Elevated TWIST1 expression in myelodysplastic syndromes/ acute myeloid leukemia reduces efficacy of hypomethylating therapy with decitabine

Myelodysplastic syndromes (MDS) comprise a heterogeneous group of myeloid neoplasms characterized by peripheral blood cytopenia, hematopoietic cell dysplasia, and transformation to secondary acute myeloid leukemia (AML) in 30% of cases.[1] Epigenetic changes are recognized as major drivers of MDS progression. A recent study indicates that DNA hypermethylation is a relevant parameter of MDS at the molecular level.[2] Treatment with demethylating compounds, decitabine, such as 5-azacytidine or 5-aza-2'-deoxycytidine (DAC) increased overall survival and delayed AML transformation.[3] However, hypomethylating agents are ineffective in a substantial proportion of patients, for reasons which remain unclear.

We showed previously that the highly conserved transcription factor TWIST1, which contains a basic helix-loop-helix structure, is aberrantly upregulated in advanced MDS.[4] TWIST1 expression is regulated in turn by microRNA-10a/10b, and inhibition of miR-10a/10b in clonal cells interfered with its proliferation and enhanced its susceptibility to apoptosis.[5] TWIST1 is associated with DNA methylation and chromosome modification in a variety of solid tumors.[6,7] The goal of the present study is to clarify the role of TWIST1 in modification of epigenetic changes and alteration of DAC sensitivity in MDS/AML.

We carried out a direct comparison of TWIST1 levels in cells from healthy individuals (HD) and MDS/AML patients who are unresponsive to DAC treatment (DAC-NR group) or are responsive to DAC treatment (DAC-R group). There was a significantly lower expression in healthy individuals than in patients with MDS/AML, consistent with our previous results. Levels of TWIST1 in DAC-R group approached those in healthy controls (*Online Supplementary Figure S1A*). More interestingly, we found that *TWIST1* levels were higher in the DAC-NR group than in the DAC-R group (Figure 1A and *Online Supplementary. Table S1*). A receiver operating characteristic (ROC) curve was created to show the threshold value for optimal sensitivity (70%) and specificity (86.4%) of *TWIST1* expression in the DAC-NR group *versus* the DAC-R group (*Online Supplementary Figure S1B*). Follow-up experiments showed that global methylation levels

