Multi-omics reveals azacitidine partially restores the hematopoiesis-supportive functions in mesenchymal stromal cells from patients with chronic myelomonocytic leukemia

Ruohao Xu
Department of Hematology, Guangdong Provincial People's Hospital (Guangdong Academy of Medical Sciences), Southern Medical University, Guangzhou, Guangdong, 510080, P.R China

Suxia Geng
Guangdong General Hospital (Guangdong Academy of Medical Sciences)

Lingji Zeng
Guangdong General Hospital (Guangdong Academy of Medical Sciences)

Chengxin Deng
Guangdong General Hospital (Guangdong Academy of Medical Sciences)

Xin Huang
Department of Hematology, Guangdong Provincial People's Hospital (Guangdong Academy of Medical Sciences), Southern Medical University, Guangzhou, Guangdong, 510080, P.R China

Minming Li
Guangdong General Hospital (Guangdong Academy of Medical Sciences)

Ping Wu
Guangdong General Hospital / Guangdong Academy of Medical Sciences

Peilong Lai
Guangdong General Hospital, Guangdong Academy of Medical Sciences

Jianyu Weng
Guangdong General Hospital / Guangdong Academy of Medical Sciences

Xin Du (✉ xind_gdph2020@163.com)
Department of Hematology, Guangdong Provincial People's Hospital (Guangdong Academy of Medical Sciences), Southern Medical University, Guangzhou, Guangdong, 510080, P.R China

Article

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Abstract

Hypomethylating agents (HMAs) including azacitidine (AZA) represent the only FDA-approved first-line treatments for patients with chronic myelomonocytic leukemia (CMML). However, the mechanism by which HMAs produce therapeutic responses (e.g., hematological improvement) remains unclear. Bone marrow mesenchymal stromal cells (MSCs) play a crucial role in regulating the self-renewal, survival and differentiation of hematopoietic stem and progenitor cells (HSPCs). Recently, the identified sensitivity of patient-derived MSCs to HMAs underlines a critical yet unexplored role of MSCs in regulating post-HMA efficacies. By utilizing high-throughput approaches including targeted exon sequencing, DNA methylation profiling, and RNA sequencing, our present study aims to delineate the modifications and consequences of AZA on CMML-MSCs. Demonstrated by integrated multi-omics analysis, our results reveal that cytogenetically independent CMML-MSCs exhibit strikingly high amenability to AZA. Through selectively de-methylating/methylating CpGs of 1395 sensitive genes, AZA re-activates HSPC-supportive signatures and enhances the protective effect of CMML-MSCs on healthy HSPCs. Along with the reconstitution of the dysregulated methylome/transcriptome, AZA partially restored the impaired functions of CMML-MSCs to support healthy hematopoiesis in long-term co-culture conditions. Our findings suggested a niche-dependent mechanism of post-HMA recovery of normal hematopoiesis. Identifying AZA-sensitive genes and functions may be of particular value in developing niche-targeting strategies in treating myeloid malignancies.

Introduction

Chronic myelomonocytic leukemia (CMML) is an aggressive myeloid malignancy characterized by persistent peripheral monocytosis, bone marrow (BM) dysplasia, and a high risk of acute myeloid leukemia (AML) transformation(1, 2). In recent decades, efforts have been made to dissect the genetic/epigenetic events within the hematopoietic stem and progenitor cells (HSPCs) in CMML(3–6). The two FDA-approved de-methylation compounds for CMML, azacitidine (AZA) and decitabine (DEC) were initially designed to restore the disordered epigenetics in malignant HSPCs, which were thought to be disease-promoting consequences of genetic lesions involving epigenetics(2). However, demonstrated by the limited inhibitory effect on malignant clones in patients, HMAs may not produce efficacies prominently through the direct anti-tumor effect(7–10). Those post-HMA therapeutic responses (e.g., hematological improvement) (11) are more likely attributed to the selective promotion of residual healthy hematopoiesis in the bone marrow.

Bone marrow mesenchymal stromal cells (BM-MSCs) are crucial niche elements regulating the self-renewal, survival and differentiation of hematopoietic stem and progenitor cells (HSPCs)(12). BM-MNCs from patients with myeloid malignancies exhibit certain aberrant phenotypes involving impaired functions(13, 14), transcriptome(15–17), and epigenetics(18–20). However, the molecular basis for these signatures remained undetermined. Although controversial, there has been no evidence of a high frequency of genetic mutations or clonal abnormalities detected in patient-derived MSCs(21–24). Under such context, the functional sensitivity of MSCs in response to DNA de-methylation underscored a
potentially targetable HMA-sensitive niche in patients with myeloid malignancies. De-methylation of specific CpG islands in patient-derived MSCs leads to selective support of healthy HSPCs over malignant clones(20, 25). The responsiveness of MSCs to HMA also correlates with the post-HMA treatment efficacies in patients(19). However, the lack of molecular insights into the HMA-mediated regulations on MSCs has impeded the development of niche-targeting methods in treating myeloid malignancies.

