An Extensive Network of TET2-Targeting MicroRNAs Regulates Malignant Hematopoiesis

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SUMMARY

The Ten-Eleven-Translocation 2 (TET2) gene, which oxidizes 5-methylcytosine in DNA to 5-hydroxymethylcytosine (5hmC), is a key tumor suppressor frequently mutated in hematopoietic malignancies. However, the molecular regulation of TET2 expression is poorly understood. We show that TET2 is under extensive microRNA (miRNA) regulation, and such TET2 targeting is an important pathogenic mechanism in hematopoietic malignancies. Using a high-throughput 3’ UTR activity screen, we identify >30 miRNAs that inhibit TET2 expression and cellular 5hmC. Forced expression of TET2-targeting miRNAs in vivo disrupts normal hematopoiesis, leading to hematopoietic expansion and/or myeloid differentiation bias, whereas coexpression of TET2 corrects these phenotypes. Importantly, several TET2-targeting miRNAs, including miR-125b, miR-29b, miR-29c, miR-101, and miR-7, are preferentially overexpressed in TET2-wild-type acute myeloid leukemia. Our results demonstrate the extensive roles of miRNAs in functionally regulating TET2 and cellular 5hmC and reveal miRNAs with previously unrecognized oncogenic potential. Our work suggests that TET2-targeting miRNAs might be exploited in cancer diagnosis.

INTRODUCTION

The recently discovered Ten-Eleven-Translocation (TET) genes are key players in epigenetic regulation, with important roles in development and cancer. All three TET proteins, including TET1, TET2, and TET3, are enzymes that catalyze the conversion of 5-methylcytosine (5mC) in genomic DNA to 5-hydroxymethylcytosine (5hmC) and its oxidative derivatives (Ito et al., 2010, 2011; Tahiliani et al., 2009; He et al., 2011). These enzymatic activities are involved in both active and passive DNA demethylation (reviewed in Wu and Zhang, 2011; Cimmino et al., 2011; Shih et al., 2012), the tight regulation of which is essential in defining and safeguarding cellular identities. Accordingly, TET gene expression and 5hmC levels are often downregulated in a wide spectrum of cancers (Lian et al., 2012; Hsu et al., 2012; Yang et al., 2013). In particular, haploinsufficient loss-of-function mutations in TET2 are frequently found in patients with a variety of hematopoietic malignancies, including acute myeloid leukemia (AML), myeloproliferative neoplasms, myelodysplastic syndromes, chronic myelomonocytic leukemia (CMML), and lymphoid malignancies (Cimmino et al., 2011; Shih et al., 2012). In mouse models, homozygous or heterozygous loss of TET2 results in enhanced hematopoietic stem cell activity and CMML-like malignant progression (Moran-Crusio et al., 2011; Quivoron et al., 2011; Li et al., 2011).

Increasing efforts are underway to incorporate TET2 mutational status in routine clinical diagnostics to inform molecular pathogenesis and therapeautic outcomes. However, genetic TET2 mutation analysis is not sufficient to completely capture TET2 functional deregulation. For example, it was found that a substantial fraction of patients with AML with wild-type TET2 shows similarly decreased 5hmC levels as TET2 mutant AMLs (Ko et al., 2010). Hence, it raises the question whether, in addition to genetic mutations and inhibition of enzyme activity (Marquis et al., 2009; Shih et al., 2012), molecular pathways regulating TET2 expression can serve as an important alternative mechanism in hematopoietic malignancies and should be considered in diagnosis.

Despite the importance of TET gene dosage control, much less is known about the mechanisms that regulate TET gene expression (Kallin et al., 2012; Wu et al., 2013; Song et al., 2013). Hence, it raises the question whether, in addition to genetic mutations and inhibition of enzyme activity (Moran-Crusio et al., 2011; Quivoron et al., 2011; Li et al., 2011).
MicroRNAs (miRNAs) are small noncoding RNAs that downregulate target gene expression by inhibiting target mRNA stability and translatability (Bartel, 2009). Target downregulation by miRNAs is primarily achieved through cognitive sites in the 3′ UTRs, with miRNA binding sites in other regions of target transcript generally contributing much less to functional regulation (Bartel, 2009). However, despite increasing understandings of how miRNAs regulate their targets, faithful identification of miRNA-mediated functional targeting still presents a major challenge.