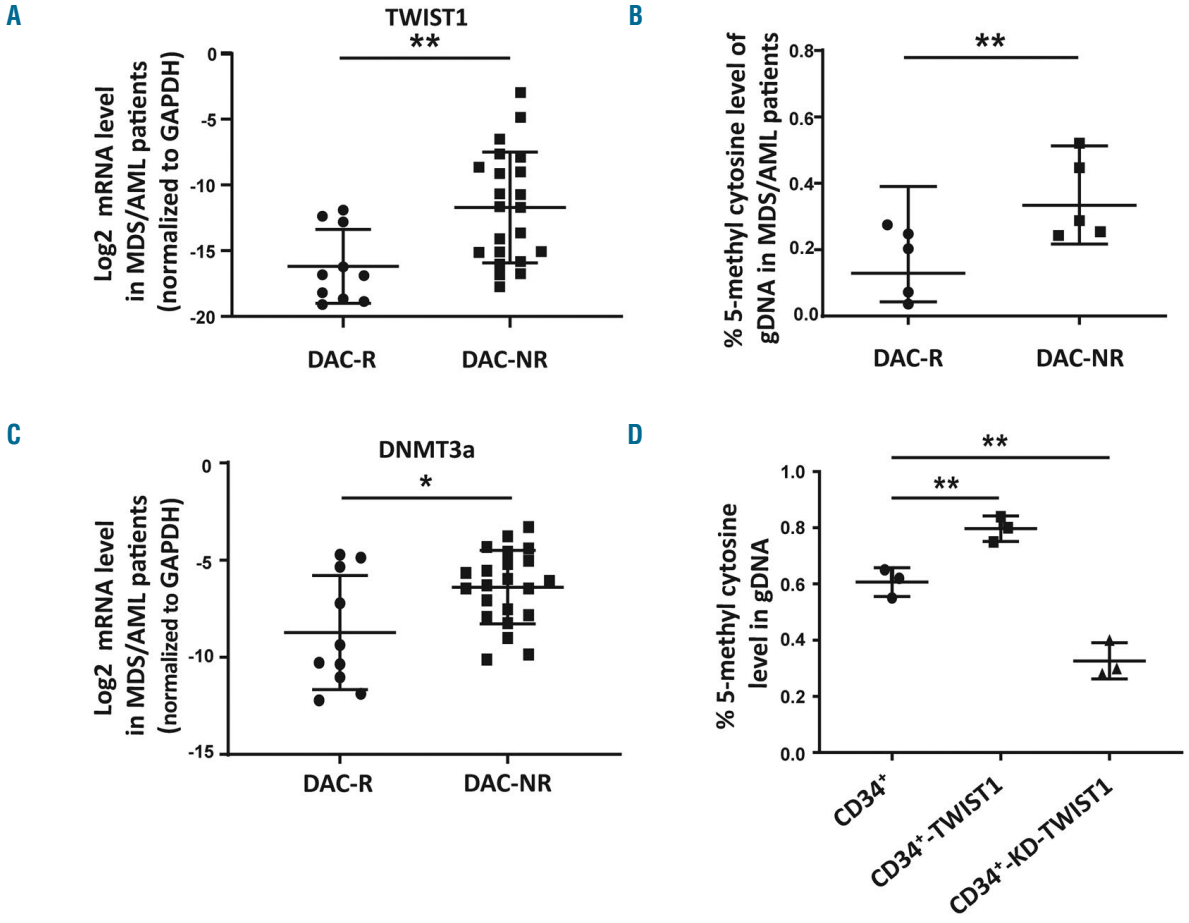


Figure 1. Increased *TWIST1* level in cells of patients unresponsive to the decitabine DAC. (A) *TWIST1* mRNA levels in cells from myelodysplastic syndromes/ acute myeloid leukemia (MDS/AML) patients were analyzed by real-time PCR. RNA was prepared from mononuclear cells from patients unresponsive to DAC (DAC-NR) (n=22) and from patients responsive to DAC (DAC-R) (n=10). (B) Global DNA methylation analysis of cells from MDS/AML patients. Genomes were prepared from mononuclear cells of DAC-NR (n=5) and DAC-R (n=5) groups. (C) *DNMT3a* mRNA levels of cells from MDS/AML patients were analyzed by real-time PCR. RNA was prepared from mononuclear cells of DAC-NR (n=22) and DAC-R (n=10) groups. (D) Global DNA methylation analysis of $CD34^+$, $CD34^+$-TWIST1 (TWIST1-overexpressing), and $CD34^+$-KD-TWIST1 (TWIST1 knockdown) cells. Data shown are mean ± standard error of the mean (SEM) from three independent experiments. *P<0.05; **P<0.01; ***P<0.001.

were significantly higher in DAC-NR than in DAC-R (Figure 1B), and that DNMT3a expression, which can initiate *de novo* methylation, was higher in DAC-NR than in DAC-R (Figure 1C). DAC-resistant KG1a cells (KG1a-DAC-R), generated by continuous culture and treatment with DAC at gradually increasing concentrations, showed enhanced DAC resistance, significantly higher TWIST1 expression, and increased global methylation levels (*Online Supplementary Figure S1C-D*), similar to findings for DAC-NR *versus* DAC-R. On the basis of these findings, we hypothesized that TWIST1 expression is associated with *de novo* DNA methylation status. Consistent with this hypothesis, TWIST1 overexpression in $CD34^+$ cells from cord blood was associated with increased DNA methylation and transcriptome suppression, whereas silencing of TWIST1 expression in these cells reduced DNA methylation (Figure 1D and *Online Supplementary Figure S1E*).