In order to delineate the regulatory targets/effects of HMA on CMML-MSCs, high-throughput DNA methylation profiling and RNA sequencing (RNA-seq) were performed on paired AZA-treated (AZA+) and AZA-untreated (AZA-) MSCs from both CMML and HD individuals. Our integrated multi-omic study identified a series of AZA-sensitive genes and functions as regulatory targets of AZA. At last, as demonstrated by short-term co-culture and long-term colony-forming cell (CFC) assays in vitro, AZA partially restored the supportive abilities of CMML-MSCs to healthy HSPCs and hematopoiesis. Targeted improvement of the hematopoiesis-supportive mesenchymal niche may be of potential therapeutic value.

Results

Dysfunctional CMML-MSCs do not carry genetic mutations or chromosomal abnormalities that detected in paired MNC samples

While the dysregulated functional and transcriptomic signatures were characterized in primary uncultured CMML-MSCs in our previous report(16), the mutational and chromosomal profiles remained uninvestigated. To address this issue, we first performed genotyping on paired MNC and primary CMML-MSCs at P0 using a targeted exon sequence method (Exon-seq) to detect exon loci covering the 130 most frequently mutated genes in myeloid malignancies (Figure 1A)(Supplemental Table 1). Clinical characteristics of enrolled CMML patients and healthy BM donors (HD) were displayed (Supplemental File 1). In brief, 52 somatic mutations (corresponding to 44 genes) were identified in all 21 CMML-MNC samples, with at least one mutation detected in each sample. In accordance with the genetic features of CMML(2), the high frequency of mutated TET2 (52.4%), ASXL1 (42.9%), SRSF2 (28.6%) and DNMT3A (28.6%) was identified, further validating the CMML nature of our study cohort (Supplemental Figure 1). Interestingly, 11 mutations with VAF range from 2.26% to 90.1% were also identified in 5/8 CMML-MSC samples (Figure 1C). These mutated loci were strictly consistent with those identified in paired CMML-MNC samples. After excluding three germline mutations confirmed by oral mucosal epithelium detections in three patients (Pt#9, Pt#11, Pt#15), the VAF of recurrent mutations in CMML-MSCs was significantly lower than that in the CMML-MNCs (39.04 vs. 4.47, P< 0.0001) (Figure 1B). Thus, these detected mutations with low VAF (<10%) in primary CMML-MSCs are more likely to derive from the contamination of mutation-bearing malignant cells during adherent culture. For chromosomal evaluation, the fluorescence in situ hybridization (FISH) assay revealed the existence of chromosomal abnormalities in 33.3% of CMML-MNC samples (7/21), including +8 (n=3), aneuploidy (n=2) and complex karyotype (n=2) (Supplemental File 1). However, no chromosomal abnormalities seen identified in CMML-MNCs were simultaneously detected in paired CMML-MSC samples (Figure 1D, E). Thus, demonstrated by the
absence of mutations/chromosomal abnormalities, these data suggested CMML-MSCs do not carry
cytogenetic abnormalities consistent with those in malignant cells.

**CMML-MSCs exhibit distinct signatures of DNA methylome compared with HD-MSCs**

DNA methylation represents one of the central epigenetic modifications controlling the transcription and
expression of genes(26). Emerging evidence suggests that BM-MSCs from patients with myeloid
malignancies exhibited a disorganized DNA methylome(19, 27) along with certain functional sensitivity
to de-methylating treatment(20, 25). In order to elucidate the regulatory effects of HMA on CMML-MSCs
at both molecular and functional levels. In brief, MSCs from CMML patients (n=10) and age-matched
healthy BM donors (n=5) were expanded to passages 2-3 and pairwisely treated with 10 μM (AZA+) or
DMSO (AZA-) for 48h in vitro. Afterward, AZA+/- MSCs were harvested and subjected to DNA methylation
profiling (n=30) and parallel RNA-seq (n=30)(Figure 2A).

After quality control and normalization processes, a total of 742212 methylation probes with quantified β
values were identified from 865918 methylation probes (Supplemental Figure 2). We first determined the
baseline DNA methylome of untreated CMML-MSCs. According to genomic locations and CpG island
(CGI) features, CMML-MSCs exhibited similar DNA methylation levels to HD-MSCs among most genomic
regions. Higher methylation levels could be seen only in the “gene body” and “opensea” regions of CGI of
CMML-MSCs (Figure 2B). In the context of similar global DNA methylation, the principal component
analysis (PCA) revealed separated clusters between primary CMML-MSCs and HD-MSCs, indicating
potential differential methylation at specific loci (Figur 2C).

