Acute myeloid leukemia

Restoration of microRNA function impairs MYC-dependent maintenance of MLL leukemia

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To the Editor:

MicroRNAs (miRNAs) are a class of small noncoding RNAs that have critical functions in gene silencing by binding to complementary mRNAs to induce their degradation or translational repression [1]. Interestingly, only a small fraction of miRNAs can exert their function in the form of miRNA-induced silencing complex (miRISC) [2]. Unfortunately, although dysregulated expression or processing of cancer-related miRNAs has been demonstrated to play a crucial role in oncogenesis, the contribution of miRNA dysfunction is still poorly understood.

MLL-rearranged leukemias generally have a poor prognosis and account for 10% of overall acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) cases [3]. MLL translocations encoding chimeric fusion proteins comprising the N-terminus of MLL in frame with various fusion partner proteins are characteristically found in MLL leukemias [3]. MLL is a histone H3 Lysine4 (H3K4) methyltransferase and is proteolytically cleaved into two distinct subunits, MLLN320 and MLLC180, which non-covalently interact to form an intramolecular complex involved in epigenetic transcriptional regulation. Interestingly enough, we recently uncovered an unexpected role for MLL protein in miRNA-mediated translational repression [4]. Our studies showed that the MLLC180 subunit alone could colocalize with miRISC components in cytoplasmic processing bodies (P-bodies), and affect the function of a subset of miRNAs such as the let-7 family [4].

Multiple miRNAs have been found to be dysregulated in MLL leukemias [5, 6]. However, since only a small fraction of miRNAs are functional, it is not clear whether the expression levels of miRNAs reflect their actual contribution to the pathogenesis of MLL leukemia. Considering that the abundance of wild-type MLL protein was reduced in MLL leukemic cells [7, 8], we reasoned that the function of a subset of miRNAs would be compromised and may play a critical role in the pathogenesis of MLL leukemia.

We first addressed how P-body formation and miRNA-mediated gene silencing were affected in MLL leukemic cells. Immunofluorescence results showed that the number of DDX6- and DCP1A-marked P-bodies in MLL leukemic cells, including RS4;11, SEM, KOPN8, and THP-1 cells, was significantly fewer than in non-MLL leukemic lines such as JM1, REH, and U937 cells (Fig. 1a (i), and Supplementary Fig. S1a–c). Furthermore, the capacity of ectopically expressed let-7a or CXCR4 to silence their bulged miRNA reporters, but not perfect siRNA reporters, was markedly reduced in MLL leukemic cells (Fig. 1a (ii) and Supplementary Fig. S1a–c). Furthermore, the capacity of ectopically expressed let-7a or CXCR4 to silence their bulged miRNA reporters, but not perfect siRNA reporters, was markedly reduced in MLL leukemic cells (Fig. 1a (ii) and Supplementary Fig. S2a, b). These results were consistent with our previous findings that MLL was required for miRNA-mediated translational repression of partially matched miRNAs, but not for cleavage of perfectly matched miRNAs [4]. These defects in the MLL leukemic cells were associated with a reduced level of MLLC180 but not P-body proteins (Supplementary Fig. S2c). Further introduction of MLLC180 restored miRNA-mediated gene silencing in MLL leukemic cells (Supplementary Fig. S2d), indicating that the impairment in miRNA-mediated gene silencing in MLL leukemic cells was caused by downregulating wild-type MLL, especially the MLLC180 subunit.
MYC represents a critical cooperation pathway and a therapeutic target in MLL-rearranged AML that is frequently upregulated in this disease [9, 10]. We further showed that the proliferation of MLL-rearranged B-ALL cells was decreased upon MYC depletion (Supplementary Fig. S3a, b) and that MYC protein abundance in MLL-rearranged B-ALL cells was much higher than in non-MLL-rearranged B-ALL cells (Supplementary Fig. S3c), implying that both AML- and B-ALL-type MLL leukemic cells are generally dependent on high levels of MYC protein. However, although

and let-7a. Proteins were detected by western blot with anti-MYC antibody at 24 h post transfection. c (i) SEM cells transduced with MLLC180, together with or without MYC were subjected to western blot assays. Antibodies were used as indicated. MLL-AF4 fusion proteins were detected using antibody specifically recognizing the amino terminus of MLL. (ii) SEM and KOPN8 cells transduced with or without MLLC180 were subjected to anti-AGO1 RIP assays. Pull-down RNAs were analyzed by qRT-PCR using primers for let-7a. *P < 0.05, **P < 0.01, ***P < 0.001. Data represent mean and s.e.m. of three independent experiments.

