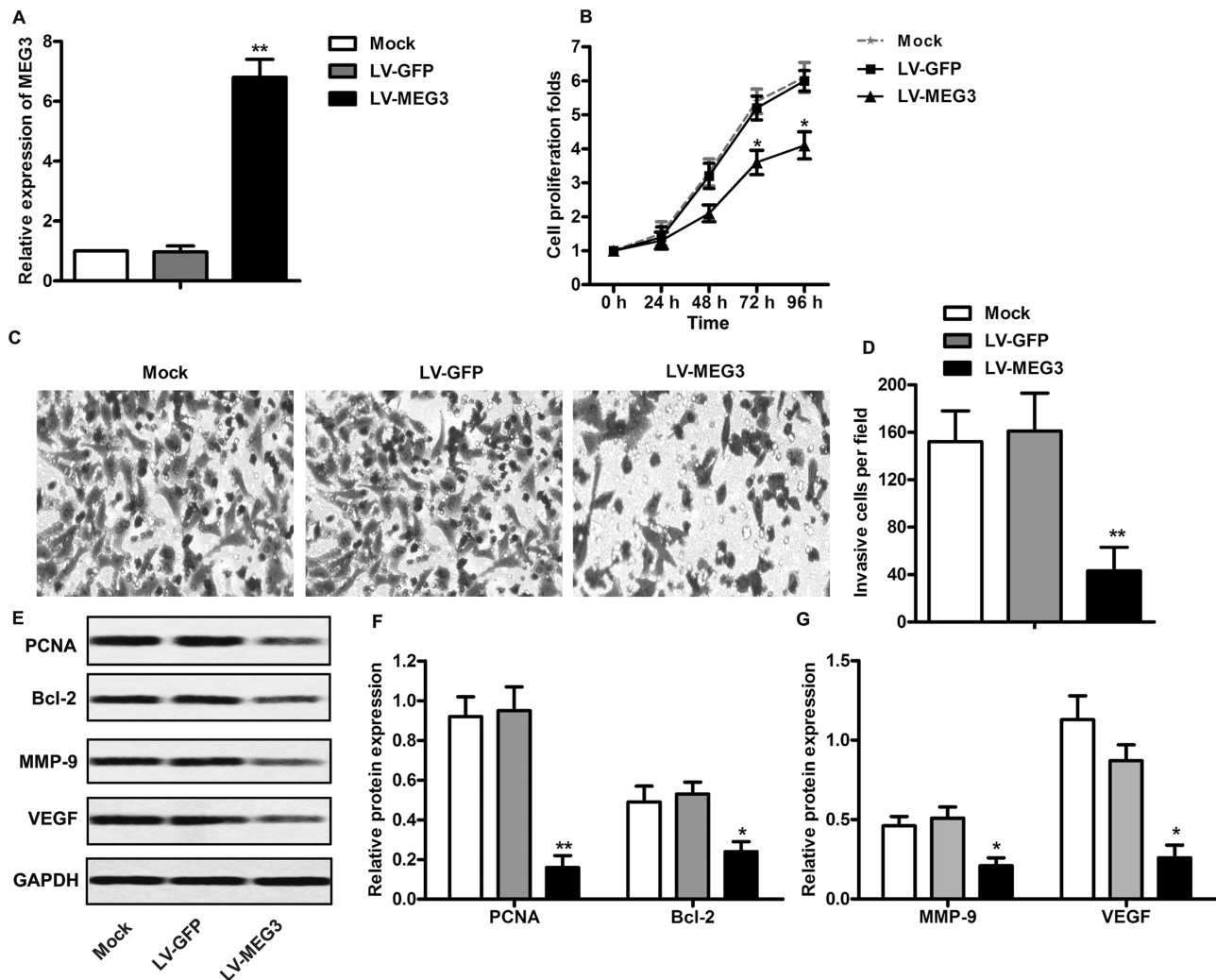
proteins were measured in LV-MEG3-treated cells. As shown in Figure 2E and F, the expression levels of proliferating cell nuclear antigen (PCNA) and B-cell lymphoma 2 (BCL-2) were significantly decreased in LV-MEG3-treated HL-60 cells ( $p < 0.01$ ). Because vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) play an important role in tumor progression by promoting migration and invasion, the effect of MEG3 overexpression on the expression levels of these proteins was determined in HL-60 cells. As anticipated, the expressions of MMP-9 and VEGF were significantly decreased after transfecting with LV-MEG3 ( $p < 0.05$ ) (Fig. 3E and G). Taken together, these findings indicate that MEG3 overexpression may inhibit the proliferation and invasion of leukemia cells through the downregulation of proliferation- and invasion-related proteins.