In this study, we systematically surveyed miRNA-mediated regulation of TET2 expression, and the roles of TET2-targeting miRNAs in abnormal hematopoiesis. Unlike biochemical identification of miRNA binding regions on target mRNA (Lipchina et al., 2011; Chi et al., 2009; Hafner et al., 2010), this approach produced functional miRNA-target relationships rather than just binding relationships. We first cloned 3′ UTR luciferase reporters of human and mouse TET2 from the corresponding full-length isoforms. Although several splicing variants of TET2 have been reported (Langemeijer et al., 2009; Moran-Crusio et al., 2011), only the full-length isoforms encode the catalytic domain in the C terminus, the importance of which was confirmed in a murine knockout study by Quivoron et al. (2011). We next successfully screening IDH1/IDH2 (Shih et al., 2012), TET2-targeting miRNAs could be useful diagnostic biomarkers and potential therapeutics.

RESULTS
A High-Throughput Reporter Screen Identifies a Large Network of miRNAs that Inhibits TET2 3′ UTR
To identify TET2-targeting miRNAs, we undertook an unbiased high-throughput screen to identify miRNA-mediated regulation of TET2 3′ UTR (Figure 1A). Unlike biochemical identification of miRNA binding regions on target mRNA (Lipchina et al., 2011; Chi et al., 2009; Hafner et al., 2010), this approach produced functional miRNA-target relationships rather than just binding relationships. We first cloned 3′ UTR luciferase reporters of human and mouse TET2 from the corresponding full-length isoforms. Although several splicing variants of TET2 have been reported (Langemeijer et al., 2009; Moran-Crusio et al., 2011), only the full-length isoforms encode the catalytic domain in the C terminus, the importance of which was confirmed in a murine knockout study by Quivoron et al. (2011). We next successfully
miniaturized a cell-based reporter assay system, with which we quantified the effects of ~460 individual miRNA constructs (expressing a single miRNA or miRNA cluster) one by one with human or mouse TET2 3′ UTR reporters in quadruplicates in 384-well plates. For the vast majority of the assayed miRNA-3′ UTR pairs, the miRNAs had either no or weak effect on the corresponding TET2 3′ UTR (Figures S1A and S1B; Table S1). In contrast, 48 miRNA-3′ UTR pairs (see Experimental Procedures) led to a >25% repressive effect (Figures 1B and S1B; Table S1). Compared to two popular computational target prediction algorithms, TargetScan and miRSVR (Grimson et al., 2007; Betel et al., 2010), these inhibitory miRNA-3′ UTR relations include only 13% (32 out of 246) of predicted relations by both algorithms or 9% (44 out of 491) of those predicted by either algorithm, suggesting that the majority of the algorithm-predicted miRNA-3′ UTR pairs had weak or no effect (Figure S1C). In addition, 4 (8.3% of all) inhibitory miRNA-3′ UTR relations were not predicted, and 12 (25%) were only predicted by one of the two algorithms, suggesting a significant level of false negatives by these computational predictions. These data support the importance of defining TET2-targeting miRNAs through experimental approaches.

The 48 miRNA-3′ UTR pairs with >25% repression consist of 32 unique miRNAs that repress either human or mouse TET2 3′ UTRs. Among such TET2-targeting miRNAs, distinct constructs from the same miRNA family (i.e., sharing the same seed sequence) often led to similar effects, such as miR-29, miR-125, and miR-26 families (Figure 1B). We also noticed that whereas many candidate miRNAs had similar levels of repression on both human and mouse TET2 3′ UTRs, some show species specificity in functional targeting. For example, the miR-7 family had a much weaker repression of mouse 3′ UTR compared to human. To validate the screen result, we repeated the luciferase reporter assays on the candidate miRNAs and obtained highly consistent data (Figure S1D). In addition, by mutagenesis, we confirmed that miR-29b, miR-125a, miR-101, and miR-26a regulate TET2 3′ UTR through specific binding sites, supporting direct regulation (Figure S2). The data above show that our high-throughput reporter assay approach can reproducibly and systematically reveal miRNA-mediated regulation of TET2 3′ UTR by an extensive network of miRNAs.

These candidate TET2-targeting miRNAs contain mostly two classes of miRNAs: (1) miRNAs that are known to be involved in hematopoietic malignancies, and (2) oncogenic miRNAs without fully understood downstream mechanisms. For example, forced expression of miR-29a induces malignant hematopoiesis, yet the relevant miR-29a target for this biology is unknown (Han et al., 2010). On the other hand, miR-29b and miR-29c are not known to be myeloid oncogenes (Han et al., 2010), and miR-29b was instead reported as a tumor suppressor in myeloid leukemia (Garzon et al., 2009a, 2009b; Huang et al., 2013). In contrast to these reports, our data (Figure 1B) suggest that miR-29b and miR-29c could regulate TET2 and function as hematopoietic oncogenes under certain circumstances. Another example is the miR-125a/125b family, which is known to induce a CMML-like disease (reviewed in Shaham et al., 2012; Guo et al., 2012) via incompletely characterized mechanisms. Our data also suggest that additional TET2-targeting miRNAs, such as miR-101, miR-7, and miR-26a/26b, may play oncogenic roles in hematopoietic malignancies, which have not been previously recognized. We thus went on to further characterize the effects of TET2-targeting miRNAs in vitro and in vivo.