To further characterize the differentially methylated DNA loci between CMML-MSCs and HD-MSCs,
differentially methylated probes (DMPs) were calculated with a |Δβ|≥0.10 (in CMML-MSCs vs. HD-MSCs)
and adjusted P value ≤0.05 as a threshold of statistical significance(28). In general, a total of 62064
DMPs were identified in CMML-MSCs compared with HD-MSCs, of which 61.0% were hyper-methylated,
and 39.0% were hypo-methylated (Figure 2D). After aligning the DMPs to the corresponding genes, these
DMPs were involved in a series of fundamental pathways regulating functions of MSC, including the
PI3K-Akt signaling and MAPK signaling pathway and WNT signaling (Supplemental Figure 3). Distinct
methy whole patterns became more apparent through hierarchical clustering and heatmap of the 500 most
differentially methylated DMPs (Supplemental Figure 4A). At last, similar genomic distributions of the
hyper-/hypo-methylated were seen in these identified DMPs (Supplemental Figure 4B).

**The DNA methylome of CMML-MSCs exhibit higher amenability to AZA than that of HD-MSCs**

Next, we sought to elucidate the regulatory effects of AZA on MSCs from both functional and molecular
levels. At the standard treatment schedule of 75 mg/m², the plasma concentration of AZA achieved in
myelodysplastic syndromes (MDS) patients ranges between 3 to 11 μM(29). Thus a concentration of 10
μM AZA was utilized for subsequent experiments. Similar to our previous observations, CMML-MSCs
were associated with significantly diminished proliferative potential compared with HD-MSCs (16). The
treatment of AZA at 10 μM for 48h did not alter the morphology, viability and proliferation of CMML-
MSCs and HD-MSCs (Figure 3A-C). By comparing the DNA methylome of AZA+ and AZA- MSCs samples, our results revealed that AZA readily induced prominent global de-methylation on both CMML-MSCs and HD-MSCs. The methylation β value was significantly down-regulated across genomic and CGI regions in AZA+ CMML-MSC and AZA+ HD-MSC samples (Figure 3D, E). Interestingly, the spectrums of DMPs differed greatly between the CMML and HD groups after AZA treatment. In brief, AZA exerted prominent de-methylation effects on HD-MSCs. Among the 20775 DMPs identified in AZA+ HD-MSCs (Figure 3F), 99.98% displayed down-regulated methylation β value. Only 0.02% exhibited higher methylation β values and were aligned to SLC44A4, DLGAP2, SMU1, TSACC and TNF genes (Figure 3H). However, AZA exerted more comprehensive regulations at specific sites in CMML-MSC samples. AZA induced 106561 DMPs in AZA+ HD-MSCs (Figure 3G), and up to 15.48% exhibited increased methylation levels after AZA treatment (Figure 3I). Furthermore, only 4.2% of the hypo-methylated DMPs and 0.6% of hyper-methylated DMPs induced by AZA were shared between the CMML and HD groups (Supplemental Figure 5). These results suggest that AZA may exert more complex, bidirectional regulations in a disordered DNA methylome context.

We next focused on the AZA-mediated regulations on CMML-MSCs. Differentially methylated regions (DMRs) are genomic regions with prominently enriched DMPs and are regarded as possible functional regions involved in transcriptional regulations(30). A total of 855 DMRs aligning for 867 genes were identified in AZA+ CMML-MSCs (Supplemental File 2). As demonstrated by a cicos plot, the genomic distribution of these DMRs highly correlated with the enrichment of DMPs (Figure 4A). However, according to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database, the 867 aligned genes of identified DMRs failed to enrich for any signature (data not shown) significantly. Among these aligned genes, the molecules TET1, SFRP1 and SFPR2 play a central role in maintaining normal hematopoiesis(31-33). Visualization of individual DMR of these genes revealed that AZA readily de-methylated their CpGs at the promoter regions (Figure 4B-D). By combining data from parallel RNA-seq, our results confirm that the transcriptomic levels negatively correlated with the CpG methylation (Figure 4E-G), thus further suggesting AZA-mediated regulations on the transcriptome through modification of DNA methylome on certain genomic regions.