To clarify the role of TWIST1 in DAC-mediated cell proliferation, TWIST1 was overexpressed in KG1a (KG1a-TWIST1) and was stably knocked down in SKM1 and OCI-AML3 (*Online Supplementary Figure S2A-B*). Cell

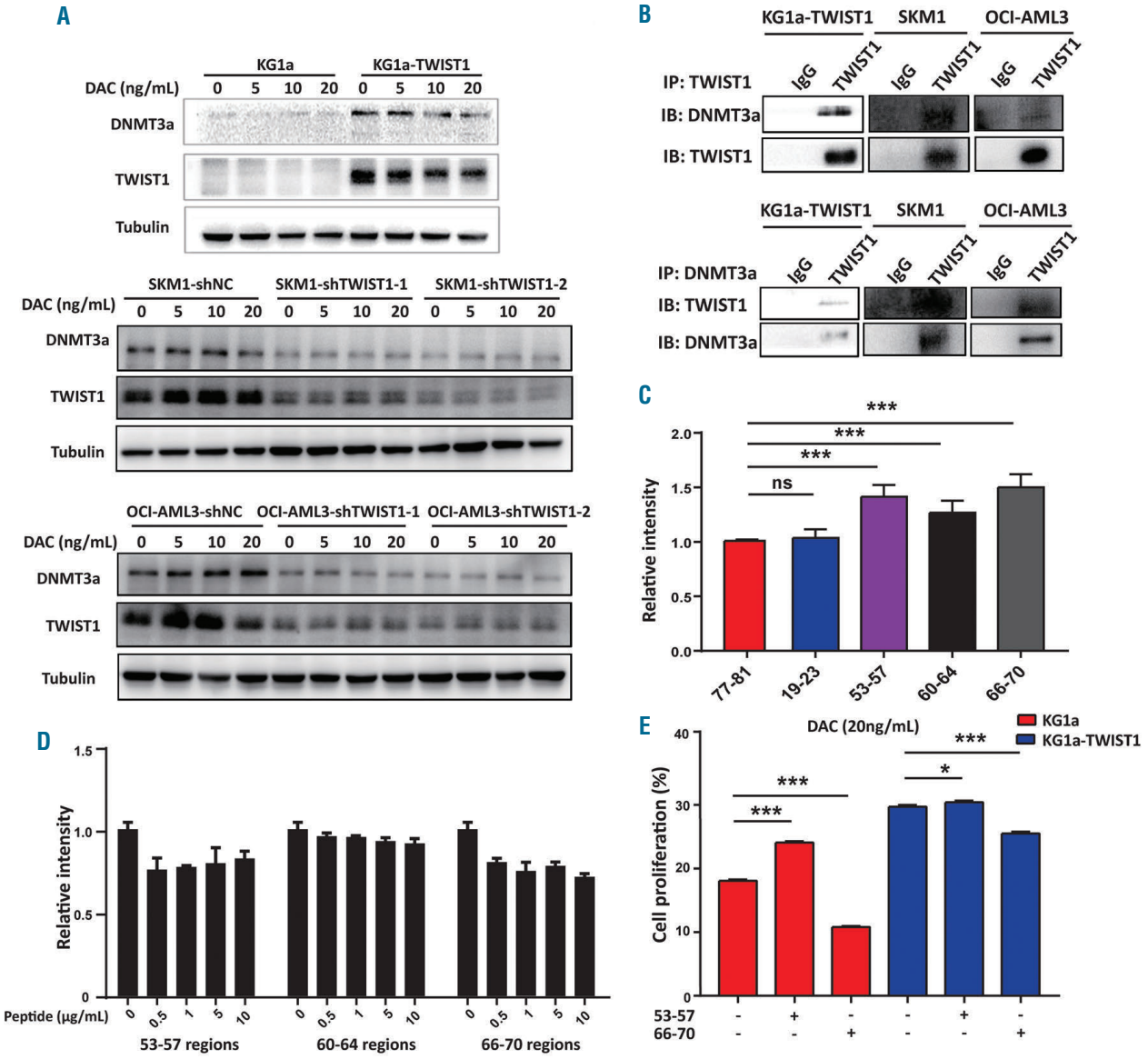
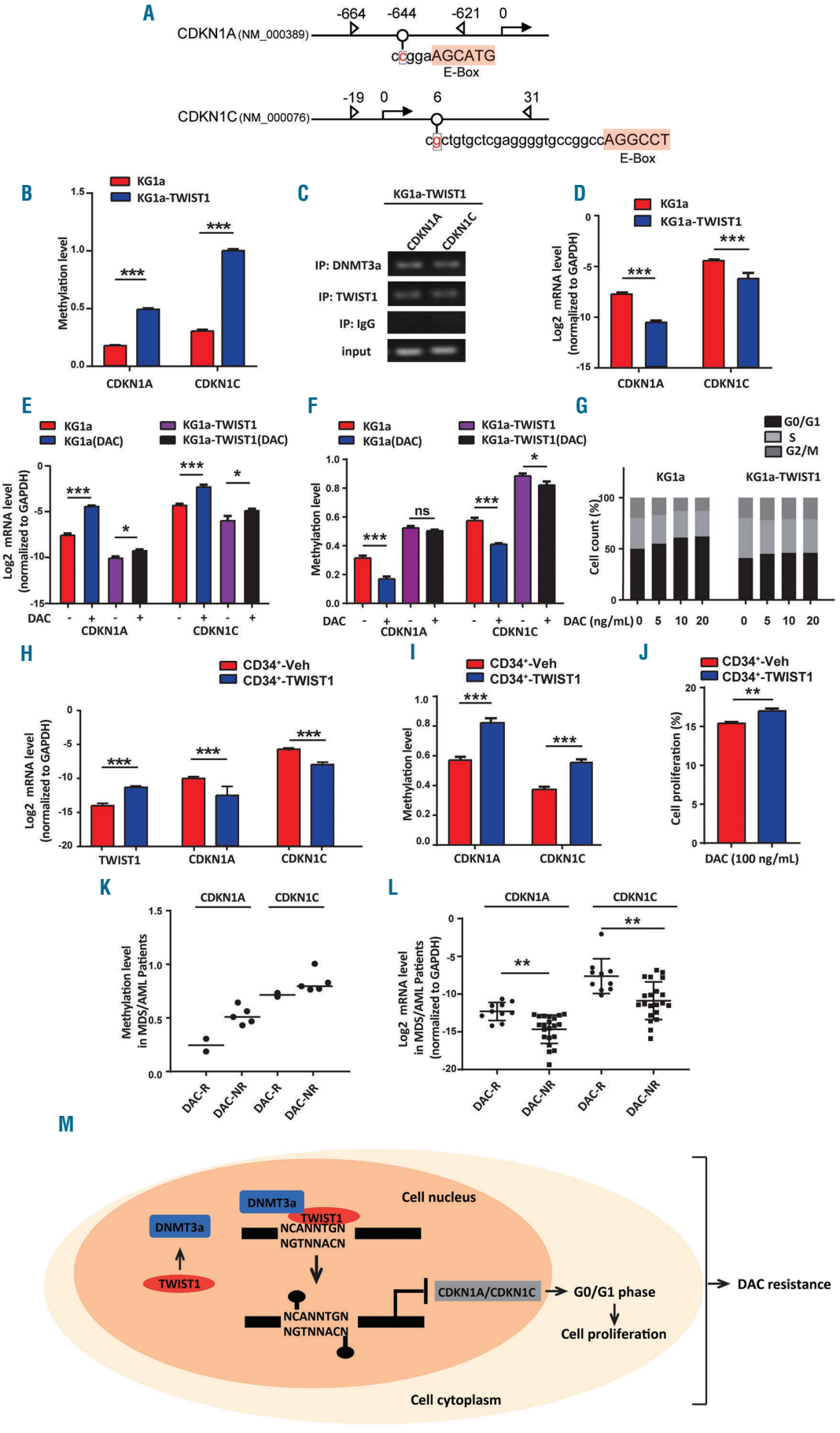