**TET2-Targeting miRNAs Downregulate TET2 Protein and 5hmC Levels in Hematopoietic Cells**

To systematically examine the functions of TET2-targeting miRNAs, we first determined whether these candidate miRNAs can regulate endogenous TET2 expression and function in hematopoietic cells. We initially examined hematopoietic cell lines BaF3 (murine) and K562 (human), which express endogenous TET2 protein at detectible levels (Figures S3A and S3B). We individually expressed ~16 candidate miRNAs in BaF3 and K562 cells, as well as a negative control miR-128b. We focused on those miRNAs that target both human and mouse 3′ UTRs, as well as a few that preferentially target the human 3′ UTR. Many of the miRNAs, including those from miR-29, miR-125, and miR-26 families, miR-101, and miR-520d significantly suppressed endogenous TET2 protein expression in both murine and human cells (Figures 2A and S3C). The miRNA-mediated downregulation of TET2 was also detected on the RNA level (Figure 2B). To confirm the regulation of TET2 in primary cells, we expressed miR-29b and miR-125a in primary murine bone marrow cells, which similarly decreased TET2 protein levels (Figure 2C). Consistent with the luciferase reporter screen data, miR-7 only reduced TET2 protein level in human but not in murine cells (Figures 2A and S3C). The decreases in endogenous TET2 protein levels were also accompanied by decreases in total cellular 5hmC levels (Figures 2D–2G and S3D), supporting that the function of TET protein(s) was compromised. Importantly, the downregulation of cellular 5hmC by miR-29b or miR-125a could be rescued by expression of a TET2 cDNA without 3′ UTR (Figures 2F and 2G), further supporting the role of a miRNA-TET pathway in the control of cellular 5hmC levels.

To determine whether the regulation by TET2-targeting miRNAs is physiologically or pathologically relevant, we first quantified the overexpression levels of miR-125a, miR-29b, miR-101, and miR-26a using quantitative RT-PCR and compared to those seen in human AML samples (see Experimental Procedures for details). Results showed that the overexpression levels of miR-125a and miR-101 were comparable to or lower than those reachable in AML samples, especially when considering the contribution of family members such as miR-125b (Figures S4C and S4D). For miR-29b and miR-26a, the levels of overexpression were ~2- to 10-fold higher than clinical AML samples (Figures S4E and S4F). We thus asked whether TET2 expression was under the control of endogenous miR-29 and miR-26 family miRNAs. We used miRNA sponges against miR-29 family or miR-26 family, which are decoy targets that inhibit miRNA function (Ebert et al., 2007). In BaF3 cells that express endogenous miR-29b and miR-26a at levels comparable to those in clinical samples (Figures S4E and S4F), both miR-29a/29b/29c sponge and miR-26a/26b sponge led to a significant increase in endogenous TET2 protein level, as well as an increase in cellular 5hmC (Figures 2H–2K). In contrast, miR-125a/125b sponge did not increase TET2 protein because endogenous miR-125a/125b expression is low in BaF3 cells (Figure S4C; Table S2) and thus...
serves as a negative control. Taken together, these data demonstrate that the endogenous TET2 can be regulated by an extensive network of miRNAs, the expression of which contributes to controlling the epigenetic landscape via targeting a central regulator of cellular 5hmC levels.

Multiple TET2-Targeting miRNAs Also Regulate TET1 and TET3

Given that there are three TET family proteins and that TET3, like TET2, is abundantly expressed in hematopoietic tissues (Ito et al., 2010), we asked whether miR-29b, miR-26a, and several other TET2-targeting miRNAs can also regulate TET1 and TET3. Indeed, expression of miR-29 and miR-26 family miRNAs resulted in an inhibition of TET1 and TET3 3’ UTR reporter activities (Figures 3A and 3B). These miRNAs also decreased endogenous TET3 RNA and protein levels in hematopoietic cells (Figures 3C and 3D). The endogenous TET1 RNA level was too low to be reliably quantified in these cells (data not shown).