**AZA modulates diverse biological functions through selective targeting series of AZA-sensitive genes in CMML-MSCs**

In order to identify the regulatory targets of the AZA-mediated regulations on CMML-MSCs, we performed a multi-omics integrated analysis by combining data from DNA methylation profiling and parallel RNA-seq. The identified DMPs (AZA+ vs. AZA-) in CMML-MSCs aligned to a total of 20438 genes. Simultaneously, the parallel RNA-seq on CMML-MSC samples identified 2487 differentially expressed genes (DEGs) in AZA+ CMML-MSC samples. Among these DEGs, 1064 up-regulated DEGs (66.3%) and 331 down-regulated DEGs (37.5%) exhibited negatively correlated expression levels with their DNA methylation levels. The expression of these genes was considered to be regulated by AZA-mediated modification on DNA methylome, thus defined as AZA-sensitive genes (Figure 5A)(Supplemental File 3). By referring to the KEGG database, the up-regulated AZA-sensitive genes enriched 46 KEGG entries, while
the down-regulated AZA-sensitive genes only enriched 2 KEGG entries (Supplemental Files 4 and 5). The 20 top-listed signatures showed that AZA up-regulated diverse functions in CMML-MSCs, such as hematopoietic support (Hematopoietic cell lineage), cell adhesion-related functions (Cell adhesion molecules; ECM-receptor interaction, Focal adhesion) and immune activation (Th1 and Th2 cell differentiation; Th17 cell differentiation). AZA also up-regulates multiple pathways such as TGF-β signaling, PI3K-Akt signaling and Wnt signaling (Figure 5B).

Results from GSEA further confirmed that AZA led to a prominently enriched signature supporting hematopoietic stem cell differentiation (Figure 5C), along with 20 other enriched gene sets, most of which indicate signatures of immune activation (Supplemental file 6). Stroma-derived cytokines play a vital role in maintaining the quiescence of HSPCs and controlling normal hematopoietic differentiation(34). AZA de-methylated the CpGs of genes encoding for a range of hematopoietic supportive factors, including ANGPT1, CXCL14 and SCF. However, the CpG methylation levels of CXCL12 showed a slight but significant increase after AZA treatment (Figure 5D). Further RT-qPCR assays confirmed the significantly elevated expression of ANGPT1 and CXCL14 in AZA+ CMML-MSCs. The relative expression of SCF and CXCL12 was not significantly changed (Figure 5E).

AZA partially restored the protective/supportive functions of CMML-MSCs to healthy HSPCs and normal hematopoiesis

Under a damaged stromal niche, HSPCs colonized in it will lose their quiescent state and undergo reactive intracellular accumulation of reactive oxygen species (ROS)(35). In this case, ROS impairs the genome stability of HSPCs and induces double-strand break (DSB), which can be localized by γH2AX staining(36). Given that AZA up-regulated the expression of hematopoiesis supportive pathways and cytokines at the transcriptomic level, we sought to explore whether AZA truly restored the protective/supportive effects of CMML-MSCs to healthy HSPCs and hematopoiesis. To this end, we established a 7-day co-culture assay to evaluate the protective effect of MSCs on healthy HSPCs. Magnetically-sorted CD34⁺ HSPCs were co-cultured with AZA+ or AZA- CMML/HD-MSCs for 7 days, then their intracellular ROS levels and DSB were then detected (Figure 6A). Our data showed that HSPCs co-cultured with AZA- CMML-MSCs produced significantly higher ROS than those with AZA- HD-MSCs. Interestingly, this elevated ROS production was significantly rescued when co-cultured with AZA+ CMML-MSCs, while still higher than those of HSPCs after co-cultured with AZA+/- HD-MSCs (Figure 6B). Consistent with these observations, after co-culture with AZA+ CMML-MSCs, HSPCs displayed significantly lower γH2AX fluorescence intensity than those with AZA- CMML-MSCs, reaching a level close to that co-cultured with HD-MSCs (Figure 6C-D). These results showed that AZA improved the protective effects of functionally impaired CMML-MSCs on healthy HSPCs under a short-term co-culture condition.

Finally, we investigated whether the reconstitution of hematopoiesis-supportive genes and functions by AZA would impact the ability to support healthy hematopoiesis in CMML-MSCs. Being a validation of the restored expression of hematopoietic support factors in CMML-MSCs (Figure 7B), the protein concentration of ANGPT1 and SCF were also significantly elevated in the supernatant of CMML-MSCs
after AZA treatment (Figure 7C). Next, the hematopoietic differentiation potential of healthy HSPCs was evaluated by CFC assays after culturing on AZA+/- MSC feeder layers. After 14 days of culture in the semi-solid methylcellulose medium, the number of CFU colonies was counted (Figure 7A). CFC colonies were successfully induced on both AZA+/- CMML-MSC feeder layers (Figure 7D). Similar to previous reports on MDS-MSCs(13, 19), the number of CFU-E, BFU-E, CFU-GM, and CFU-GEMM formed by healthy HSPCs was significantly lower following co-culture on AZA- CMML-MSCs than that on AZA- HD-MSCs (Figure 7E). Surprisingly, the number of colony-forming unit-erythroid (CFU-E), burst-forming unit-erythroid(BFU-E), granulocyte-macrophage colony-forming unit (CFU-GM), and colony-forming unit-granulocyte, erythroid, macrophage, megakaryocyte (CFU-GEMM) formed on the AZA+ CMML-MSC feeder layer was significantly higher than on the AZA- CMML-MSCs. The number of CFC colonies formed by HSPCs co-cultured on AZA+ CMML-MSC feeder layer recovered to a level close to the HD-MSC group. Demonstrated by the increased number of BFU-E and CFU-E in the AZA+ HD-MSC group, our data show that AZA also promotes the hematopoiesis-supportive effect of HD-MSCs to a certain extent.