Figure 2. TWIST1 interacts with DNMT3a. (A) TWIST1 and DNMT3a expression levels, determined by Western blotting. Cells were treated with 0, 5, 10, or 20 ng/mL decitabine DAC for 96 hours (h). Lysates (25 μg) were loaded on SDS-PAGE and blotted with anti-TWIST1 and anti-DNMT3a Abs (tubulin as loading control). (B) Immunoprecipitation (IP) analysis of DNMT3a and TWIST1 binding affinity in KG1a-TWIST1, SKM1 and OCI-AML3. Lysates (1 mg) were incubated overnight with anti-TWIST1 or anti-DNMT3a antibody (Ab) (1:100). Control: lysates were incubated overnight with mouse or rabbit IgG. (C) Verification of TWIST1/DNMT3a binding site by enzyme-linked immunosorbent assay (ELISA). Peptides (19-13, 53-57, 60-64, 66-70, 77-81) of TWIST1 were based on respective wells prior to addition of purified protein His-DNMT3a. Anti-DNMT3a Ab and secondary Ab were added sequentially. Peptide 77-81 was used as a negative control, and relative intensity was normalized to intensity of peptides 77-81. (D) Competitive ELISA to verify TWIST1/DNMT3a binding site(s). His-DNMT3a was based on the well prior to addition of various concentrations (0, 10, 5, 1, 0.5 μg/mL) of amino acid region 53-57, 60-64, or 77-81. Purified GST-TWIST1, anti-GST Ab, and secondary Ab were added to each well sequentially. Relative intensity was normalized to the intensity of non-peptide treated well. (E) Proliferation of KG1a and KG1a-TWIST1 under DAC and peptide treatments. Peptides were packaged by lipofectamine to facilitate peptide intake. The cells were treated with DAC and peptide for 96 h. Data and statistical notations as in Figure 1.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-644
-621
0
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E-Box
CDKN1C (NM_000076)
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cgctgtgctcgaggggtgccggccAGGCCT
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Methylation level
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Cell proliferation (%)
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Methylation level in MDS/AML Patients
DAC-R
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Log2 mRNA level in MDS/AML Patients (normalized to GAPDH)
DAC-R
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DAC-R
DAC-NR
M
Cell nucleus
DNMT3a
TWIST1
NCANNTGN
NGTNNACN
DNMT3a
TWIST1
NCANNTGN
NGTNNACN
CDKN1A/CDKN1C
G0/G1 phase
Cell proliferation
DAC resistance
Cell cytoplasm

