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Addiction of t(8;21) and inv(16) Acute Myeloid Leukemia to Native RUNX1

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SUMMARY

The t(8;21) and inv(16) chromosomal aberrations generate the oncoproteins AML1-ETO (A-E) and CBFβ-SMMHC (C-S). The role of these oncoproteins in acute myeloid leukemia (AML) etiology has been well studied. Conversely, the function of native RUNX1 in promoting A-E- and C-S-mediated leukemias has remained elusive. We show that wild-type RUNX1 is required for the survival of t(8;21)-Kasumi-1 and inv(16)-ME-1 leukemic cells. RUNX1 knockdown in Kasumi-1 cells (Kasumi-1$^{RX1\text{-}KD}$) attenuates the cell-cycle mitotic checkpoint, leading to apoptosis, whereas knockdown of A-E in Kasumi-1$^{RX1\text{-}KD}$ rescues these cells. Mechanistically, a delicate RUNX1/A-E balance involving competition for common genomic sites that regulate RUNX1/A-E targets sustains the malignant cell phenotype. The broad medical significance of this leukemic cell addiction to native RUNX1 is underscored by clinical data showing that an active RUNX1 allele is usually preserved in both t(8;21) or inv(16) AML patients, whereas RUNX1 is frequently inactivated in other forms of leukemia. Thus, RUNX1 and its mitotic control targets are potential candidates for new therapeutic approaches.

INTRODUCTION

Acute myeloid leukemia (AML) is characterized by a block in early progenitor differentiation leading to accumulation of immature, highly proliferative leukemic stem cells (LSCs) in bone marrow (BM) and blood (Rosenbauer et al., 2005). Chromosome-21-encoded transcription factor (TF) RUNX1 (a.k.a. AML1) is a frequent target of various chromosomal translocations (Lam and Zhang, 2012). The most prevalent translocation in AML is t(8;21) (Hatlen et al., 2012; Müller et al., 2008), which creates a fused gene product designated AML1-ETO (A-E). It contains the DNA-binding domain of RUNX1 (the runt domain [RD]) linked to the major part of the chromosome-8-encoded protein ETO (Erickson et al., 1992; Miyoshi et al., 1993), which by itself lacks DNA-binding capacity (Davis et al., 2003).

RUNX1 is a key hematopoietic gene expression regulator in embryos and adults (Cameron and Neil, 2004; de Bruijn and Speck, 2004; Wang et al., 1996). Its major cofactor, core-binding factor β (CBFβ), is essential for RUNX1 function (Miller et al., 2001, 2002). On the other hand, ETO is a transcriptional repressor (Davis et al., 2003) that is known to interact with corepressors such as NCoR/SMRT, mSin3a, and histone deacetylases (HDACs) (reviewed in Hug and Lazar, 2004). Of note, although the *ETO* gene is normally expressed in the gut and CNS (Lam and Zhang, 2012), the t(8;21) translocation places it under transcriptional control of *RUNX1* regulatory elements (Bee et al., 2009; Ghozi et al., 1996; Levanon et al., 2001). This occurrence evokes expression of A-E in the myeloid cell lineage.

The prevailing notion is that A-E binds to RUNX1 target genes and acts as dominant-negative regulator, thereby producing conditions that resemble the RUNX1$^{-/-}$ phenotype (reviewed in Goyama and Mulloy, 2011; Hatlen et al., 2012; Lam and Zhang, 2012; Licht, 2001). Consistent with this concept, mice expressing an A-E knockin allele (Okuda et al., 1998; Yergeau et al., 1997) display early embryonic lethality and hematopoietic defects similar to those observed in Runx1$^{-/-}$ mice (reviewed in Goyama and Mulloy, 2011; Hatlen et al., 2012; Lam and Zhang, 2012; Licht, 2001). However, it has also been shown that A-E-mediated leukemogenicity involves other events that affect gene regulation, in addition to repression of RUNX1 targets (Bakshi et al., 2008; Hatlen et al., 2012; Hyde and Liu, 2010; Lam and Zhang, 2012; Okumura et al., 2008). Reduction of A-E expression in leukemic cells by siRNA restores myeloid differentiation (Dunne et al., 2006; Heidenreich et al., 2003; Martinez et al., 2004) and delays in vivo tumor formation (Martinez Soria et al., 2009). Depletion of A-E in t(8;21)$^{+}$ AML cells causes genome-wide changes in chromatin structure leading to redistribution of RUNX1 occupancy and inhibition of cell self-renewal capacity (Ptasinska et al., 2012).