These data reveal miRNA-mediated regulation of TET1 and TET3 and suggest that these specific miRNAs can function as
lineage, as well as hematopoietic expansion (Moran-Crusio et al., 2011; Pronier et al., 2011). We used a myeloid bias index to reflect the biased differentiation, which was calculated by ratios of myeloid (Mac1+) versus nonmyeloid cells in peripheral blood transduced GFP+ versus nontransduced GFP− populations (see Experimental Procedures). To quantify hematopoietic expansion, we followed the peripheral blood GFP+ percentage from 3 weeks posttransplantation and on. Among TET2-targeting miRNAs, the known oncogenic miR-125a served as a positive control, which led to both increased myeloid bias index, indicating skewed differentiation into myeloid lineage, and an increase in GFP+ percentage over time, indicating hematopoietic expansion (Figures 4A–4C). On the other hand, miR-144, which does not strongly affect TET2 3′ UTR, behaved similarly as the control vector (Figures 4A–4C). Consistent with miR-29b functioning as a bona fide oncogene, we observed both increased myeloid bias index and hematopoietic expansion (Figures 4A–4C). In addition, miR-29b mice had splenomegaly, expression led to a significant myeloid bias. Interestingly, although the GFP+ cell expansion was not statistically significant in the miR-101 cohorts, two out of ten miR-101 recipients showed persistent hematopoietic expansion, suggesting incomplete penetrance. In contrast, these phenotypes were never observed in cohorts of 12 control recipients (Figures 4A–4C). Another example is miR-26a, which led to a reproducible transient myeloid bias at 3.5 weeks posttransplantation (Figures 4A–4D), with one out of ten mice showing hematopoietic expansion. Multiple other tested miRNAs (Figures S5A–S5D) also led to variable degrees of myeloid bias and hematopoietic expansion, often with incomplete penetrance. Of note, the levels of overexpression in vivo were similar to those observed in vitro (Figures S4C–S4F), and we confirmed that 5hmC levels in vivo could be suppressed by TET2-targeting miR-29b and miR-125a (Figure S5E). The differential phenotypes induced by these TET2-targeting miRNAs may reflect the different degrees of repression on TET2, the level of overexpression, and/or the effects from additional targets. Taken together, we identified previously unrecognized functions for miRNAs in derailing normal hematopoiesis. They converge on inhibiting TET2 and induce phenotypes associated with hematopoietic malignancy.

Expression of TET2-Targeting miRNAs Leads to Malignant Hematopoietic Traits

To determine whether TET2-targeting miRNAs can cause malignant hematopoiesis, we next examined the effect of ten miRNAs in vivo. Forced expression of control or candidate miRNAs was delivered through viral transduction of wild-type bone marrow cells, with GFP marking transduced cells, followed by bone marrow transplantation into lethally irradiated host mice. We paid special attention to two hematopoietic traits associated with TET2 loss, namely biased differentiation into the myeloid lineage, as well as hematopoietic expansion (Moran-Crusio et al., 2011; Quivoron et al., 2011; Li et al., 2011; Ko et al., 2010; Pronier et al., 2011). We used a mieloid bias index to reflect the biased differentiation, which was calculated by ratios of myeloid (Mac1+) versus nonmyeloid cells in peripheral blood transduced (GFP+) versus nontransduced (GFP−) populations (see Experimental Procedures). To quantify hematopoietic expansion, we followed the peripheral blood GFP+ percentage from 3 weeks posttransplantation and on. Among TET2-targeting miRNAs, the known oncogenic miR-125a served as a positive control, which led to both increased myeloid bias index, indicating skewed differentiation into myeloid lineage, and an increase in GFP+ percentage over time, indicating hematopoietic expansion (Figures 4A–4C). On the other hand, miR-144, which does not strongly affect TET2 3′ UTR, behaved similarly as the control vector (Figures 4A–4C). Consistent with miR-29b functioning as a bona fide oncogene, we observed both increased myeloid bias index and hematopoietic expansion (Figures 4A–4C). In addition, miR-29b mice had splenomegaly, with increased percentage of GFP+/Ly6C+Ly6G− monocytes in bone marrow, revealing a CMMILike disease (Figures 4E and 4F). Other candidate miRNAs examined, except for miR-33, displayed these hematopoietic phenotypes to variable severities, and sometimes in unique manners (summarized in Figure S5D). For example, miR-101

expression led to a significant myeloid bias. Interestingly, although the GFP+ cell expansion was not statistically significant in the miR-101 cohorts, two out of ten miR-101 recipients showed persistent hematopoietic expansion, suggesting incomplete penetrance. In contrast, these phenotypes were never observed in cohorts of 12 control recipients (Figures 4A–4C). Another example is miR-26a, which led to a reproducible transient myeloid bias at 3.5 weeks posttransplantation (Figures 4A–4D), with one out of ten mice showing hematopoietic expansion. Multiple other tested miRNAs (Figures S5A–S5D) also led to variable degrees of myeloid bias and hematopoietic expansion, often with incomplete penetrance. Of note, the levels of overexpression in vivo were similar to those observed in vitro (Figures S4C–S4F), and we confirmed that 5hmC levels in vivo could be suppressed by TET2-targeting miR-29b and miR-125a (Figure S5E). The differential phenotypes induced by these TET2-targeting miRNAs may reflect the different degrees of repression on TET2, the level of overexpression, and/or the effects from additional targets. Taken together, we identified previously unrecognized functions for miRNAs in derailing normal hematopoiesis. They converge on inhibiting TET2 and induce phenotypes associated with hematopoietic malignancy.