### *MEG3 Downregulates miR-184 Expression in Leukemia*

Many studies have reported that miR-184 is highly expressed in various tumors<sup>10,12</sup>, and our bioinformatics analysis indicated that MEG3 and miR-184 have a targeted correlation (Fig. 3A). Based on this, we established a luciferase reporter containing exact or mutant miR-184 binding sites. The results showed that the miR-184 mimic dose-dependently decreased the luciferase activity of wild-type MEG3 reporter plasmid ( $p < 0.01$ ) (Fig. 3B). However, the reducing effect was abolished on mutant MEG3 reporter plasmid. We next investigated the expressions of miR-184 in serum from 57 patients. The results showed that the expressions of miR-184 in patients with leukemia were significantly higher than those in healthy control ( $p < 0.05$ ) (Fig. 3C). As before, we subsequently



**Figure 1.** The expression of maternally expressed gene 3 (MEG3) is decreased in leukemia patients and cell lines. (A) The mRNA levels of MEG3 in serum from 57 leukemia patients were assayed by quantitative real-time polymerase chain reaction (qRT-PCR). (B) The mRNA levels of MEG3 in THP-1, HL-60, CCL-240, and CRL-1582 cells were assayed by qRT-PCR. All the experiments were repeated at least three times. \* $p < 0.05$ , \*\* $p < 0.01$  versus normal cells or serum.



