Dysfunction of the WT1-\textit{MEG3} signaling promotes AML leukemogenesis via p53-dependent and -independent pathways

Y Lyu$^{1,2,7}$, J Lou$^{1,3,7}$, Y Yang$^{1,2,7}$, J Feng$^{1,7}$, Y Hao$^{1}$, S Huang$^{1}$, L Yin$^{1,2}$, J Xu$^{1}$, D Huang$^{1,2}$, B Ma$^{1,3}$, D Zou$^{1}$, Y Wang$^{1,2}$, Y Zhang$^{1}$, B Zhang$^{1}$, P Chen$^{4}$, K Yu$^{5}$, EW-F Lam$^{6}$, X Wang$^{1}$, Q Liu$^{1}$, J Yan$^{1,2}$ and B Jin$^{1}$

Long non-coding RNAs (lncRNAs) play a pivotal role in tumorigenesis, exemplified by the recent finding that IncRNA maternally expressed gene 3 (\textit{MEG3}) inhibits tumor growth in a p53-dependent manner. Acute myeloid leukemia (AML) is the most common malignant myeloid disorder in adults, and \textit{TP53} mutations or loss are frequently detected in patients with therapy-related AML or AML with complex karyotype. Here, we reveal that \textit{MEG3} is significantly downregulated in AML and suppresses leukemogenesis not only in a p53-dependent, but also a p53-independent manner. In addition, \textit{MEG3} is proven to be transcriptionally activated by Wilms' tumor 1 (\textit{WT1}), dysregulation of which by epigenetic silencing or mutations is causally involved in AML. Therefore \textit{MEG3} is identified as a novel target of the \textit{WT1} molecule. Ten–eleven translocation-2 (\textit{TET2}) mutations frequently occur in AML and significantly promote leukemogenesis of this disorder. In our study, \textit{TET2}, acting as a cofactor of \textit{WT1}, increases \textit{MEG3} expression. Taken together, our work demonstrates that \textit{TET2} dysregulated \textit{WT1-MEG3} axis significantly promotes AML leukemogenesis, paving a new avenue for diagnosis and treatment of AML patients.


development of human cancer, including AML.\textsuperscript{10} Dysregulation of the \textit{WT1} gene, located on chromosome 11p13, encodes a transcriptional regulator that is capable of activating or repressing gene transcription.\textsuperscript{14,15} The precise role of \textit{WT1} in hematopoiesis and its contribution to leukemogenesis are open for speculation. It is reported that \textit{WT1} mutations are associated with an extremely poor outcome,\textsuperscript{15} and they can lead to progression of leukemia by confronting drug resistance.\textsuperscript{16,17} Dysregulation of the \textit{WT1} gene by epigenetic modifications or mutations might promote leukemic cell proliferation and impair differentiation.\textsuperscript{18,19} However, the role of \textit{WT1} in regulating cancer-related gene expression remains largely unknown, especially whether \textit{WT1} controls \textit{MEG3} activity remains to be explored.

The ten–eleven translocation (\textit{TET}) family proteins \textit{TET1}, \textit{TET2} and \textit{TET3} constitute a novel family of dioxygenases, whose functions are to demethylate DNA sequence by converting 5-methylcytosine to 5-hydroxymethylcytosine.\textsuperscript{20} Pathologically, \textit{TET2} is frequently mutated in hematopoietic malignancies of the myeloid lineage, particularly in AML.\textsuperscript{21} Most recently, \textit{WT1} is found to physically interact with \textit{TET2} and recruit it to the target genes of

1Department of Hematology, the Second Affiliated Hospital, Institute of Cancer Stem Cell, Cancer Center, Dalian Medical University, Dalian, China; 2Department of Hematology, the Second Affiliated Hospital, Institute of Hematopoietic Stem Cell Transplantation of Dalian Medical University, Liaoning Hematopoietic Stem Cell Transplantation Medical Center, Dalian Key Laboratory of Hematology, Dalian Medical University, Dalian, China; 3Department of Neurosurgery, the Second Affiliated Hospital of Dalian Medical University, Dalian, China; 4Department of Obstetrics and Gynecology, the Second Xiangya Hospital, Central South University, Changsha, China; 5Department of Cellular Biology and Anatomy, Augusta University, Augusta, GA, USA and 6Department of Surgery and Cancer, Imperial College London, London, UK. Correspondence: Professor B Jin or Professor J Yan or Professor Q Liu or Dr X Wang, Department of Hematology, the Second Affiliated Hospital, Institute of Cancer Stem Cell, Cancer Center, Dalian Medical University, No.9 West Section, Lvshun South Road, Dalian 116023, China.
E-mail: jinbilian@dmu.edu.cn or yanjsdmu@126.com or luop@mail.sysu.edu.cn or wangxs281@dmu.edu.cn

These authors contributed equally to this work.