Expression of TET2-Targeting miRNAs Regulate Additional TET Family Members

Given the in vivo phenotypes of TET2-targeting miRNAs, we asked whether TET2 expression can rescue miRNA-induced malignant hematopoiesis. We particularly focused on miR-125a, which potently induces a CMMILike disease in mice (Guo et al., 2012; and reviewed in Shaham et al., 2012), and miR-29b, which we characterized above as a new oncogene. Compared to other TET2-targeting miRNAs, another reason to
focus on miR-125a and miR-29b was that both miRNAs induced strong malignant phenotypes in vivo, and it would be more challenging to revert such strong phenotypes. Because in vivo hematopoietic expansion is often correlated with increased hematopoietic colonies in serial methylcellulose cultures in vitro, we first examined the effect of TET2 expression on colony formation in the presence of miR-125a or miR-29b. Specifically, wild-type bone marrow cells were transduced with control or indicated miRNAs, and transplanted into recipient mice, with GFP-labeling transduced cells. Peripheral blood was analyzed in recipients at the indicated time points, with each dot representing one recipient. (A) Myeloid bias index (frequency ratios of (%GFP+Mac1+/%GFP+Mac1−)/(%GFP−Mac1+/%GFP−Mac1−)) was calculated to reflect the biased presence of myeloid cells in transplanted population. (B) Normalized GFP ratios were also calculated (by taking the ratio of GFP+/GFP− cells and normalized to the average at 3.5 weeks) to reflect hematopoietic expansion. Numbers of mice per group are indicated in parentheses. The short horizontal bars represent median levels. (C and D) Representative flow cytometry plots of recipients at ~7 weeks posttransplantation (C) or 3.5 weeks posttransplantation (D) show myeloid marker Mac1 and GFP. (E) Splenomegaly in miR-29b recipients is shown. A representative image is shown on the left, with pooled spleen weight data shown on the right (n = 4 for Ctrl and n = 5 for 29b). (F) Bone marrow cells from Ctrl or miR-29b recipients were analyzed for granulocyte (Ly6G+Ly6C−) and monocytes (Ly6G−Ly6C+). Representative flow cytometry plots are shown, after gating on transduced myeloid cell populations (GFP+Mac1+ population). Note the increased monocytic frequency in miR-29b-transduced cells. Error bars represent SDs. *p < 0.05.

See also Figure S5.
significance (Figure 5F). This incomplete rescue of miR-29b-induced myeloid bias may be due to additional miR-29b targets or suboptimal stoichiometry of TET2 during rescue. Thus, our data demonstrate the importance of TET2 targeting in the oncogenic activities of miR-125a and miR-29b and suggest that increasing TET2 expression could be a potential strategy to combat certain groups of hematopoietic malignancies.

**TET2-Targeting miRNAs Are Preferentially Overexpressed in TET2-Wild-Type AMLs**

We assessed the miRNA-TET2 mechanism in the pathogenesis of human leukemia. Because decreased TET2 function could be a result of either genetic TET2 mutations or elevated expression of its targeting miRNAs, we reasoned that these two mechanisms likely occur independently, rather than redundantly. If true, we would expect TET2-targeting miRNAs to be more frequently overexpressed in TET2-wild-type leukemia, as compared to those harboring TET2 mutations. To test this possibility, we profiled miRNA expression for a cohort of 67 cases of cytogenetically normal AMLs, among which 16 patients carry protein sequence-altering TET2 mutations (Table S3). To assess miRNA overexpression outliers, we used a method similar to COPA (Tomlins et al., 2005) and quantified the frequency of TET2-wild-type and TET2-mutant samples with outlier overexpression levels, using false discovery rate <0.15 to define significant association (see Experimental Procedures). Among the 17 TET2-targeting miRNAs that we measured and passed detection threshold, overexpression of miR-125b, miR-29b, miR-29c, miR-101, and miR-7 was more frequently observed in TET2-wild-type cases than TET2-mutant cases, at two different outlier cutoffs (Figure 6; Table S4). Interestingly, the overexpression spectra of these miRNAs were not fully overlapped in TET2-wild-type AMLs (Figure S6E), suggesting that TET2-targeting miRNAs are differentially utilized in different AMLs in a largely nonredundant manner. Other TET2-targeting miRNAs were significant at a single outlier cutoff (miR-30e), had a single strong expressing sample in the TET2-wild-type but not TET2-mutant cohort (e.g., miR-520a-5p and miR-202), or were not significant in this cohort (Figures S6A–S6D; Table S4).

