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The oncogenic microRNA miR-22 targets the TET2 tumor suppressor to promote hematopoietic stem cell self-renewal and transformation

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SUMMARY

MicroRNAs are frequently deregulated in cancer. Here we show that miR-22 is upregulated in myelodysplastic syndrome (MDS) and leukemia, and its aberrant expression correlates with poor survival. To explore its role in hematopoietic stem cell function and malignancy, we generated transgenic mice conditionally expressing miR-22 in the hematopoietic compartment. These mice displayed reduced levels of global 5-hydroxymethylcytosine (5-hmC) and increased hematopoietic stem cell self-renewal, accompanied by defective differentiation. Conversely, miR-22 inhibition blocked proliferation in both mouse and human leukemic cells. Over time, miR-22 transgenic mice developed MDS and hematological malignancies. We also identify TET2 as a key target of miR-22 in this context. Ectopic expression of TET2 suppressed the miR-22-incuced phenotypes. Downregulation of TET2 protein also correlated with poor clinical outcomes and miR-22 overexpression in MDS patients. Our results therefore identify miR-22 as a potent proto-oncogene, and suggest that aberrations in the miR-22-TET2 regulatory network are common in hematopoietic malignancies.

INTRODUCTION

Epigenetic mechanisms such as DNA methylation and histone modification drive stable and clonally propagated changes in gene expression, and have recently been shown to serve as molecular mediators of pathway dysfunction in neoplasia (Esteller, 2008; Rodriguez-

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Paredes and Esteller, 2011). Indeed, both myelodysplastic syndrome (MDS) and leukemias are characterized by frequent epigenetic abnormalities and aberrant DNA hypermethylation has been linked to poor prognosis in cases of MDS, as it is associated with more rapid progression to acute myeloid leukemia (AML) (Shen et al., 2010; Shih et al., 2012). Importantly, genome-wide discovery efforts in patients with myeloid malignancies have led to the identification of a number of causal genetic abnormalities that impact the epigenetic landscape. These genes include *TET2*, *IDH1*, *IDH2*, *DNMT3a* and *EZH2*, all of which affect DNA and/or histone lysine methylation (Shih et al., 2012).

Recent studies have examined in detail one such gene, *TET2* (ten-eleven-translocation gene 2), which is located in 4q24, and whose mutation or deletion is extremely frequent in hematological malignancies, affecting 19% of patients with MDS, 12% of patients with myeloproliferative neoplasm (MPN), and 24% of patients with secondary AML (Abdel-Wahab et al., 2009; Delhommeau et al., 2009; Jankowska et al., 2009; Tefferi et al., 2009). *TET2* mutations are observed in $CD34^+$ progenitor cells and detected most frequently after MPN and MDS progression to AML (Abdel-Wahab et al., 2010).

TET2 catalyzes the oxidation of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) and is known to control hematopoiesis, presumably by regulating gene expression through its effect on DNA methylation (Branco et al., 2012; Ito et al., 2011; Ko et al., 2010; Shih et al., 2012). *Tet2* deficiency in mice impairs 5-mC hydroxylation and leads to skewed differentiation, as well as enhancement of the self-renewal and repopulating capacity of hematopoietic stem cells (HSCs). This in turn promotes malignant transformation in mice, causing disorders resembling MDS and MPN, as well as chronic myelomonocytic leukemia (CMMoL) (Moran-Crusio et al., 2011; Quivoron et al., 2011). Recent studies have likewise shown that leukemia-associated Isocitrate Dehydrogenase (IDH) 1 or 2 mutant enzymes can induce carboxylation of glutamine-derived α-ketoglutarate (α-KG), with a concomitant increase in synthesis of 2-hydroxyglutarate (2-HG), an inhibitory metabolite of TET2 (Figueroa et al., 2010; Sasaki et al., 2012). Collectively, these studies implicate functional and mutational losses of *TET2* as a key event underlying aberrant hematopoiesis and leukemogenesis, in turn suggesting that the inactivation of TET2 function by additional upstream cues could play a critical role in hematological malignancies.

MicroRNA (miRNA) deregulation is also known to contribute to hematological malignancies, including MDS and AML (Bartel, 2004; Chen et al., 2004; Gangaraju and Lin, 2009). A combined approach involving assessment of miRNA expression (using microarrays) and bioinformatic prediction of mRNA targets has revealed that distinct miRNA signatures fine-tune each step of hematopoiesis, including the reconstitution potential of HSCs (Arnold et al., 2011; Gangaraju and Lin, 2009). In turn, miRNAs that target critical leukemia suppressors could act as powerful proto-oncogenes in the hematopoietic compartment (Calin and Croce, 2006).

In this study, we demonstrate that miR-22 controls hematopoiesis and HSC maintenance by negatively regulating TET2 protein levels. Importantly, we identify miR-22 as an epigenetic modifier and the key oncogenic determinant for the pathogenesis of MDS and hematological malignancies *in vivo*, findings with novel prognostic and critical therapeutic implications for a large number of hematological disorders.