Received 26 August 2016; revised 16 March 2017; accepted 4 April 2017; accepted article preview online 12 April 2017.
WT1, suggesting that TET2 may be involved in the transcriptional activity of WT1 in AML.

In this study, we demonstrate that inactivation of MEG3 promotes AML leukemogenesis in a p53-dependent as well as a p53-independent mode. Further analyses show that WT1 specifically binds to the MEG3 promoter and activates its transcription, and thereby, inducing a reduction of its downstream molecule MDM2. We further investigate the possible association between TET2 and WT1 in AML, and the results suggest that TET2 acts as a cofactor of WT1 to promote MEG3 transcription. Given the importance of the WT1-MEG3 axis in suppressing tumor growth, our findings suggest that targeting this axis may represent a novel approach for effective AML treatment.

MATERIALS AND METHODS

More detailed information on materials and methods can be found in the Supplementary Information.

Patient and tissue samples

Forty-two AML patient samples were analyzed at the time of diagnosis in this study. AML patients were classified according to French-American-British. All patients gave their written informed consent. The study has been approved by the Ethics Committee of the Institute. Mononuclear cells were isolated by density gradient centrifugation using Lymphoprep, and cryopreserved. In addition, 15 potential donors for allogeneic bone marrow transplantation were used as normal controls. Highly enriched human CD34+ cells (>90%) were derived from bone marrow mononuclear cells using MiniMACS (Miltenyi Biotech, Bergisch Gladbach, Germany) following the manufacturer’s instructions. Confirmation of bone marrow-derived CD34+ cells phenotype and purity was assessed by immunophenotypic analysis using CD34-FITC (BD Biosciences, San Diego, CA, USA) coupled with flow cytometry. All patients’ samples and controls were provided by the Second Affiliated Hospital of Dalian Medical University.

Statistical analysis

Student’s t-test (two-tailed), t-test with Welch’s correction, Log-rank (Mantel–Cox) test, F-test were performed to analyze the data using GraphPad Prism 6.0 software (GraphPad software Inc., San Diego, CA, USA). P-values < 0.05 were considered statistically significant. *P < 0.05; **P < 0.01; ***P < 0.001.

RESULTS

LncRNA MEG3 is downregulated in AML

To gain insights into the patterns of activation of MEG3 gene in the AML pathogenesis, we examined its expression in normal CD34+ bone marrow cells and AML patient samples with different WT1 or TET2 mutation status. Given some variations were usually not regarded as true missense mutations (including P29R, I1762V, V218M, L1721W and H1778R) in TET2, only nonsense (4/42, 9.5%) and frameshift mutations (9/42, 21.4%) were used in our analysis. WT1 mutations (frameshift) were detected in two patients (2/42, 4.8%; Supplementary Tables 1 and 2). Real-time quantitative PCR results indicated that MEG3 was robustly expressed in bone marrow CD34+ cells, significantly downregulated in all AML samples, particularly in the WT1- or TET2-mutant AML subtypes (Figures 1a and b).