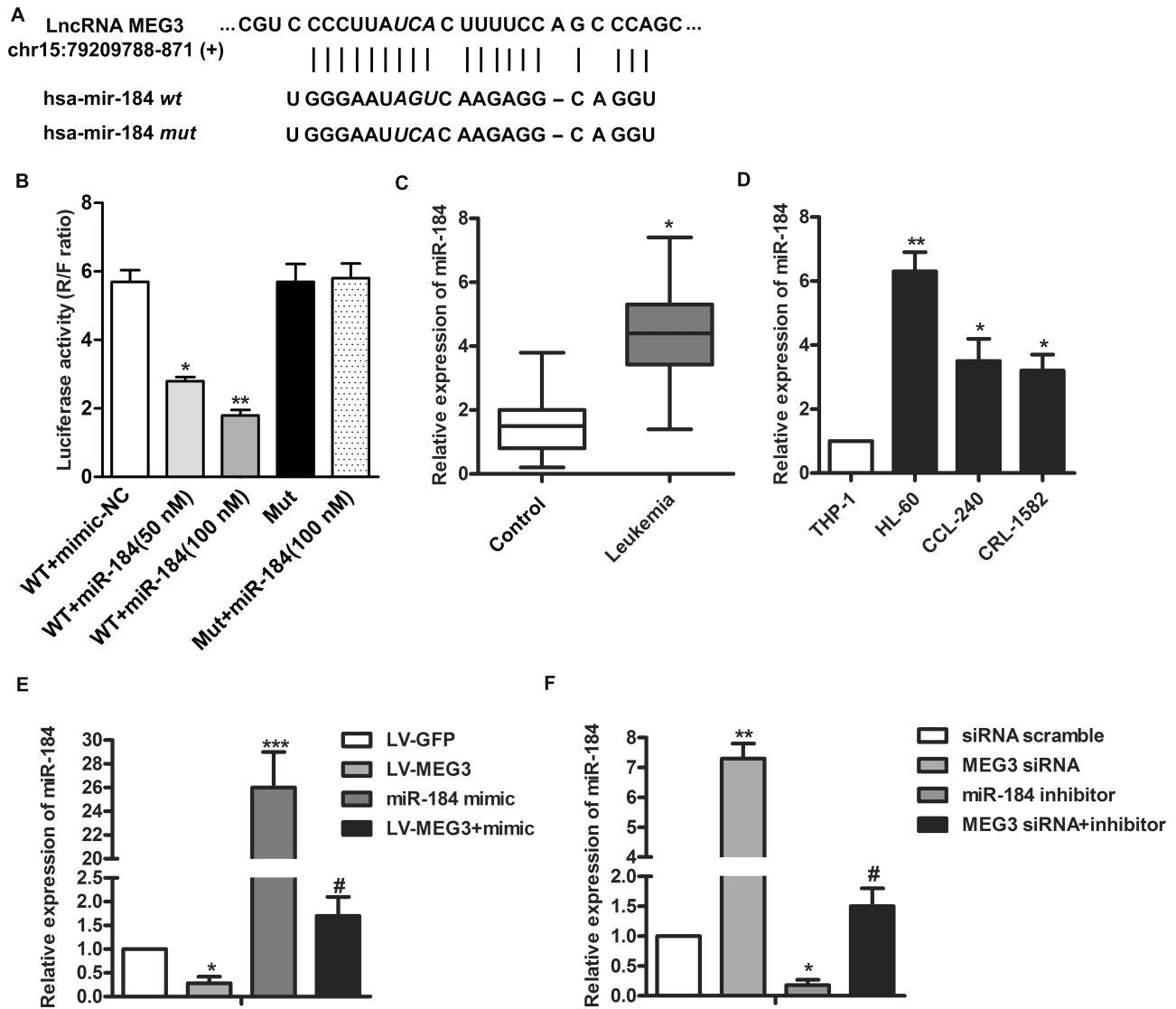
**Figure 2.** MEG3 overexpression suppresses proliferation and invasion of leukemia cells. (A) HL-60 cells were transfected with LV-GFP or LV-MEG3 for 24 h, and the mRNA levels of MEG3 were measured by qRT-PCR. (B) HL-60 cells were transfected with LV-GFP or LV-MEG3 for 24, 48, 72, and 96 h, and the cell viability was assayed by the CCK-8 kit. (C) Matrigel invasion assay. (D) Quantifications of (C). (E) Protein levels of proliferating cell nuclear antigen (PCNA), B-cell lymphoma 2 (BCL-2), matrix metalloproteinase 9 (MMP9), and vascular endothelial growth factor (VEGF) were assayed by Western blot. (F, G) Quantifications of (E). All the experiments were repeated at least three times, and the representative data are shown. GAPDH was used as loading control. \* $p < 0.05$ , \*\* $p < 0.01$  versus mock.

compared the expressions of miR-184 in THP-1 cells and leukemia cell lines (HL-60, CCL-240, and CRL-1582). As shown in Figure 3D, the mRNA expression levels of miR-184 were significantly elevated in leukemia cells compared with THP-1 cells ( $p < 0.05$ ). To further determine the relationship between MEG3 and miR-184, the expression levels of miR-184 in cells transfected with LV-MEG3 and/or the miR-184 mimic were detected. As shown in Figure 3E, the expression levels of miR-184 were significantly decreased in HL-60 cells transfected with LV-MEG3 ( $p < 0.05$ ). LV-MEG30 also partially downregulated miR-184 mimic-induced overexpression

of miR-184 ( $p < 0.05$ ). Then inhibition of MEG3 and miR-184 was carried out. The results showed that MEG3 siRNA markedly increased the expression of miR-184 ( $p < 0.01$ ) (Fig. 3F), but the promotion was reduced in the presence of the miR-184 inhibitor ( $p < 0.05$ ) (Fig. 3F). These results suggest that MEG3 may downregulate the expression of miR-184.