Figure 5. **TET2 Expression Rescues Malignant Phenotypes by Oncogenic miRNAs**

Bone marrow cells were transduced with Ctrl or miR-125a or miR-29b in combination with a cDNA Ctrl vector or TET2. The same number of sorted transduced cells was transplanted into each recipient in each experiment.

(A and B) For miR-125a/TET2 rescue, GFP+ percentage and myeloid bias index at 3.5 weeks posttransplantation are shown, with each dot representing one recipient. (C) Representative flow cytometry plots for (A) and (B) are presented.

(D) Bone marrow cells from miR-125a+Ctrl or miR-125a+TET2 recipients were analyzed for granulocyte (Ly6G+Ly6C–) and monocytes (Ly6G–Ly6C+). Representative flow cytometry plots are shown, after gating on transduced myeloid cell populations (GFP+Mac1+ population). Note that the monocytic bias in the miR-125a+Ctrl recipient was largely corrected by TET2 expression.

(E and F) For miR-29b/TET2 rescue, GFP+ percentage and myeloid bias index at 3.5 weeks posttransplantation are shown, with each dot representing one recipient. (G) Representative flow cytometry plots for (E) and (F) are shown. The short horizontal bars represent median levels. The p values are indicated. See also Figure S5.
An alternative explanation for the preferential overexpression of TET2-targeting miRNAs in TET2-wild-type leukemia is that TET2 functionally upregulates these miRNAs. To examine this possibility, we overexpressed TET2 cDNA in BaF3 and K562 cells and knocked down TET2 in BaF3 cells (Figures S3B, 2D, and S3D) and then profiled miRNA expression. Results showed that although TET2 level modulation altered the expression of some miRNAs, the five TET2-targeting miRNAs that correlated with TET2 mutational status were not positively regulated by TET2 (Table S2). Another possibility is that many other miRNAs can score significantly in this statistical test, and our observation of the association with TET2-targeting miRNAs is solely random. To exclude this possibility, we examined >580 miRNAs profiled in this cohort. Excluding the TET2-targeting miRNAs, there were 196 miRNAs passing detection threshold, among which 9 miRNAs were similarly associated with TET2 wild-type status (Table S5). These nine miRNAs include miR-99a, which is located in the same genomic cluster as miR-125b-2 and, thus, coexpressed with the TET2-targeting miR-125b (Figure S6F). In addition, three of the miRNAs (miR-18a, miR-18b, and miR-19a) are known to be coexpressed and were correlated with each other in our data set (Table S5). Even without eliminating such influences, our observation of 5 out of 17 (29%) TET2-targeting miRNAs scoring in this test is significant (as compared to 9 out of 196, or 4.6%; \( p < 0.003 \), Fisher’s exact test). Taken together, these data support the notion that overexpression of a subset of TET2-targeting miRNAs identified in this study can be an important mechanism in human leukemogenesis.

**DISCUSSION**

Using a high-throughput reporter screen, our study systematically identified miRNA-mediated regulation of TET2 through its 3’ UTR and revealed the roles of TET2-targeting miRNAs in malignant hematopoiesis. We found that in a cohort of cytogenetically normal human AMLs, multiple TET2-targeting miRNAs, including miR-29b, miR-101, miR-125b, miR-29c, and miR-7, were preferentially overexpressed in TET2-wild-type specimens than those with TET2 mutations. These data support a role for miRNA-TET2 pathway in the pathogenesis of human AML and other malignancies, adding a new layer to the existing paradigms of loss-of-function mutations in TET2 and gain-of-function mutations in the IDH genes. Our data also argue that in addition to routine genetic mutational analyses on TET2 and related IDH1/IDH2 genes, which are currently being developed and implemented in clinics, the expression status of our identified TET2-targeting miRNAs could be considered as an additional diagnostic parameter to inform the deregulation of the TET2 pathway. In this regard, measuring TET2-targeting miRNAs has advantages over directly measuring TET2 protein or mRNA levels, due to the limited range of differential TET2 expression and difficulty in its protein measurements. For example, we noticed that the range of differential TET2 RNA expression in AML samples is ~3-fold (data not shown) and thus is susceptible to interference by measurement noise. TET2-protein measurements suffer from the same restraints, and western-based measurements require a large number of cells. In contrast, TET2-targeting miRNAs displayed a much larger dynamic range of expression. For example,
miR-125b expression has a range over 10,000-fold in the same data sets (Figure S4C). Our findings also raise several important questions to be further examined in future diagnostic and prognostic studies. For example, do TET2-targeting miRNAs contribute differently in the pathogenesis of single-allelic TET2 mutants versus biallelic mutants (which our study cohort was not statistically powered to address, given that a much larger cohort will be needed)? In addition, it is important to point out that other TET2-targeting miRNAs demonstrated in this study may also have a role in human hematopoietic malignancies, even though they were not significantly associated in this cyogenetically normal AML cohort because they may be involved in other AML types or other hematopoietic malignancies (Shih et al., 2012).