For subsequent cell model experiments, we also investigated the genetic backgrounds (Supplementary Tables 1 and 2) and p53 levels (Supplementary Figure 1A) of eight representative cell lines derived from AML (K562, TF-1, MOLM-13, U937, NB4, Kasumi-1, KG-1 and HL-60) and the correlations between WT1, TET2 mutation status/expression levels and MEG3 inactivation in these cells (Supplementary Figure 1B). As expected, MEG3 displayed negligible expression in the WT1-mutant AML cell lines (U937) relative to WT1- and TET2-wild-type AML cells (Figure 1c). Taken together, these findings demonstrate that dysregulation of MEG3 expression definitely correlates with WT1 or TET2 mutations, which thus probably plays an important role in AML pathogenesis.
Figure 2. Effect of MEG3 on cell proliferation and apoptosis in vitro. (a) Western blotting analysis of p53 protein levels in samples with/without TET2 or WT1 mutations. (b) RT-qPCR analysis of MEG3 RNA expression in MOLM-13 cell line. (c) MTT assay of the proliferation of MOLM-13 cell line. (d) The bar chart represented the percentage of cells in G0/G1, S or G2/M phase, as indicated. (e) The apoptotic rates of cells were detected by flow cytometry. (f) Western blotting analysis of MDM2, p53, GDF15 and p21 after pCDH-MEG3 and control transfection. (g) Induction of TP53 promoter activity by MEG3 in MOLM-13 cell line. (h) RT-qPCR analysis of MEG3 RNA expression in U937 and HL-60 cell lines. (i) MTT assay of the proliferation of U937 and HL-60 cell lines. (j) The bar chart represented the percentage of cells in G0/G1, S or G2/M phase, as indicated. (k) The apoptotic rates of cells were detected by flow cytometry. (l, m) Western blotting analysis of MDM2, AKT, PI3K, RB, hypophospho-RB (S249/T252) and DNMT3A after pCDH-MEG3 and control transfection in U937 and HL-60 cell lines. (n) RT-qPCR analysis of DNMT3A mRNA expression in U937 and HL-60 cell lines. Results shown were from three independent experiments. ACTB or GAPDH protein was used as an internal control for western blotting analysis. *P < 0.05; **P < 0.01; ***P < 0.001. NS, not significant; RT-qPCR, real-time quantitative PCR.
MEG3 suppresses tumor growth through a p53-dependent pathway

MEG3 has been widely demonstrated to exert its biological functions via p53 signaling.\(^{24-26}\) We reasoned that MEG3 should module AML pathogenesis to some extent in a similar way. On the basis of the data of genetic status of AML samples (Supplementary Tables 1 and 2), we discovered that p53 protein levels were dramatically reduced in samples with TET2 or WTI mutations (Figure 2a). Then we examined the regulatory roles of MEG3 in cellular phenotypes and key signaling molecules in TP53wt AML cell line. Overexpression of MEG3 in MOLM-13 (WT1wt, TET2wt and TP53wt) cell line was found to significantly suppress cell

Figure 3. MEG3 inhibits AML leukemogenesis in vivo. (a) Relative MEG3 RNA expression in U937 cells stably transfected with CTRL and MEG3-OE. (b) Relative MEG3 RNA expression in bone marrow of PBS (n = 3), U937-CTRL (n = 6) and U937-MEG3-OE (n = 8) at 4 weeks. (c) Peripheral blood WBC count at 4 weeks. (d) CD45\(^+\) and CD38\(^+\) immunophenotype of PBS, U937-CTRL and U937-MEG3-OE treated for 4 weeks. (e) Bone marrow morphology in PBS, U937-CTRL and U937-MEG3-OE at 4 weeks. Scale bar, 10 \(\mu\)m. (f) H&E of liver, lung and spleen of PBS, U937-CTRL and U937-MEG3-OE at 4 weeks. Scale bars represented 100 \(\mu\)m. (g) IHC of liver with CD45 antibody of PBS, U937-CTRL and U937-MEG3-OE at 4 weeks. (h) The weight of spleen of mice treated with PBS, U937-CTRL and U937-MEG3-OE. *P < 0.05; ***P < 0.001. H&E, hematoxylin and eosin; IHC, immunohistochemistry; NS, not significant; PBS, phosphate-buffered saline; WBC, white blood cells.
proliferation and induce G0/G1 cell cycle arrest and apoptosis when compared with controls (Figures 2b–e). In addition, MEG3 was found to positively regulate the expression of p53 and p53 target genes (including p21 and GDF15), but has no effect on BAX, NOXA and PUMA, and negatively regulate MDM2 expression (Figure 2f; Supplementary Figure 2A). Furthermore, luciferase reporter assays confirmed the p53 transcriptional activity in the MOLM-13 cells (Figure 2g). Intriguingly, forced expression of MEG3 had no obvious effect on PI3K, AKT, RB and hypophosphorylated RB (S249/T252) expression levels (Supplementary Figure 2B).