Figure 3. (previous page) TWIST1/DNMT3a complex facilitates methylation of CDKN1A/C, and reverses cell cycle arrest. (A) Schematic representation of distributions of E-box and of CG dinucleotides in promoter regions of *CDKN1A/C* genes in KG1a and KG1a-TWIST1. The red represents methylation of CpG site. (B) Methylation-specific PCR analysis of the *CDKN1A/C* promoters in KG1a and KG1a-TWIST1. (C) Chromatin immunoprecipitation (ChIP) analysis of DNMT3a and *TWIST1* binding to *CDKN1A/C* promoters. The assay was performed using digested chromatin from KG1a-TWIST1 cells. Purified DNA immunoprecipitated by anti-TWIST1 and anti-DNMT3a antibody (Ab) was analyzed by standard PCR methods, with purified DNA IP'd by immunoglobulin G (IgG) as negative control. (D) *CDKN1A/C* mRNA levels in KG1a and KG1a-TWIST1, analyzed by real-time PCR. (E) *CDKN1A/C* mRNA levels of KG1a and KG1a-TWIST1 treated for 96 h with 20 ng/mL DAC, determined by real-time PCR. (F) Methylation-specific PCR analysis of *CDKN1A/C* promoter under DAC treatment. (G) Cell cycle analysis in KG1a and KG1a-TWIST1 by fluorescence-activated cell sorting (FACS). Cells were treated for 96 hours (h) with various DAC concentrations (0, 5, 10, 20 ng/mL). Cell cycle distribution was analyzed by propidium iodide (PI) staining, and results were fitted by FlowJo software program. (H) mRNA levels of *TWIST1* and *CDKN1A/C* in normal $CD34^+$ cells ($CD34^+$-Veh) and TWIST1-overexpressing $CD34^+$ ($CD34^+$-TWIST1) cells, analyzed by real-time PCR. RNA was prepared from $CD34^+$-TWIST1 and unmodified $CD34^+$ cord blood cells. All cells were transfected with TWIST1 for 96 h and collected after 48 h puromycin screening. (I) Methylation levels of *CDKN1A/C* promoters in $CD34^+$-Veh and $CD34^+$-TWIST1 cells, determined by methylation PCR. (J) Cell proliferation of $CD34^+$-Veh and $CD34^+$-TWIST1 cells following 96 h DAC treatment. Cell proliferation was analyzed by flow cytometry. (K) Methylation levels of the *CDKN1A/C* promoter in mononuclear cells from responsive to the decitabine DAC (DAC-R) (n=2) and unresponsive to DAC (DAC-NR) (n=5) groups. (L) *CDKN1A/C* mRNA levels of cells from MDS/AML patients were analyzed by real-time PCR. RNA was prepared from mononuclear cells from DAC-NR (n=22) and DAC-R (n=10) groups. (M) Proposed model of the role of TWIST1 in reversal of G0/G1 phase cell cycle arrest. Data and statistical notations as in Figure 1.