While this work was being revised, it was published that miR-22 targets TET2 through 3’ UTR and regulates hematopoietic stem cells (Song et al., 2013a, 2013b). While our work systematically complements and extends these findings on TET2 regulation, it is also interesting to note that we did not detect a repressive effect of miR-22 on TET2 3’ UTR (Table S1), even though we confirmed that miR-22 was overexpressed >12-fold (Figure S4G). In our AML cohort, miR-22 did not show significant association with TET2 mutational status (Table S5). The difference in 3’ UTR data may be due to the use of full-length TET2 3’ UTR in our study versus a much shorter 500 bp 3’ UTR fragment (Song et al., 2013b). Because it is recognized that the location of the miRNA binding site within the 3’ UTR and target RNA structure can determine the effectiveness of miRNA binding sites (Bartel, 2009; Long et al., 2007), it raises the possibility that additional mechanisms regulate the presentation of the miR-22 binding site.

Our data also uncovered multiple miRNAs with unrecognized oncogenic potential and revealed TET2 targeting as a relevant mechanism of previously known oncogenic miRNAs. For example, miR-29b was previously recognized as a tumor suppressor in myeloid leukemia (Garzon et al., 2009a, 2009b), but our study demonstrated an opposite oncogenic role of this miRNA. The differences observed may be related to the level of miR-29b expression or the duration of expression. Interestingly, the miR-29 family and miR-26 family miRNAs regulate TET1 and TET3 in addition to TET2, suggesting a miRNA-mediated master regulatory program in shaping cellular 5hmC landscape. The in vitro and in vivo data presented here also showed that miRNAs, such as miR-101, miR-29c, miR-26a/26b, and miR-520d, can function as previously unappreciated oncogenes by derailing normal hematopoietic differentiation processes and provided a new molecular mechanism for the known oncogenic miRNAs, including the miR-125 family and miR-29a. It is also interesting to note that although all these miRNAs were capable of targeting TET2, their in vivo overexpression phenotypes were variable. One possibility for such differences is the involvement of other targets of the specific miRNAs. For example, miR-125 family miRNAs regulate multiple pathways (Shaham et al., 2012), such as enhancing growth factor signaling and inhibiting apoptosis (Guo et al., 2010, 2012), which may cooperate with TET2 repression by this miRNA. Similarly, miR-101 can also regulate the PRC2 component EZH2 (Varambally et al., 2008), suggesting a broad effect of this miRNA in regulating multiple enzymes that control the epigenome. Another example is the miR-26 family, which led to transient myeloid differentiation bias but only caused hematopoietic expansion in a small number of mice. Notably, miR-26 has been shown to target cyclin D2 and E2, and inhibit cell cycle in other cancer types (Kota et al., 2009), a mechanism that may modify the TET2-targeting effect of this miRNA. Alternatively, the different efficiencies of TET2 targeting by different miRNAs may themselves contribute to the varying phenotypes. In this regard, it is important to note that minor changes in TET2 expression cannot only lead to functional consequences in malignant hematopoiesis but also be associated with longer latency or incomplete penetrance. For example, heterozygous TET2 knockout in mouse, which led to ~50% loss of TET2 gene expression (Li et al., 2011), results in significant but slower and less-frequent malignant transformation than double-allele knockout (Li et al., 2011; Quivoron et al., 2011; Moran-Crusio et al., 2011). As a third possibility, it is also conceivable that such in vivo phenotype differences were due to different levels of overexpression of TET2-targeting miRNAs (Figure S4). It will be interesting to dissect these possibilities in the future.