MEG3 suppresses tumor growth through a p53-independent pathway

Given that TP53 mutations or loss are frequently detected in patients with therapy-related AML or AML with complex karyotype,27,28 we therefore next explored whether MEG3 plays a role as tumor suppressor in AML cell lines absent of TP53. As in the TP53wt AML cell line, stable overexpression of MEG3 in the two TP53mut AML cell lines U937 (WT1mut, TET2wt) and HL-60 (WT1wt, TET2wt) significantly suppressed cell proliferation, induced G0/G1 cell cycle arrest and apoptosis when compared to controls (Figures 2h–k). In contrast, knockdown of MEG3 in K562 (WT1wt, TET2wt) and TF-1 (WT1wt, TET2wt) cell lines resulted in a significant increase in cell proliferation, a failure to restrict cell cycle progression and induce apoptosis (Supplementary Figures 2C–F). These results suggest that MEG3 also suppresses tumor growth through a p53-independent pathway.

Subsequently, we interrogated the molecular mechanism by which MEG3 negatively regulates cell proliferation in a p53-independent manner by examining the expression of MDM2, PI3K, AKT and RB. Gain- or loss-of-function experiments in the above selected AML cell lines revealed that MEG3 negatively regulates MDM2 at the protein expression level (Figure 2l; Supplementary Figure 2G). Although the changes in AKT and PI3K expression were not obvious, RB and hypophosphorylated RB (S249/T252) protein levels correlated positively with MEG3 abundances (Figures 2l and m; Supplementary Figures 2G and H).

Extensive studies have suggested that RB functions as a classic tumor suppressor and inhibits cell proliferation mainly by negatively regulating the transcription of certain genes that are required for cell cycle progression. Depletion of MDM2 increases the total RB and the active form of hypophosphorylated RB at S249/T252.13 RB lacks a DNA-binding domain and is tethered to promoters through its interaction with other sequence-specific transcription factors such as members of the E2F family of proteins. RB binds to the transcription activation domain of E2F and blocks its activity. More importantly, the RB–E2F complex also actively represses transcription on promoters that contain E2F sites.29 Indeed, DNMT3A is one of genes whose promoters contain E2F sites. To examine whether MEG3 affects the expression of DNMT3A by promoting hypophosphorylation of RB at S249/T252, we determined the expression levels of DNMT3A in AML cells after silencing or overexpressing MEG3. The results showed that both mRNA and protein expression levels of DNMT3A negatively correlated with MEG3 abundances (Figures 2m and n; Supplementary Figures 2H and I). Taken together, these data demonstrate that DNMT3A is a MEG3 downstream gene and that MEG3 probably plays crucial roles in inhibiting tumor growth by downregulating DNMT3A via the MDM2/RB signaling pathway in AML cells in the absence of p53.

MEG3 inhibits AML leukemogenesis in vivo

To investigate whether MEG3 affects AML leukemogenesis in vivo, two types of mouse models were established. We first examined the effects of MEG3 on the robust engraftment of AML in NOD-SCID mice. U937 cells transfected with pCDH-HEG3 (MEG3-OE) or control vector (CTRL; Figure 3a) were injected into 6–8-week-old mice. Four weeks after tail injection, the mice transplanted with U937-CTRL showed higher frequencies of serious paralysis and accumulation of urine in bladders than those with U937-MEG3-OE (Supplementary Figure 3A). After U937-MEG3-OE injection, MEG3 was highly expressed in the peripheral blood and bone marrow, and the number of white blood cells decreased dramatically (Figures 3b and c; Supplementary Figure 3B). Bone marrow assay showed that a significant decrease in CD45+/CD38+ cells in mice injected with U937-MEG3-OE, indicating that MEG3 inhibits the percentage of engraftment (Figure 3d). A large amount of monocellular cells with megakaryocytes was observed in AML mice (Figure 3e). Leukemic infiltration was also observed in multiple organs after robust NOD-SCID engraftment of numerous AML cells (Figures 3f and g; Supplementary Figure 3C). We also detected the more serious splenomegaly in mice injected with U937-CTRL compared with U937-MEG3-OE (Figure 3h; Supplementary Figure 3D). Furthermore, our data reveal that MEG3 expression prolongs the survival of AML mice (Figure 3i).

We next injected U937 cells transfected with pCDH-MEG3 or control into female athymic nude (nu/nu) mice. Twenty-one days after the injection, we observed that the tumors formed in the U937-MEG3-OE group were substantially smaller compared to those from the U937-CTRL group (Supplementary Figures 3E and F). The average tumor weights were markedly lower in the U937-MEG3-OE group than the control group at the end of the experiment (Supplementary Figure 3G). As predicted, the expression levels of MEG3 in tumor tissues of the U937-MEG3-OE group were significantly higher than those of the control group (Supplementary Figure 3H). Moreover, hematoxylin and eosin and immunohistochemistry showed that proliferating cell nuclear antigen levels in tumor tissues of the U937-MEG3-OE group exhibited decreased positivity for proliferating cell nuclear antigen than in those of the U937-CTRL group (Supplementary Figures 3I and J). Taken together, these results suggest that MEG3 limits the proliferation of AML cells in vivo.