In summary, data from our multi-omics analysis and functional experiments suggest that AZA partially restores the hematopoiesis-supportive abilities through modulating the DNA methylation in CMML-MSCs (Figure 8).

Discussion

Primary MSCs from patients with myeloid malignancies exhibit aberrant phenotypes involving cellular functions(13, 14), transcriptome(15–17), and epigenetics(18–20). Inspired by these groundbreaking studies, an open question remains whether these abnormal signatures of patient-derived MSCs could be attributed to the cooccurrence of genetic lesions similar to those in malignant HSPCs. Although controversial, there has been no evidence of a high frequency of genetic mutations or clonal abnormalities detected in patient-derived MSCs(21–24). Some studies have reported various mutations or chromosomal abnormalities in the BM-MSCs from MDS/AML patients(21, 22). However, little validation was done to address whether these detected molecular/cytogenetic lesions were secondary events responding to a stressful cell culture procedure(23). In other studies, more comprehensive whole exome sequencing methods are used to detect mutations in MSC samples. Although genetic alterations were also detected, these lesions are revealed to be non-specific, and were most likely induced by stressful cell culture procedures(23, 24).

We assessed the genotype of CMML-MSCs using a targeted Exon-seq method covering the 130 most frequently mutated genes in myeloid malignancies (Fig. 1A). To avoid potential deleterious effects on the genomic stability of MSCs(23), CMML-MSCs at passage P0 were used for detections. Similar to previous reports(21, 22), gene mutations were detected in some CMML-MSC samples (62.5%). It is worth noting that these mutations in MSC samples were consistent with those detected in the CMML-MNCs, thus underlining a possibility that MSCs may share a common mutational origin with malignant cells. However, after excluding germline mutations with high VAF by oral mucosal testing, the remaining somatically mutated sites were associated with extremely lower VAFs in CMML-MSCs (Fig. 1B).
Furthermore, consistent with previous studies indicating normal chromosomes in MDS-MSCs(19), no chromosomal abnormalities were seen in these CMML-MSCs (Fig. 1D, E). Thus, being a supplement to previous reports suggesting normal cytogenetics in patient-derived MSCs(23, 24), our data do not support the existence of cytogenetically clonal MSCs in CMML patients. These detected mutations with unusually low VAFs seemed to originate from the contamination of mutation-bearing malignant cells in the primary culture.

Given that results from previous studies(23, 24) and present detections found no evidence of cytogenetically clonal aberrant MSCs, the dysregulated signatures in CMML-MSCs may be partially attributed to epigenetics. DNA methylation represents one of the central epigenetic modifications controlling transcription and gene expression(26). Indeed, our data from high-throughput DNA methylation profiling revealed a significant differential distribution of hyper-/hypo-methylated DMPs between CMML-MSC and HD-MSC samples (Fig. 2 and Supplemental Fig. 4). Enrichment of genes corresponding to these identified DMPs revealed involvements of a wide range of signaling critical for MSCs functions, including PI3K-Akt signaling, MAPK signaling pathway and WNT signaling(37) (Supplemental Fig. 3). Importantly, the theoretical reversibility of these abnormal DNA methylome signatures underlined the potential sensitivity of CMML-MSCs to de-methylating treatment.

As a validation of this hypothesis, our in vitro experiments revealed that low-dose AZA readily induced global DNA de-methylation in MSCs from both CMML and HD without affecting their proliferation and viability. Interestingly, CMML-MSCs exhibited strikingly higher amenability to AZA than HD-MSCs at the epigenetic level (Fig. 3). While causing global de-methylation, AZA exerted more comprehensive regulations at specific CpG sites of the methylome in CMML-MSC samples. Up to 15.48% of DMPs showed increased methylation levels in CMML-MSCs after AZA treatment, compared with 0.02% in HD-MSC samples. Furthermore, only 4.2% and 0.6% of the hyper-/hypo-methylated DMPs were shared between those identified in AZA + CMML-MSC and AZA + HD-MSC samples. Thus, these results suggest that AZA may exert more complex, bidirectional regulations in a disordered DNA methylome context. For instance, AZA de-methylated the hyper-methylated CpG of DNA methylase, TET1(38), and simultaneously increased its transcript level in CMML-MSCs (Fig. 5B, E). The re-expression of the epigenetic regulators may potentially contribute to the differential or even opposite epigenetic modifications, thus, at least in part, amplifying the uncertainty of de-methylating effects in CMML-MSCs.

Although AZA was reported to alter the transcriptome and cellular functions of patient-derived MSCs(20), being a globally de-methylating drug, its regulatory targets in MSCs have not yet been elucidated.