proliferation was higher in KG1a-TWIST1 than in parental KG1a, while inhibition of TWIST1 in SKM1 and OCI-AML3 resulted in reduced cell proliferation (*Online Supplementary Figure S2C-D*). Under treatment with various DAC concentrations, proliferation beginning at 24 hours (h) was significantly enhanced in KG1a-TWIST1 relative to KG1a, suggesting that TWIST1 facilitated DAC resistance in KG1a-TWIST1. In contrast, TWIST1 knockdown in SKM1 and OCI-AML3 was associated with reduced DAC-mediated proliferation (*Online Supplementary Figure S2E-F*). Consistent with findings described in the preceding paragraph, our *in vivo* studies strongly supported the hypothesis that TWIST1 overexpression enhances DAC resistance of myeloid cells (*Online Supplementary Figure S2G-J*).

DNMT activity and DNMT3a expression were significantly upregulated in KG1a-TWIST1 and downregulated in TWIST1 knock-down cells (Figure 2A and *Online Supplementary Figure S3A*). Studies using a TAP tag-TWIST1 fusion protein and mass spectrometry allowed us to identify a set of proteins, including DNMT3a, that interact directly with TWIST1 (*Online Supplementary Figure S3B-C*). Direct interaction between TWIST1 and DNMT3a was observed in both KG1a-TWIST1 and SKM1. Co-immunoprecipitation (IP) assay and immunofluorescence staining showed that TWIST1 also interacted with mutant DNMT3a in OCI-AML3 cells, which harbor a heterozygous DNMT3a mutation (R882C)(8); i.e., presence of mutant DNMT3a had no effect on TWIST1/ DNMT3a interaction (Figure 2B and *Online Supplementary Figure S3D-E*).

The TWIST1/ DNMT3a binding site was determined by epitope mapping assay. The total 202 amino acids of TWIST1 protein were broken down into 95 peptides, 15-mer peptides each, overlapping by 13 residues, and covalently bound to nitrocellulose membrane (Online Supplementary Figure S3F and *Online Supplementary Table S4*). Among the 95 peptides, DNMT3a bound to peptides 19-23, 53-57, 60-64, and 66-70 (*Online Supplementary Figure S3G*). Results of ELISA assay and competitive inhibition binding assay showed that peptides 53-57 and 66-70 suppressed TWIST1/ and DNMT3a interaction *in vitro* (Figure 2C-D). DAC treatment of peptide 66-70 for 96 h significantly suppressed proliferation of KG1a and KG1a-TWIST1 (Figure 2E). These findings indicate that TWIST1 amino acid sequence PSDKLSK (peptide 66-70) is the probable DNMT3a-binding site.