RESULTS

miR-22 is highly expressed in human hematological malignancies and increases the replating ability of hematopoietic stem cells

In order to build on the insights of microRNA biology into epigenetic alterations in myeloid malignancies, we attempted to identify those miRNAs playing a critical role in the pathogenesis of the stem cell disorder myelodysplastic syndrome (MDS), and its progression to overt leukemia. We first analyzed a previously reported miRNA microarray profiling database and found that miR-22 is markedly upregulated in patients with MDS (Figures S1A and S1B and Ref. (Dostalova Merkerova et al., 2011)). These results prompted us to perform expression analysis by *in situ* hybridization in a large set of patient samples, which included bone marrow with no sign of disease (n=37), early stage MDS (refractory anemia (RA), 5q syndrome (5q-), refractory cytopenia with multilineage dysplasia (RCMD) and RA with ringed sideroblasts (RARS)) (n=40) and RAEB (RA with excess blasts) (n=67). Our comprehensive *in situ* hybridization analysis revealed that miR-22 is highly expressed in patients with MDS (Figure 1A **and** Figure S1C). We next estimated the survival rates of the sets of MDS patients by using a Kaplan-Meier analysis, and applied a log-rank test to compare the survival rates of MDS patients on the basis of miR-22 expression levels. Remarkably, we found that aberrant expression of miR-22 directly correlates with poor survival rates in patients (Figure 1B). It is noteworthy that the distribution of blasts into each WHO (World Health Organization) category of MDS stages was not directly associated with the expression level of miR-22, but a significant correlation between miR-22 levels and poor survival rates was observed within each WHO classification, as well as among MDS patients harboring a normal karyotype (Figures S1D–S1F). This indicates that the poorer survival expectancy linked to miR-22 overexpression does not simply reflect confounding factors such as blast count and cytogenetic karyotype. Taken together, these findings led us to investigate the role of miR-22 in the hematopoietic system and the genesis of hematological malignancies.

To explore the function of miR-22 in hematopoiesis and leukemia, we first transduced mouse hematopoietic stem/progenitor cells (HSPCs; c-Kit^{pos}Sca-1^{pos}Lin^{neg} hereafter referred to as KSL cells) isolated from wild-type donor mice with either a retroviral vector encoding miR-22 and a green fluorescent protein (GFP), or a control vector encoding GFP only. The GFP-expressing KSL cells were then re-sorted by flow cytometry and plated in methylcellulose for an *in vitro* CFU (colony-forming unit) assay (Figure S1G). We observed in the first plating that the cells expressing miR-22 produced fewer colonies compared to control cells; however, when GFP$^+$KSL cells were re-sorted and re-plated, miR-22–transduced cells produced more colonies in the second plating (Figure S1H). Strikingly, these cells also maintained this ability throughout serial replatings – whereas control cells only formed colonies in the first two platings, miR-22–expressing KSL cells were able to produce colonies after being re-plated at least seven times (Figure S1H). Microscopic analysis of the cells from these colonies revealed a homogenous blast-like morphology (Figure S1I, **top)**. Consistent with these results, miR-22–expressing KSL cells maintained their characteristics and c-Kit expression (a marker of undifferentiated cells) during their replatings (Figure S1I, **bottom and** Figures S1J and S1K). To ensure that miR-22–transduced and control cells were subjected to the same experimental treatment, we also performed an *in vitro* competitive colony assay by mixing GFP$^+$KSL cells expressing miR-22 and GFP$^-$ control KSL cells (Figure S1L, **top)**. This analysis confirmed that miR-22–expressing GFP$^+$, but not GFP$^-$, KSL cells remained mostly in colonies through consecutive replatings (Figure S1L, **bottom)**. Taken together, these data suggest that miR-22 can drive aberrant replating capacity of HSPCs and may contribute to malignant transformation.

miR-22 affects hematopoietic stem cell biology *in vivo*

We next sought to examine the biological effects of miR-22 *in vivo* and generated a conditional transgenic mouse model overexpressing miR-22 in the hematopoietic compartment (Figure S2A). miR-22 expression was induced by *Mx1-Cre*–mediated excision of a *LoxP* flanked transcriptional STOP cassette by administrating seven doses of polyinosine-polycytidine (pIpC) over 14 days (Figures S2B and S2C). Two weeks after pIpC administration, we isolated KSL cells from *miR-22*$^{F/+}$;*Mx1-Cre* mice and *Mx1-Cre* littermate controls and analyzed their properties by using CFU and LTC-IC (long-term culture-initiating cell) assays (Figures 1C–1G and Figure S2D). In agreement with our previous retroviral transduction experiments, we found that KSL cells from *miR-22*$^{F/+}$;*Mx1-Cre* transgenic mice displayed an enhanced replating potential (Figures 1D and 1E). Furthermore, ectopic expression of miR-22 caused a robust increase in the number of both KSL and mature myeloid cells, as determined by LTC-IC assay, indicating that miR-22 enhances HSPC maintenance and predisposes those cells to differentiate into the myeloid lineage (Figures 1F and 1G).