Using an integrated multi-omics strategy, combined data from DNA methylation (DMPs) and RNA-seq data (DEGs) to identify the transcriptomic targets of AZA through methylation modifications. Our data revealed that AZA selectively re-activated a series of specific cellular functions and signaling pathways by identifying and annotating AZA-sensitive genes. Similar to the report by Wenk C et al.(25), AZA prominently rescued the expression of genes participating in HSPC-supportive functions, including the activation of hematopoietic cell lineage and HSPC differentiation (Fig. 6B, O). De-methylation of the CpG
promoters and elevated expressions of a series of hematopoiesis-supportive secretory factors further suggested that AZA switched CMML-MSCs into hematopoietic-supporting phenotypes. At the same time, these AZA-sensitive genes also enriched for the WNT signaling pathway and TGF-β signaling, both of which are critical for regulating HSPC functions(20, 31).

The SFRP molecule family are well-characterized WNT secretory antagonists in the BM niche(31, 39). Epigenetic silencing of these antagonists in BM-MSCs may result in the overactivation of the WNT pathway in HSPCs, thus contributing to the exhaustion of normal HSPCs(31) and the propagation of malignant clones(20). Being a supplement to our previous report(16), the present data suggested that the decreased expression of SFRPs (SFRP1, SFRP2) in CMML-MSCs may be at least partly attributed to the hyper-methylated CpGs in these genes. Importantly, AZA readily de-methylated the CpGs and up-regulated the expression of SFRP1 and SFRP2 (Fig. 4), thus suggesting potential niche-dependent therapeutic mechanisms of AZA in regulating normal and malignant HSPCs.

At last, demonstrated by short-term/long-term co-culture and CFC assays, AZA partially restored the protective/supportive effects of CMML-MSCs to healthy HSPCs and normal hematopoiesis (Fig. 8). Enhanced clonogenicity potentials were seen in HSPCs co-cultured with AZA + CMML-MSCs feeder layer than those co-cultured with AZA- feeder layer. The number of CFU-E, BFU-E, and CFU-GM in the AZA-treated CMML-MSC group recovered to a level close to that of the HD-MSC group (Fig. 7E). These results corroborate the previous reports that AZA improves the support of MDS-MSCs for the expansion of healthy HSPCs(19). In another study, short-term AZA pretreatment of healthy or MDS-MSCs provides additional supportive effects on normal HSPCs over malignant HSPCs(25). Our results suggested that AZA also promotes the ability of HD-MSCs to support healthy hematopoiesis to a certain degree.

In sum, this comprehensive study may provide insights into the molecular basis of CMML-MSCs. The restoration of hematopoiesis-supportive functions in MSCs further suggested a potential niche-dependent therapeutic mechanism of AZA in treating CMML. Targeted improvement of the hematopoiesis-supportive mesenchymal niche may be of potential therapeutic value in treating patients with myeloid malignancies.

Methods

Please see Supplemental Methods

Declarations

Data Availability

The Gene Expression Omnibus (GEO) accession number for DNA methylation profiling data reported in this paper is GSE221269. All data generated in this study are available in the supplemental material files or available from the corresponding authors under reasonable request.
Code Availability

The code used in this manuscript is publicly available from the corresponding authors under reasonable request.

Supplemental Material

Refer to the Web version on PubMed for supplemental materials

Acknowledgments

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Author contributions

XD designed the research. RHX performed most of the experiments, analyzed the data and wrote the manuscript. SXG, and LJZ assisted with the DNA methylation profiling and RNA-seq. CXD, XH, PW and MML assisted in collecting clinical samples. JYW contributed to the manuscript discussion. All authors read and approved the final manuscript.

Competing Interests

All authors declare there is no competing interest.

References


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Figures
Figure 1

Genotyping and FISH detections on pairwise bone marrow MNC and MSC samples from patients with CMML

A. Experimental design. CMML-MNC and paired MSC samples were subjected to targeted Exon-seq (130-gene panel) and chromosomal detections using fluorescence in situ hybridization (FISH).
B. VAF of somatic mutations (after excluding germline mutations) in CMML-MNC and paired MSC samples.

C. Mutations with corresponding VAFs in CMML-MNC and MSC paired samples. By performing targeted Exon-seq using mucosal epithelium samples, three mutations with high VAFs (Pt#9: PPM1D-p.I486V; Pt#11: ZRSR-c.937+1G>A; Pt#15: GATA2-p.S429T) in MSC samples were revealed to be germline mutations.

D. Chromosomal evaluations of CMML-MNC and MSC samples from eight CMML patients. Chromosomal abnormalities were identified in eight CMML patients (chromosomal aneuploidy in two patients, +8 chromosome in three patients and complex karyotype in two patients), while no chromosomal lesion was seen in their MSCs.