An additional AML subtype associated with altered RUNX1 activity involves the chromosomal aberrations inv(16)(p13q22) and t(16;16)(p13q22) (abbreviated as inv(16)). This inversion

fuses the chromosome 16q22-encoded CBFβ gene with the MYH11 gene, which resides at 16p13 and encodes smooth-muscle myosin heavy chain (SMMHC). The resulting chimeric oncoprotein is known as CBFβ-SMMHC (Arthur and Bloomfield, 1983; Le Beau et al., 1983). Similarly to A-E, CBFβ-SMMHC (C-S) is a dominant inhibitor of RUNX1 activity (Castilla et al., 1996; Lukasik et al., 2002) that impairs myeloid differentiation and contributes to AML development (Castilla et al., 2004; Kuo et al., 2006).

Although the central role of A-E in the leukemic process has been extensively studied, the potential role of the wild-type (WT) RUNX1 allele in t(8;21) AML etiology remains unclear (Goyama and Mulloy, 2011). Similarly, the function of RUNX1 in the development of inv(16) AML is completely unknown. We used the AML cell lines t(8;21) Kasumi-1 (Asou et al., 1991) and inv(16) ME-1 (Yanagisawa et al., 1991) to evaluate the possible involvement of WT RUNX1 in A-E- and C-S-mediated leukemogenesis. We show that t(8;21) Kasumi-1 and inv(16) ME-1 AML cells are physiologically dependent on RUNX1 activity for their survival. The broad medical significance of this leukemic cell addiction to native RUNX1 is underscored by clinical data showing that an active *RUNX1* allele is maintained in both t(8;21) and inv(16) AML patients, whereas *RUNX1* is frequently inactivated in other forms of AML (Schnittger et al., 2011; Tang et al., 2009; reviewed in Goyama and Mulloy, 2011).

Knockdown (KD) of RUNX1 in Kasumi-1 cells (Kasumi-$1^{RX1\text{-}KD}$) results in A-E-mediated, caspase-dependent apoptosis. This apoptosis is triggered by the dysregulated expression of a gene subset that is crucial for mitotic functions, including mitotic checkpoint signaling. Significantly, KD of A-E in Kasumi-$1^{RX1\text{-}KD}$ cells evades this apoptosis and rescues Kasumi-$1^{RX1\text{-}KD}$, whereas KD of A-E diminishes the leukemogenic cell phenotype. Thus, a delicate balance between A-E and WT RUNX1 activities is required to maintain the malignant phenotype of Kasumi-1 cells. We elucidated the mechanism underlying this RUNX1/A-E balance by using combined differential gene expression and genome-wide chromatin immunoprecipitation sequencing (ChIP-seq) analyses. This analysis identified a subset of RUNX1/A-E common responsive genes that are inversely regulated by these two TFs and singled out the most critical RUNX1 targets. Our findings uncovered a previously unrecognized role of RUNX1 in the regulation of mitotic checkpoint events, through which it prevents the inherent apoptotic process in t(8;21) cells and facilitates leukemogenesis. These data implicate RUNX1 and its downstream mitotic regulators as potential targets for new therapeutic treatments of t(8;21) and inv(16) leukemias.