Finally, our data raise the prospect of enhancing TET2 expression to combat certain subgroups of hematopoietic malignancies, and implicate modulating TET2-targeting miRNAs as a strategy for both solid and hematopoietic cancers. Recently, decreased TET gene expression and cellular 5hmC levels have been found as a hallmark of multiple solid cancer types (Yang et al., 2013; Lian et al., 2012; Hsu et al., 2012), whereas elevating TET1 or TET2 gene expression has been proposed as a strategy against melanoma and breast cancer (Lian et al., 2012; Hsu et al., 2012). When we expressed TET2 together with oncogenic miR-29b and miR-125a, we observed strong suppression of miRNA-mediated malignant phenotypes. In the case of miR-125a, TET2 expression not only suppressed hematopoietic expansion but remarkably also corrected multiple differentiation biases induced by miR-125a. These data suggest that targeting mechanisms that inhibit TET2 gene expression may be a useful strategy to overcome certain hematopoietic malignancies. Our findings of an extensive network of TET2-targeting miRNAs and several pan-TET-inhibitory miRNAs raise the possibility and opportunity for future therapeutic intervention in this pathway.

**EXPERIMENTAL PROCEDURES**

**Luciferase Reporter Assay and Analysis**

Reporter assays were carried out in 384-well plates. Specifically, 460 miRNA constructs were individually assayed in combination with 3’ UTR luciferase reporters. 293T cells were transfected with 6 ng of 3’ UTR reporter and 54 ng of a miRNA construct in each well. After 2 days, luciferase assays were performed using the Dual-Glo Luciferase kit (Promega). We built three types of control miRNA construct in each well. After 2 days, luciferase assays were performed using the Dual-Glo Luciferase kit (Promega). We built three types of control miRNA construct in each well. After 2 days, luciferase assays were performed using the Dual-Glo Luciferase kit (Promega). We built three types of control miRNA construct in each well. After 2 days, luciferase assays were performed using the Dual-Glo Luciferase kit (Promega).
identify TET2-targeting miRNAs, we excluded constructs for clusters of miRNAs and categorized the same mature miRNA appearing at different genome loci as only one TET2-targeting miRNA. See Extended Experimental Procedures for more details.

Murine Bone Marrow Transplantation and Related Experiments
All mouse experiments were approved by Yale IACUC and followed federal, state, and institutional guidelines. Bone marrow transplantation with single miRNAs cloned into the pMIRWAY-GFP-based vectors was performed as described previously (Guo et al., 2012). These miRNAs included miR-29b-1, miR-125a, miR-26a-1, miR-101-1, miR-767, miR-520d, miR-33, miR-153-2, miR-144, and a vector control.

For Tet2 rescue experiments, 5-FU-primed bone marrow cells were cotransduced with mouse Tet2cDNA (with puromycin marker), or a corresponding vector control together with a specific miRNA expression construct in pMIRWAY-GFP backbone. Transduced cells were cultured, selected with puromycin, and sorted for GFP+ cells for transplantation. A total of 50,000 cells (per mouse) were injected for miR-125a-related rescue and control groups, and 10,000 cells (per mouse) were injected for the miR-29b rescue and control groups.

Assessment of hematopoietic phenotypes was performed as previously described (Guo et al., 2010, 2012; Adams et al., 2013). The myeloid bias index was used to quantify biased differentiation into myeloid lineages, which was calculated by: (%GFP×Mac1+)/(%GFP×Mac1−)/(%GFP×Mac1−/(%GFP×Mac1−/(%GFP×Mac1−)). To examine monocytic differentiation bias, Mac1+ cells from GFP+ fraction (transduced) were gated before examining Ly6C and Ly6G distribution. To examine hematopoietic expansion, peripheral %GFP+GFP− ratios were taken and normalized to the ratio at 3.5 weeks posttransplantation. All flow cytometry antibodies were from BD Biosciences or eBioscience.

For assessment of in vivo effect of miRNA overexpression levels or 5hmC levels, Mac1+GFP+ cells was FACS-sorted from recipient mice and subjected to qRT-PCR or 5hmC analysis.

Clinical Samples and TET2 Sequence Analysis
All human AML samples were obtained with informed consent and approved by the Ethics Committee of the First Affiliated Hospital of Soochow University (FAHSU). A total of 67 Chinese patients with AML with normal cytogenetics by the Ethics Committee of the First Affiliated Hospital of Soochow University (FAHSU). A total of 67 Chinese patients with AML with normal cytogenetics were analyzed by PCR amplification of the entire coding region spanning exon 3 to exon 11 followed by direct bidirectional DNA sequencing, as previously described by Delhommeau et al. (2009). For analysis purpose, samples with nonsynonymous coding sequence alterations were classified as TET2 mutant, whereas those without amino acid-altering coding region changes were defined as TET2-wild-type (Table S3).

Statistical Analysis
The Student’s t test was used to assess statistical significance, unless otherwise stated. The false discovery rate was calculated following the Benjamini Hochberg method.

Additional Procedures
Constructs, computational target analysis, cell culture, western blot and dot blot analyses, quantitative RT-PCR, colony formation assays, and miRNA profiling and data analyses are described in Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Extended Experimental Procedures, six figures, and six tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2013.08.050.

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