MEG3 is transcriptionally regulated by WT1 in AML

WT1 mutations or inactivation have been implicated in the leukemogenesis of AML.5,30 The correlation between WT1 and MEG3 expression in clinical samples and AML cell lines therefore led us to hypothesize that the WT1 transcription factor regulates MEG3 expression. To test this conjecture, we performed in silico analysis on the MEG3 promoter sequence and identified a binding site of WT1 in this region (Supplementary Figure 4A). Relatively low-expression levels of WT1 were detected in U937 and NB4 cells (Supplementary Figures 4B and C), but a significant increase in MEG3 expression levels was observed following the transfection of wt WT1 into the two cell lines (Figure 4a). In contrast, WT1 depletion dramatically decreased MEG3 expression in KG-1 cells (WT1wt, TET2wt) (Supplementary Figures 4D and E). To examine whether WT1 can activate the transcription of MEG3, we constructed a WT1 luciferase reporter by inserting the sequence of MEG3 promoter region into the pGL4 vector. As shown in Figure 4b, compared with controls, the luciferase reporter assays revealed that overexpression of WT1 induced the transcriptional activation of MEG3, whereas knockdown of WT1 significantly reduced its transcription activity (Supplementary Figures 4F). To verify the prediction for the WT1 binding site, we performed chromatin immunoprecipitation assay in U937 and HL-60 cell lines using WT1-specific antibody after transfection of wt WT1 into the two cell lines. The chromatin immunoprecipitation results confirmed the direct binding of WT1 on the endogenous MEG3 promoter (Figure 4c). These findings provide compelling evidence that WT1 binds to MEG3 promoter and thus activates MEG3 transcription.

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WT1–TET2 complex regulates LncRNA MEG3 in AML

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MEG3 expression is associated with its first intron methylation mediated by TET2

Previous study has demonstrated that MEG3 gene expression is under epigenetic control, and aberrant CpG methylation occurs in AML patients. Downregulation of MEG3 in our TET2-mutant AML subtype samples implies that TET2 is likely to modulate MEG3 expression. We found that overexpression of MEG3 in U937 and NB4 cell lines, but not in HL-60 cells, resulted in the downregulation of MEG3 (Supplementary Figures 5A and B). Furthermore, we confirmed that the catalytic domain was required for the effect of TET2 on activating MEG3 expression, as the TET2 catalytic inactive mutant (TET2CM) could not upregulate MEG3 expression (Supplementary Figures 5C and D). DNA methylation is a common mechanism for promoter repression, and it also contributes to inactivation of enhancer elements usually existing in regions outside of promoters, particularly in introns. It is also noteworthy that expression of the MEG3-DLK1 locus may be regulated by differentially methylated regions (DMRs). Therefore, the methylation status of multiple cis elements (including promoter, enhancer, DMR and the first intron) of MEG3 gene was assayed to determine whether TET2 affects the expression of MEG3 through the epigenetic mechanism. Interestingly, after knockdown or overexpression of TET2 in K562 cells, no apparent changes in the DNA methylation levels were detected in the promoter, enhancer and DMR regions of MEG3, but the significant changes were observed in the first intron (Figure 4e; Supplementary Figures 6A and B). The same results were observed in HL-60 cell line (Supplementary Figure 6C). In agreement, the luciferase reporter assays showed that overexpression of TET2CD, but not the TET2CM, induced the transcriptional activity of the MEG3 promoter in U937 cells (Figure 4f). Collectively, these results suggest that TET2 can activate MEG3 transcription, which is associated with the ability of TET2 to mediate the MEG3 first intron DNA methylation.