#### *MEG3 Downregulates miR-184 and Mitigates the Progression of Leukemia*

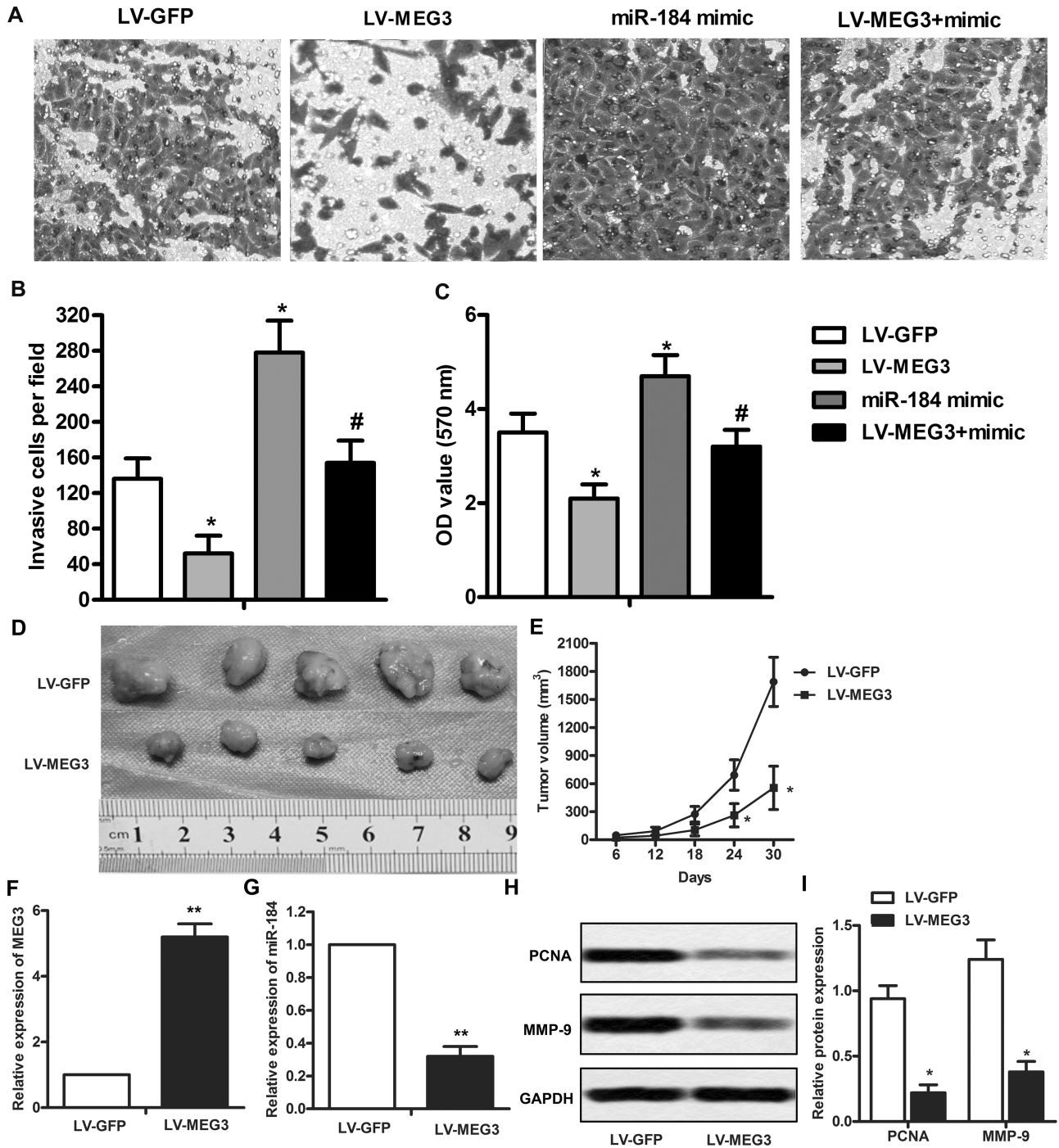
To further determine the biological significance of miR-184 in the progression of leukemia, cell invasion