MYC is regarded as a downstream target of MLL-fusion proteins [9], the expression level of MYC mRNA was not proportionally increased (Supplementary Fig. S3c), which was also validated using publicly available microarray datasets of well-characterized primary ALL and AML patient samples (Supplementary Fig. S3d), suggesting that translational repression of MYC mRNA was reduced in MLL leukemic cells.

Since our previous study demonstrated that MLL was required for let-7a-mediated translational repression, and
**MYC** is one of the most well-established let-7a targets, we reasoned that the inability of endogenous let-7a to repress translation of its target mRNAs may partly contribute to the high expression level of MYC protein in MLL leukemic cells. Therefore, we determined whether the translational repression function of endogenous let-7a was impaired in MLL leukemic cells. Since translational suppression of mRNA targets by mature miRNAs preferentially requires AGO1 [11], we performed AGO1 RNA immunoprecipitation (RIP) experiments and showed that the binding of both let-7a and MYC mRNA to AGO1 were reduced in MLL leukemic cells (Fig. 1b (i), and Supplementary Fig. S4a (i–ii), b). The pull-down assay using biotinylated let-7a further validated that the binding of AGO1 to let-7a was reduced in MLL leukemic cells (Supplementary Fig. S4c). Moreover, the protein levels of MYC decreased significantly after let-7a transfection in non-MLL-rearranged REH and JM1 cells, but not in MLL-rearranged SEM, RS4;11, and KOPN8 cells (Fig. 1b (ii) and Supplementary Fig. S4d, e). These results suggested that the expression of MYC proteins could escape translational repression by let-7a in MLL leukemic cells.

To confirm that the impaired translational repression of MYC by let-7a and the high level of MYC in MLL leukemic cells were caused by reduced MLL-C180 expression, we evaluated how restoring MLL-C180 affected miRNA function and MYC expression. The introduction of MLL-C180 in SEM and KOPN8 cells decreased MYC expression and impaired cell proliferation, which could be recovered by MYC overexpression (Fig. 1c (i) and Supplementary Fig. S5a, b). However, the expression level of MLL-AF4 (Fig. 1c (i) and MLL-ENL (Supplementary Fig. S5a) proteins was similar between the control and MLL-C-rescued MLL leukemic cell lines, suggesting the decreased MYC protein level in the MLL-C-rescued cells was not associated with a lower level of MLL-fusion proteins. In addition, immunofluorescence results showed that introduction of MLL-C180 increased the number of P-bodies (Supplementary Fig. S5c). The binding of let-7a and MYC mRNA to AGO1 in SEM and KOPN8 cells after MLL-C180 introduction was also enhanced as revealed by RIP assays (Fig. 1c (ii) and Supplementary Fig. S5d, e). These data indicated that the reduction of MLL-C180 played a causal role in the miRNA functional deficiency in MLL leukemic cells.

LIN28A and LIN28B can be transcriptionally activated by MYC [9], and in turn enhance the expression of MYC by blocking the expression of let-7a [12], thus forming a regulatory circuit. Although the introduction of MLL-C180 could significantly downregulate the MYC expression, it had little effect on the levels of LIN28A/LIN28B and mature let-7a (Supplementary Fig. S6a, b). Furthermore, depletion of endogenous LIN28A/LIN28B showed little effects on the binding of let-7a and MYC mRNA to AGO1 (Supplementary Fig. S6c (i–ii), d (i–ii)). These results ruled out the contribution of LIN28 in the context of MLL-C180 regulated let-7a dysfunction, highlighting the dominant role of MLL-C180 in controlling let-7a-mediated MYC suppression.