The transactivation activity of WT1 on MEG3 is modulated by TET2 in AML

As described above, both WT1 and TET2 affect the transcriptional activity of MEG3. Both of them are found to be mutated, which are believed to play key roles in AML leukemogenesis. However, their mutations are mutually exclusive, indicating they may function in the same pathway to suppress AML. Moreover, WT1 has recently been demonstrated to physically bind and recruit TET2 to its target genes. This led us to propose that TET2 may serve as a cofactor of WT1 in its regulation of MEG3 transcription, as TET2...
lacks a DNA-binding domain. To validate this idea, we first determined the WT1–TET2 association by coupled immunoprecipitation and western blotting (IP-western) in NB4 and 293 T-cell lines. As shown in Supplementary Figure 7A, we demonstrated that the WT1–TET2 interaction could readily be detected in the two cell lines. In addition, WT1 was transiently expressed either alone or with shTET2 in U937 and NB4 cell lines. The cells only transfected with the WT1 vector alone expressed higher MEG3 levels than those transfected with both the WT1 and shTET2 vectors (Figure 4g; Supplementary Figures 7B and C). To elucidate the influence of the WT1 and TET2 interaction on the transcriptional activity of MEG3, the luciferase assay was carried out to test the activity of MEG3 promoter. Figure 4h showed that cells transfected only with the WT1 vector exhibited higher luciferase activity than those transfected simultaneously with the WT1 and shTET2 vectors. Moreover, silencing of WT1 expression with short hairpin RNA together with transfection with TET2CD vector in KG-1 cells resulted in higher luciferase activities compared with WT1 short hairpin RNA vector only (Supplementary Figure 7D). Subsequently, chromatin immunoprecipitation and real-time quantitative PCR assays were used to further explore whether regulation of MEG3 promoter by WT1 requires TET2. Consistently, WT1 was found to be less enriched on the MEG3 promoter in cells simultaneously transfected with WT1 and shTET2 vectors than those transfected only with WT1 vector (Figure 4i). Taken together, these results suggest that TET2 serves as a cofactor for WT1 in the regulation of MEG3 transcriptional activation in AML cells.

**DISCUSSION**

Mounting evidence has supported the notion that IncRNAs are involved in tumorigenesis. LncRNA MEG3, silenced in various types of cancer, is commonly regarded as a tumor suppressor. Although MEG3 low expression and promoter hypermethylation have been found to be markers of poor prognosis in AML patients, the functional consequence of MEG3 downregulation and underlying mechanisms involved have remained elusive. In this study, we show that MEG3 suppresses AML leukemogenesis through both p53-dependent and p53-independent pathways. More importantly, our work reveals MEG3 as a novel target of WT1 and that TET2 interacts with WT1 to co-regulate MEG3 transcriptional activation.

Our works show that ectopic expression of MEG3 suppresses cell proliferation and induces G0/G1 cell cycle arrest in AML, consistent with previous observations in other solid tumors. The inhibitory roles of MEG3 in AML leukemogenesis are further verified in two independent in vivo mouse models. It is well-known that MEG3 protects the p53 from degradation, and thus inhibits tumor growth in a p53-dependent fashion. Our experiments extend our understanding of the molecular events leading to AML leukemogenesis, where decreased expression of MEG3 significantly restricts the increase of the tumor suppressor p53 protein. These observations often further support for a common tumor suppressive mechanism, whereby MEG3 functions as a tumor suppressor, at least partly via the activation of p53 in multiple cancers.

MEG3 is capable of inducing MDM2 degradation, thereby stabilizing the p53 protein. However, p53 is often lost or mutated in patients including therapy-related AML or AML with complex karyotype. It has been demonstrated that MDM2 contributes to tumor initiation and progression even when p53 is no longer active. Our data confirm that MEG3 inhibits AML cell growth under genetic backgrounds of TP53 mutation, deletion or depletion, by decreasing MDM2 protein level. We discover that MDM2 functionally interacts with its target RB in AML. MEG3 promotes MDM2 degradation, and eventually increases the total RB level and the active form of hypophosphorylated RB. After activation, RB interacts with transcription factor E2F to negatively regulate expression of their target genes. We uncover that DNMT3A, a very important target of E2F complex, is markedly downregulated by MEG3. Given the crucial role of DNMT3A in AML, it is most probably that MEG3 inhibits tumor growth through RB-DNMT3A pathway. Apart from the p53-dependent pathway, our results unveil a novel p53-independent mechanism by which MEG3 exerts its tumor-suppressive function in AML.