**Figure 3.** MEG3 downregulates miR-184 expression in leukemia. (A) Bioinformatics analysis of MEG3 and miR-184. (B) HL-60 cells were transfected with luciferase reporter plasmid that contained the wild-type or mutant binding site of miR-184 and cotransfected with different levels of the miR-184 mimic for 24 h. Cell lysates were assayed for luciferase activity. (C) The mRNA levels of miR-184 in serum from 57 leukemia patients were assayed by qRT-PCR. (D) The mRNA levels of miR-184 in THP-1, HL-60, CCL-240, and CRL-1582 cells were assayed by qRT-PCR. (E) HL-60 cells were transfected with the LV-MEG3 and/or miR-184 mimic for 24 h, and the mRNA levels of miR-184 were assayed by qRT-PCR. (F) HL-60 cells were transfected with the MEG3 siRNA and/or miR-184 inhibitor for 24 h, and the mRNA levels of miR-184 were assayed by qRT-PCR. All the experiments were repeated at least three times. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  versus normal cells or scramble. # $p < 0.05$  versus miR-184 mimic or inhibitor treated alone.

and proliferation assays were carried out. As shown in Figure 4A and B, LV-MEG3 significantly reduced miR-184 overexpression-induced cell invasion ( $p < 0.05$ ). Similar results were found in the cell proliferation assay ( $p < 0.05$ ) (Fig. 4C). We next assessed the effect of MEG3 overexpression on tumor growth and metastasis in mice injected with HL-60 cells. As shown in Figure 4D and E, the tumor volumes in the MEG3 overexpression mouse model were markedly smaller than those in the scrambled

model at 24–30 days ( $p < 0.05$ ). In addition, the expression of MEG3 was significantly increased in tumor tissues ( $p < 0.01$ ) (Fig. 4G), whereas the expression of miR-184 was decreased ( $p < 0.01$ ) (Fig. 4H). Furthermore, Western blot analysis showed that the expressions of PCNA and MMP9 were significantly decreased in the MEG3 overexpression model (Fig. 4I and J). Taken together, these results suggest that the interaction between MEG3 and miR-184 may contribute to the progression of leukemia.



**Figure 4.** MEG3 downregulates miR-184 and mitigates the progression of leukemia. HL-60 cells were transfected with the LV-MEG3 and/or miR-184 mimic for 24 h. (A) Matrigel invasion assay. (B) Quantifications of (A). (C) Cell viability was assayed by the CCK-8 kit. (D) Mice were injected intraperitoneally with MEG3 overexpression or scramble HL-60 cells, and the tumor size was measured by ruler at 30 days. (E) Calculation of tumor volume at 6, 12, 18, 24, and 30 days after injection. (G, H) The expressions of MEG3 and miR-184 in tumor tissues were assayed by qRT-PCR. (I) Western blot analysis of PCNA and MMP9 in the tumor tissues. (J) Quantitation of (I). All the experiments were repeated at least three times. \* $p < 0.05$ , \*\* $p < 0.01$  versus scramble. # $p < 0.05$  versus miR-184 mimic treated alone.

## DISCUSSION

In this study, we have investigated the role of MEG3 in leukemia and made several novel observations. MEG3 has a low expression in leukemia cells. miR-184 is highly expressed in leukemia and closely related to tumor progression. MEG3 can target and downregulate the expression of miR-184.

CML is a malignant hematopoietic stem cell disorder whose most important characterization is Philadelphia chromosome translocation. The translocation results in a tyrosine kinase driving expansion of myeloid progenitors<sup>18</sup>. At least one third of patients will eventually fail imatinib treatment, resulting in rapid disease relapse, and a significant proportion of these will progress toward blast crisis (BC), which is usually rapidly disastrous<sup>19</sup>. Increased proliferation and apoptosis resistance are among the mechanisms of myeloproliferative disorder<sup>20</sup>. Thus, there has been increasing interest in finding new strategies to override apoptosis resistance and consequently improve treatment outcome in CML patients.