To further strengthen our findings, we examined whether miRNA-mediated gene silencing was impaired in primary MLL leukemic cells. Compared with the control cells, the MLL-AF9-transduced primary mouse bone marrow progenitor cells showed a marked reduction in the number of P-bodies (Fig. 2a (i)). In addition, the capacity for silencing the miRNA reporters (Supplementary Fig. S7a, b) and the binding of let-7a and MYC mRNA to AGO1 (Supplementary Fig. S7c) were also significantly reduced. These defects were correlated with a high expression level of MYC protein in primary MLL leukemic cells (Supplementary Fig. S7d, e). Furthermore, ectopically expressed MLL-C180 could partially rescue let-7a-mediated gene silencing in MLL-AF9-transduced primary cells (Supplementary Fig. S7a, c) and significantly delay the development of leukemia in the xenografted mice (Fig. 2a (ii) and Supplementary Fig. S7f).

A previous report revealed that pharmacologically inhibiting the IRAK pathway could substantially improve survival of mice with MLL leukemia by stabilizing the wild-type MLL protein [13]. We hypothesized that IRAK inhibition could rescue the function of let-7a and decrease the expression of MYC via restoration of MLL protein levels. We first examined the effect of an IRAK1/4 inhibitor (IRAK1/4i) on miRNA-mediated gene silencing in MLL-AF9-transduced primary cells. We observed that the capacity of let-7a for silencing the miRNA reporters (Supplementary Fig. S8a) and the binding of let-7a and MYC mRNA to AGO1 (Fig. 2b (i) and Supplementary Fig. S8b) were significantly increased upon IRAK1/4i treatment. In contrast, casein kinase II (CKII) inhibitor CX-4945, which increases the level of full-length MLL protein by blocking taspase1-dependent MLL processing [14], had very little effect in these assays. Furthermore, treatment with IRAK1/4i, but not CX-4945, led to significant MLL-C180 induction and MYC reduction (Fig. 2b (ii) and Supplementary Fig. S8c, d). These results further support our previous finding that only free MLL-C180 was implicated in miRNA-mediated translational suppression [4].

**MYC** represents a critical therapeutic target in MLL-rearranged leukemias [10]. Bromodomain and extraterminal (BET) protein inhibitors have shown profound efficacy against MLL-rearranged leukemia by inhibiting several key targets including MYC [10]. In fact, MYC restoration constitutes one of the major mechanisms of BET inhibitor resistance [15]. We therefore reasoned that IRAK1/4i may improve the efficacy of BET inhibitors by synergistically downregulating MYC in MLL leukemias.
Indeed, in line with this hypothesis, concomitant IRAK1/4 and BET inhibition synergistically led to a significant reduction of MYC protein in MLL-AF9 primary leukemia cells (Fig. 2c (i)), and synergistically inhibited the development of MLL-rearranged leukemia in a murine xenograft model (Fig. 2c (ii) and Supplementary Fig. S8e), suggesting combination therapy with IRAK1/4 and BET inhibitors can achieve better efficacy against MLL leukemias.

In summary, our results demonstrated that the dysfunction of let-7a caused by a reduced level of MLL was essential for maintaining MYC protein at a high level and sustaining the survival of MLL leukemic cells. Thus, our work has uncovered a functional link between miRNA dysfunction and MLL-rearranged leukemia. Moreover, our work revealed that IRAK1/4 inhibition can improve the efficacy of BET inhibitors in MLL leukemias by restoring...
the protein level of MLL and the MYC-suppressing function of *let-7a*. We thus propose a novel rationale for synergistically targeting MYC with both IRAK and BET inhibitors as part of a comprehensive therapeutic approach for treatment of MLL leukemias.

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**Author contributions** SHZ, XYC, RHW, and YTT designed and performed most of experiments, analyzed the data and wrote the draft paper; MLG and QYX performed some experiments; YS and DL provided technical assistance for the mouse experiments; CJZ and SJC provided expertise and extensively edited the paper; HL contributed grant support, designed the entire project, wrote the paper, and supervised the project. All authors discussed the results and commented on the paper.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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