Targeted genes possibly modulated by the TWIST1/DNMT3a complex were screened by methylation chip sequencing assay. Two cyclin-dependent kinase (CDK) inhibitors (*CDKN1A, CDKN1C*), the potential targeted genes of TWIST1, were highly methylated in their promoter regions in KG1a-TWIST1 cells (*Online Supplementary Figure S4A*). Enhancement of methylation levels of *CDKN1A/C* promoter regions in KG1a-TWIST1 was confirmed by methylation-sensitive PCR (Figure 3A-B). ChIP assay demonstrated that both TWIST1 and DNMT3a are capable of interaction with *CDKN1A/C* promoter regions, suggesting that TWIST1/DNMT3a complex is required for methylation of these two genes (Figure 3C). High methylation, particularly of cytosine bases in CpG sites, is one of several epigenetic mechanisms capable of suppressing gene expression. Our experiments revealed suppression of *CDKN1A/C* mRNA levels in KG1a-TWIST1 (Figure 3D). We observed significant increase of mRNA expression and reduction of methylation level of these two genes in KG1a relative to KG1a-TWIST1 following DAC treatment (Figure 3E-F). CDKN1A/C can function as CDK inhibitors involved in control of cell cycle progression.[9] Flow cytometric analysis showed that DAC treatment resulted in G0/G1 phase arrest in KG1a, and to a lesser degree in KG1a-TWIST1 (Figure 3G). These findings indicate that cellular sensitivity to DAC treatment is changed by alterations of *CDKN1A/C* promoter methylation and of G0/G1 arrest following TWIST1 overexpression.

To extend the above findings to primary cells and clinical samples, we analyzed changes in DNA methylation of *CDKN1A/C* induced by TWIST1 overexpression in $CD34^+$ cord blood cells. *CDKN1A* and *CDKN1C* were notably downregulated relative to retroviral vector-containing control cells within 96 h following TWIST1 transfection (Figure 3H), and methylation levels of their promoter areas increased in TWIST1-overexpressing $CD34^+$ (Figure 3I). Following treatment with 100 ng/mL DAC for 96 h, DAC resistance of TWIST1-overexpressing $CD34^+$ was significantly greater than that of unmodified $CD34^+$ cells (Figure 3J). In MDS/AML patients, methylation levels of *CDKN1A/C* promoter areas were higher in DAC-NR than in DAC-R group (Figure 3K), and *CDKN1A/C* expression was lower in DAC-NR (Figure 3L).

Demethylating drugs such as decitabine have been widely applied in the therapy of MDS/AML patients. However, a substantial proportion of patients undergo relapse subsequent to therapy, and the mechanisms underlying such relapse are largely unknown. The function of *TWIST1* as an oncogene has been well documented in development of many solid cancers.[10] We observed in the present study that TWIST1 is highly expressed in MDS/AML patients unresponsive to DAC therapy. J. Xu's group reported recently that TWIST1 is involved in arsenic trioxide resistance in acute promyelocytic

leukemia (APL) patients.[11] This observation and ours, taken together, suggest possible association of aberrant TWIST1 expression with therapeutic failure in hematopoietic malignancies. Our detailed analysis reveals that TWIST1 can interact with both wild-type and mutated DNMT3a and disrupt DNMT3a/TWIST1 interaction can block induction of DAC resistance.

Involvement of DNMT3a in *de novo* DNA methylation and its interaction with transcription factors (e.g., MYC) to facilitate methylation of target DNA were demonstrated in previous studies.[12,13] We investigated the ability of the TWIST1/DNMT3a complex to regulate the methylation status of TWIST1 targeted genes. TWIST1 overexpression increased methylation levels of promoter areas of *CDKN1A/C* in cell lines and primary cells. *CDKN1A/C* are CDK inhibitors that strongly affect various G1 cyclin/CDK complexes and thereby negatively regulate cell proliferation. *CDKN1A/C* are downregulated in MDS and AML *via* DNA methylation, and such abnormal methylation levels in MDS/AML are associated with poor prognosis.[14,15] Results of the present study indicate that TWIST1 recruits DNMT3a to methylate promoter regions of *CDKN1A/C*, resulting in inhibition of *CDKN1A/C* expression and consequent reversal of G0/G1 arrest.

In conclusion, TWIST1/DNMT3a complex appears to play an essential role in alterations of *CDKN1A/C* promoter methylation and of G0/G1 arrest, and in consequent changes in cellular sensitivity to DAC treatment (Figure 3M). Our findings provide new insights into the role of TWIST1 as a determinant of responses to demethylating therapy, and identification of novel factors affecting therapeutic responses. Analysis of gene profiles in cells with modified TWIST1 expression will further clarify the role of TWIST1 in promotion, suppression, or activation of genes involved in MDS pathogenesis and progression *via* its interaction with epigenomic modifiers.

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