Accumulating evidence suggests that lncRNAs play diverse roles in human carcinoma. Many lncRNAs have been linked to tumorigenesis, either as oncogenes or tumor suppressors. While the underlying mechanism of many of these lncRNAs remains to be elucidated, it is clear that lncRNAs contribute to dysregulation of gene expression in leukemia, which then results in cancer initiation, development, and progression. For example, lncRNA HULC is frequently overexpressed in CML cells and positively correlated with malignancy of patients<sup>16</sup>. Another lncRNA, MIAT, is upregulated in established leukemia/lymphoma cell lines and functions to protect malignant mature B cells from cell apoptosis<sup>21</sup>. Xing et al. found that HOTAIR knockdown inhibits cell growth and colony formation and also induces the apoptosis of leukemia cells<sup>22</sup>.

Unlike the majority of lncRNAs, MEG3 has been verified in several tumors to function as a tumor suppressor. MEG3 inhibits the proliferation of cervical carcinoma cells through the induction of cell cycle arrest and apoptosis<sup>23</sup>. Upregulation of MEG3 induces autophagy to inhibit tumorigenesis and progression of epithelial ovarian carcinoma<sup>24</sup>. Recently, MEG3 was found downregulated in AML. Moreover, there was a hypermethylation of the MEG3 promoter in AML patients, which may result from the decreased Tet methylcytosine dioxygenase 2 (TET2) activity<sup>25,26</sup>. In the present study, the role of MEG3 in leukemia was investigated in more depth. The results showed that MEG3 expression was lower in CML patients and leukemia cell lines than in the controls. In addition, overexpression of MEG3 notably decreased cellular proliferation and invasion in HL-60 cells, which may be attributed

to the downregulation of proliferation- and invasion-related proteins. This provides strong evidence that MEG3 acts as a tumor suppressor in human leukemia.

Although much remains to be learned, emerging evidence suggests that miRNAs play an important role in the pathogenesis of tumor development by regulating target mRNA. As one newly discovered miRNA family member, miR-184 has been found to be abnormally expressed in various tumor cells, either as an oncogene or a tumor suppressor. Gao et al. showed that the expression of miR-184 was upregulated in hepatocellular carcinoma tissues<sup>10</sup>. Inhibition of miR-184 suppressed HepG2 cell proliferation and induced HepG2 apoptosis by regulating INPPL1 expression<sup>10</sup>. Su et al. demonstrated that overexpression of miR-184 repressed renal cancer cell migration, inhibited cell proliferation, and induced cell apoptosis<sup>27</sup>. In this study, the bioinformatics analysis revealed that MEG3 contains a potential miRNA response element (MRE) for miR-184 in its 3'-UTR. Moreover, we validated miR-184 as a target of MEG3 using overexpression, knockdown, and reporter plasmid techniques. Furthermore, miR-184 was found overexpressed in leukemia patients and cell lines. These observations strongly indicate that MEG3 acts as an oncogene and downregulates by MEG3 in human leukemia.

MMPs are a proteolytic enzyme metal ion-dependent Zn superfamily, and the main function is to degrade a variety of extracellular matrix (ECM), especially the basement membrane in ECM tumor invasion. MMP2 and MMP9 are two important factors in the MMP family and are closely related with tumor invasion and metastasis<sup>28-31</sup>. A recent study showed that overexpression of MEG3 can inhibit the invasion and migration of the pancreatic cancer cell line PANC-1 by inhibiting the expression of MMP2 and MMP9<sup>32</sup>. In our study, we found that MEG3 overexpression effectively inhibited the expression of miR-184 and subsequently mitigated the proliferation and invasion of leukemia cells by downregulating PCNA and MMP9 in vitro and in vivo. These findings not only indicated the regulatory role but also presented a potential downstream target of MEG3 in leukemia.

In summary, we found that MEG3 is downregulated in leukemia and proposed a new mechanism through which MEG3 exerts its antitumor role at the level of miRNAs. Our results revealed that MEG3 may competitively bind miR-184 and regulate the progression of leukemia. Understanding the precise molecular mechanism is vital for exploring new potential strategies for early diagnosis and therapy. Our experimental data also suggested that targeting the MEG3-miR-184 axis may represent a novel therapeutic application in leukemia.

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