E. Microscopic fluorescence images (100×) of FISH probes for +8 chromosome in MNC and paired MSC samples of Pt#8 and Pt#16. Yellow arrows indicate cells with +8 chromosomes in MNCs. No chromosomal abnormality was seen in MSCs.
Figure 2

Characterization of global DNA methylome and identification of DMPs in CMML-MSC compared with HD-MSC

A. Experimental design. In vitro expanded MSCs at passages 2–3 from CMML patients (n=10) and HDs (n=5) were pre-treated with 10 μM AZA (AZA+) or DMSO (AZA-) for 48h. Afterward, AZA+ paired AZA- samples were subjected to parallel DNA methylome profiling (n=30) and RNA-seq (n=30), respectively.

B. Genomic and CGI-based distributions of DNA methylome in both AZA- CMML-MSC and AZA- HD-MSC samples. Violin plots representing the methylation β value from 742212 probes after stratified quantile
normalization (SQN) and plotted concerning genomic features (left) and CpG regions (right).

C. Unsupervised PCA analysis based on the distribution of normalized DNA methylome in AZA- CMML-MSC (n=10) and AZA- HD-MSC (n=5) samples.

D. Volcano plot displaying DMPs in AZA- CMML-MSCs compared with AZA- HD-MSCs according to $\Delta\beta$-value and FDR-adjusted P value. Red dots represent hyper-methylated DMPs, and blue dots represent hypo-methylated DMPs. Grey dots represent CpG probes not significantly regulated in CMML-MSCs. Texts in the boxes indicate the gene name corresponding to each DMP.
CMML-MSCs exhibited higher amenability to AZA at the epigenetic level than HD-MSCs

(A) Representative light microscopy images showing the morphology of AZA+/AZA- MSCs at indicated culture conditions (magnification ×10).
(B) MSC viability assessed by flow cytometry using Annexin V/PI after a 48h culturing. Shown are the mean ± SEM of Annexin V-/PI- cells measured in triplicates for n = 6 HD-MSCs and n = 5 CMML-MSCs.

(C) Cell counts of MSCs determined by trypan blue staining at indicated time points. Values represent mean ± SEM value normalized to the cell count before the AZA/DMSO treatment at day 0.

(D) Genomic and CGI-based distributions of DNA methylome in AZA- CMML-MSC and AZA+ CMML-MSC samples. Violin plots representing the methylation β value from 742212 probes after stratified quantile normalization (SQN) and plotted concerning genomic features (left) and CpG regions (right).

(E) Genomic and CGI-based distributions of DNA methylome in AZA- HD-MSC and AZA+ HD-MSC samples. Violin plots representing the methylation β value from 742,212 probes after stratified quantile normalization (SQN) and plotted concerning genomic features (left) and CpG regions (right).

(F) Volcano plot displaying DMPs identified in AZA+ HD-MSCs (AZA+ vs. AZA-). Blue dots represent hypo-methylated DMPs, and red dots represent hyper-methylated DMPs. Grey dots represent CpG probes not significantly regulated in CMML-MSC. Texts in the boxes indicate the gene names corresponding to each DMP.

(G) Volcano plot displaying DMPs identified in AZA+ CMML-MSCs (AZA+ vs. AZA-). Blue dots represent hypo-methylated DMPs, and red dots represent hyper-methylated DMPs. Grey dots represent CpG probes not significantly regulated in CMML-MSC. Texts in the boxes indicate the gene names corresponding to each DMP. (H) Pie chart indicating the proportions of hyper-/hypo-methylated DMPs identified in AZA+ HD-MSCs (AZA+ vs. AZA-).

(I) Pie chart indicating the proportions of hyper-/hypo-methylated DMPs identified in AZA+ CMML-MSCs (AZA+ vs. AZA-).
Figure 4

Identification of DMRs and their correlation with CpG methylation/gene expression in AZA+ CMML-MSCs

(A) Circos plot indicating strong concordancy of the genomic distribution of 855 identified DMRs with DMPs in AZA+ CMML-MSCs (AZA+ vs. AZA-). The ridge pot in red/blue indicates the relative density of the genomic distributions of hyper-methylation DMPs. The scatterplots in red/blue indicate the genomic distributions of hyper-/hypomethylated DMRs.

(B-D) Individual DMR plots indicating regional methylation differences of DMR 299 (aligned to TET1), DMR 374 (aligned to SFRP1) and DMR 114 (aligned to SFRP2). The methylation β value is shown on the y-axis, and the relative chromosomal positions of CpG probes are shown on the x-axis. The β value of CpGs corresponding to aligned genes was displayed and compared.

(E-G) Scatterplots showing the correlation of CpG methylation with gene expression by fragments per kilobase of exon model per million mapped fragments (FPKM) of aligned genes. The methylation β value is shown on the y-axis, and the FPKM gene expression is shown on the x-axis. Red dots represent AZA+ CMML-MSC.
CMML-MSC samples, and blue dots represent AZA+ CMML-MSC samples, with the Pearson correlation coefficient and P value indicated.