RESULTS

Expression of WT RUNX1 Is Essential for t(8;21) AML Kasumi-1 Cell Survival

We assessed the cell-phenotypic consequences of RUNX1 KD in Kasumi-1 cells to directly address the possibility that native RUNX1 function is required for the leukemogenic process in t(8;21) AML cells. Specific siRNA-oligo nucleotides that target *RUNX1* regions absent from the A-E transcript were used to attenuate the expression of RUNX1 (Figures 1A and S1A). Cell-cycle analysis of Kasumi-$1^{RX1\text{-}KD}$ cells revealed a prominent increase in the proportion of cells bearing subG1 DNA content and a significant decrease in the proportions of S and G2/M phases as compared with cells transfected with control nontargeting (NT) siRNA (Kasumi-1^{Cont}; Figures 1B, 1C, S1B, and S1C). This abnormal Kasumi-$1^{RX1\text{-}KD}$ cell cycle was associated with an elevated percentage of both Annexin-V^+ viable and nonviable cells (Figure 1D) and a ~7-fold decrease in the total number of viable cells (Figure 1E). These results indicated that KD of RUNX1 induces apoptotic cell death in Kasumi-$1^{RX1\text{-}KD}$. Transfection of an alternative siRNA oligo directed against a different *RUNX1* region (indicated by the orange bar in Figure 1A) confirmed that apoptosis resulted from decreased RUNX1 activity (Figure S2) and ruled out the possibility of a siRNA-specific off-target effect.

We next sought to determine whether Kasumi-$1^{RX1\text{-}KD}$ cell death involves the mitochondrial permeability transition (MPT). Flow-cytometry imaging (ImageStream System) analysis demonstrated that increased Kasumi-$1^{RX1\text{-}KD}$ cell apoptosis was associated with loss of mitochondrial membrane potential (Figures 1F and 1G), suggesting the involvement of MPT in inducing cell death. To assess whether this RUNX1 KD-triggered apoptosis involved caspase activation, we analyzed Kasumi-$1^{RX1\text{-}KD}$ and Kasumi-1^{Cont} cell cycles in the presence of the broad-spectrum caspase inhibitor *Z-VAD-FMK*. Significantly, *Z-VAD-FMK* completely blocked apoptosis in Kasumi-$1^{RX1\text{-}KD}$ cells, as reflected in a profound decrease of the subG1 fraction to a level similar to that of Kasumi-1^{Cont} cells (Figure 1H). Of note, the majority of *Z-VAD-FMK*-rescued Kasumi-$1^{RX1\text{-}KD}$ cells accumulated at cell-cycle G1 and G2/M phases (Figure 1H), suggesting that RUNX1 KD-evoked apoptosis involved impaired G2/M-to-G1 and G1-to-S transitions. Using *Z-VAD-FMK*-treated cells, we also recorded reduced RUNX1 protein levels in Kasumi-$1^{RX1\text{-}KD}$ cells (Figure 1I). Taken together, the results of the cell-cycle analysis, Annexin-V staining, viability assay, ImageStream analysis, and *Z-VAD-FMK* experiments demonstrate that attenuation of WT RUNX1 expression in Kasumi-1 cells triggers pronounced caspase-dependent apoptosis associated with changes in mitochondrial permeability. The most likely implication of these data is that WT RUNX1 plays an antiapoptotic role in t(8;21) AML cells and its activity is compromised by the oncogenic A-E bearing the RUNX RD. Therefore, the remaining WT RUNX1 activity is indispensable for cell viability.

A-E KD Rescues Kasumi-$1^{RX1\text{-}KD}$ Cells from Apoptosis

To further investigate the involvement of WT RUNX1 in the development of A-E-mediated t(8;21) AML, we used siRNA specific for the translocated transcripts to KD A-E (Kasumi-$1^{AE\text{-}KD}$) expression (Figures 2A and S3A, top). Kasumi-$1^{AE\text{-}KD}$ cells displayed decreased proliferation (Figure S3A, bottom) and increased myeloid differentiation (Figures S3B and S3C), as was previously noted (Dunne et al., 2006; Heidenreich et al., 2003; Martinez et al., 2004; Ptasinska et al., 2012), as well as a marked reduction in the proportion of $CD34^+CD38^-$ leukemogenic cell population (Figures S3B and S3C). We next examined the impact of A-E KD on the cell phenotype of Kasumi-$1^{RX1\text{-}KD}$. Interestingly, the double-KD cells (Kasumi-$1^{RX1/AE\text{-}KD}$) displayed an apoptotic level similar to or even lower than that of control cells (Figures