Currently, the observations that WT1 is expressed in the majority of AML cases, and that it is mutated in a proportion of AMLs have led to a number of studies to decipher the mechanistic role of WT1. The WT1 mutations clustering in exons 7 and 9 are correlated with lower complete remission rates, higher relapse rates, shorter disease-free survival and overall survival. The specific genes that are regulated by WT1 and become deregulated by WT1 mutation remain an area of intense investigation.

Our findings that WT1 positively modulates the transcription of MEG3 provide an explanation for the mechanism of leukemogenesis in WT1-mutant AML.

It is long known that promoter hypermethylation is a common mechanism contributing to transcriptional repression. Moreover, DNA methylation has been found to correlate negatively with enhancer activity and gene expression, possibly by interfering with transcription factor binding. These functionally relevant binding sites for transcription factors, however, usually exist in regions outside of promoters, particularly in introns. Gene expression is usually regulated by the methylation status of DNA cis elements such as promoters, enhancers and introns. It is also noteworthy that expression of the MEG3-DLK1 locus may be regulated by DMRs. Our results show that the promoter, enhancer, DMR and the first intron of MEG3 display aberrant methylation, consistent with others findings. However, only the first intron displays significant change in methylation levels upon TET2 expression, meaning that the methylation of this region might be in a dynamic state. Differential methylation at intronic enhancers has been previously reported to affect gene expression. Our results show that the induction of MEG3 expression by TET2 is associated with methylation of its first intron. Collectively, these results suggest the presence of putative regulatory elements in the first intron of MEG3 and MEG3 expression may thus be repressed by hypermethylation as a result of lack of functional TET2, contributing to MEG3 epigenetic silencing occurred specifically in the TET2-mutant AML subtype. TET2 is able to inhibit leukemogenesis, but it is frequently mutated in AML. TET2 is found to be mutated in a mutually exclusive manner with WT1, indicating that they both may suppress AML through the same pathway. Most recently, WT1 has been demonstrated to physically interact with TET2 and recruit it to the target genes of WT1, implying that TET2 may be involved in modulating the transcriptional activity of WT1. As expected, our data confirm that WT1 interacts directly with TET2, and we further demonstrate that TET2 serves as a cofactor of WT1 to activate MEG3 expression. Not surprisingly, the mutation of TET2 substantially diminishes the transcription activity of WT1 on MEG3. Taken together, these results suggest there exists a linear TET2-WT1-MEG3 axis in the suppression of AML leukemogenesis, and therefore dysfunction of this axis; such mutations to either TET2 or WT1 may be a key contributor to AML. Evidently, our study uncovers a novel mechanism by which WT1–TET2 plays a critical role through the regulation of MEG3 gene expression.

In summary, our work demonstrates that the loss of lncRNA MEG3 leads to AML leukemogenesis, indicating that its overexpression can suppress AML development via both p53-dependent and p53-independent pathways. Furthermore, the discovery that WT1 cooperates with TET2 to upregulate MEG3 expression places TET2-WT1-MEG3 signaling axis as a central tumor suppressive pathway in AML, therefore emphasizing the potentials of this axis in AML diagnosis and therapy.
CONFLICT OF INTEREST
The authors declare no conflict of interest.

ACKNOWLEDGEMENTS
This work was supported by grants from National Natural Science Foundation of China (Nos. 81372713, 81672497, 81570124, 81207066, 81402048 and 81372741), Liaoning Provincial Natural Science Foundation of China (No. 2014023010), the Reformation Project in the Key Clinical Departments of Provincial Hospitals on Construction of Diagnosis and Treatment Capacity in Liaoning Province (LNNCC-A02-2015), Medical Research Council UK (No. MR/N020971/1), Cancer Research UK (No. A1/2011) and Breast Cancer Now (Nos. 2012MayPR070, 2012NovPhD016). We would like to thank Dr Wei Cheng (ICSC Core Facility, Dalian Medical University) for the material support.

AUTHOR CONTRIBUTIONS
Conception and design by YL, JL, YY, JF, XW, QL, JY and BJ; development of methodology by YL, JL, YY, JF; acquisition of data by YL, JL, YY, JF, YH, SH, LY, JX, DH, BM, DZ, YW, YZ, KY, BZ, PC; analysis and interpretation of data (for example, statistical analysis, flow cytometry interpretation) by YL, JL, JF, writing, review and/or revision of the manuscript by YL, JL, YY, JF, XW, EW-FL, QL, JY and BJ; administrative, technical or material support: BJ, JY, QL and XW; study supervision: BJ, JY, QL and XW.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)