Figure 5

Identification of AZA-sensitive genes and functions in CMML-MSCs

(A) Venn diagrams showing the number of AZA-sensitive genes in CMML-MSCs. AZA-sensitive genes were defined as genes whose DNA methylation changes were strictly inversely correlated with expression changes in AZA+ CMML-MSCs. This strategy yielded a total of 1,395 AZA-sensitive genes (1064 up-regulated and 331 down-regulated in gene expression).

(B) Integrated KEGG analysis based on the identified AZA-sensitive genes in CMML-MSCs. Briefly, KEGG results enriched in those up-regulated AZA-sensitive genes (n = 1064) were defined as the up-regulated AZA-sensitive functions (red), while results in those down-regulated AZA-sensitive genes (n = 331) were
defined as the down-regulated AZA-sensitive functions (blue). The top 20 AZA-sensitive functions, corresponding Padj values, and the number of included genes were displayed.

(C) GSEA indicates significant enrichment of supportive signatures of hematopoietic stem cell differentiation in AZA+ CMML-MSCs. GSEA was performed based on transcriptomic data from parallel RNA-seq.

(D) Changes in the CpG methylation levels of genes encoding for hematopoiesis-supportive cytokines including ANGPT1 (i), CXCL14 (ii), SCF (iii) and CXCL12 (iv) in AZA+/- CMML-MSCs

(E) RT-qPCR validation on ANGPT1 (i), CXCL14 (ii), SCF (iii) and CXCL12 (iv) in AZA+ CMML-MSCs compared with AZA- CMML-MSCs.

Figure 6

AZA restores impaired protective effects of CMML-MSCs to HSPCs in short-term co-cultures

(A) Experimental design. In vitro expanded MSCs were pre-treated with 10 μM AZA or DMSO for 48h. After treatment, magnetic sorted CD34+ HSPC from HDs were co-cultured with AZA+ HD-MSCs (n = 3), AZA-HD-MSCs (n = 3), AZA+ CMML-MSCs (n = 5) and AZA- CMML-MSCs (n = 5) for 7 days. After co-culture, HSPCs were harvested and subjected to ROS and double-strand DNA damage detections (γH2AX).

(B) Flow cytometry detection for intracellular ROS of HSPCs co-cultured with AZA+ (blue) or AZA- (red) MSCs and quantitative statistics of fluorescence intensity.
(C) Flow cytometry detection for the intracellular γH2AX foci in HSPCs co-cultured with AZA+ (blue) or AZA- (red) MSCs and quantitative statistics of fluorescence intensity.

(D) Representative immunofluorescence image at 100× for the nuclei staining in co-cultured HSPCs with DAPI in blue and histone γH2AX foci content in green. HSPCs were co-cultured with AZA+/- CMML-MSC before immunofluorescence assays.
Figure 7

Restoration of impaired hematopoiesis-supportive functions of AZA- CMML-MSCs by long-term co-culture and CFC assays

(A) Experimental design. In vitro expanded MSCs from CMML patients and HDs were pre-treated with 10 μM AZA or DMSO for 48h. After treatment, magnetic sorted CD34+ HSPC from HDs were co-cultured with AZA+ HD-MSCs (n = 6), AZA- HD-MSCs (n = 6), AZA+ CMML-MSCs (n = 6) and AZA- CMML-MSCs (n = 6) for 14 days. After co-culture, HSPCs were harvested and subjected to 14-day CFC assays.

(B) Heatmap showing the expression for seven stroma-derived hematopoiesis-supportive cytokines in AZA+/– CMML-MSCs.

(C) Concentrations of ANGPT1 and SCF protein assessed by ELISA assays using the culture supernatant of AZA+/– CMML/HD-MSCs.

(D) Representative light microscopy image showing the morphology of BFU-E, CFU-E, CFU-GM and CFU-GEMM formed by HSPCs after co-culturing with AZA+/– MSCs.

(E) Quantitative statistics of the number of BFU-E, CFU-E, CFU-GM, and CFU-GEMM formed by HSPCs from the following culture conditions: 1) HSPCs cultured alone (dark grey); 2) HSPCs co-cultured with AZA- HD-MSCs (light grey); 3)HSPCs co-cultured with AZA+ HD-MSCs (black); HSPCs co-cultured with AZA- CMML-MSCs (red); HSPCs co-cultured with AZA+ CMML-MSCs.
Figure 8

Schematic illustration of the present study

(A) Study objects and high-throughput methodology.

(B) Identification of regulatory targets of AZA using a multi-omics strategy.

(C) AZA partially restores the hematopoiesis-supportive functions in MSCs from patients with CMML

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementalFile1.xlsx
- SupplementalFile2.csv
- SupplementalFile3.csv
- SupplementalFile4.csv
- SupplementalFile5.csv
- SupplementalFile6.csv
• SupplementalMaterials.docx