Next, we examined the ability of miR-22–overexpressing HSPCs to compete with wild-type counterparts *in vivo* by utilizing an *in vivo* competitive transplantation assay. KSL cells from *miR-22*$^{F/+}$;*Mx1-Cre* and *Mx1-Cre* mice (Ly45.2) were purified 2 weeks after pIpC administration, mixed with Ly45.1/Ly45.2 competitor bone marrow mononuclear cells (BM MNCs) and transplanted into lethally irradiated Ly45.1 congenic recipient mice. The ability of donor cells to contribute to the reconstitution was then determined by flow cytometric analysis of peripheral blood stained for Ly45.1 and Ly45.2. Within 3 weeks of transplantation, donor cells from *miR-22*$^{F/+}$;*Mx1-Cre* mice exhibited a slight increase in chimerism compared to those from control animals (Figure S2E). Over time this difference became more pronounced as *miR-22*$^{F/+}$;*Mx1-Cre* KSL cells outcompeted their wild-type counterparts (Figures 2A–2C and Figure S2F). At 6 weeks of post-transplantation, an increased chimerism was observed in all BM MNCs as well as within the myeloid fraction, and by 9 weeks this effect was also evident in the c-Kit^{pos} fraction (Figures 2A–2F). Taken together, these data strongly suggest that miR-22 enhances the self-renewal capacity of HSPCs *in vivo*.

miR-22 triggers MDS-like syndromes and hematological malignancies *in vivo*

To further test whether increased aberrant hematopoiesis caused by miR-22 overexpression ultimately progresses to malignant hematological disease, we continued to follow up primarily transplanted recipients. Notably, at 12 weeks of post-transplantation, recipient mice transplanted with KSL cells from *miR-22*$^{F/+}$;*Mx1-Cre* donors, but not from *Mx1-Cre* donors, developed an illness which closely resembled human MDS, as characterized by defective erythroid maturation and anemia, splenomegaly with myeloid infiltration, low white blood cell (WBC) counts and dysplastic myeloid cells in the peripheral circulation (Figure 3 and Figures S2G and S2H). To determine whether this disorder is transplantable, the donor-derived KSL cells re-sorted from primary recipient mice were subjected to a secondary transplantation using fresh competitor cells. In agreement with our earlier results, all secondary recipients developed a lethal hematological disease within four weeks after secondary transplantation (Figure S2I).

Furthermore, we also monitored disease onset and progression to malignancy in miR-22 transgenic mice. The development of disease similar to that found in our chimeric transplantation model was observed in *miR-22*$^{F/+}$;*Mx1-Cre* mice but not *Mx1-Cre* littermate controls at 8 months after pIpC administration: the hallmarks of this distress included dysplasia in multi-lineage including erythroid, myeloid and platelet, splenomegaly and an increased number of c-Kit-positive undifferentiated blastic cells in the peripheral blood

(Figures 4A–4C). By 16 months after pIpC administration, all miR-22 transgenic mice had developed MDS, affecting multi-lineage hematopoiesis. Importantly, ~13% of mice succumbed to myeloid leukemia as well as T cell lymphoma by 11 months, and all miR-22 transgenic mice suffered from hematological abnormality by 16 months. Finally, ~70% of miR-22 transgenic mice developed myeloid leukemia within two years (Figures 4D–4G).

We also confirmed that the disease was triggered by miR-22 from KSL cells sorted from miR-22–expressing colonies. To this end, miR-22–expressing cells were subjected to consecutive colony replating and at the 6th replating, the residual KL (c-Kit^{pos}Lin^{neg}) cells were transplanted into lethally irradiated recipient mice (Figure S3A). Consistent with our *in vivo* miR-22 transgenic mice data, by 14 weeks after transplantation, recipient mice transplanted with miR-22–expressing cells developed myeloid leukemia, as characterized by increased myeloid blasts in the peripheral blood, defective erythroid maturation and anemia, splenomegaly with the infiltration of myeloid blasts, and increased c-Kit-positive blasts in bone marrow (Figures S3B–S3H). Taken together, these data strongly suggest that miR-22 can enhance HSPC function and contribute to leukemia development *in vivo.*

miR-22 directly targets TET2 and affects the epigenetic landscape of hematopoietic compartment *in vivo*

To identify relevant molecular targets of miR-22, we next undertook a bioinformatic analysis using TargetScan 6.0 (http://targetscan.org) (Lewis et al., 2005), microRNA.org (http://www.microrna.org) (Betel et al., 2008) and miRBase (http://www.mirbase.org) (Griffiths-Jones et al., 2006). Among various potential targets, we focused on TET2 (ten eleven translocation 2) as it possesses critically conserved nucleotides indicative of a legitimate target (Figure S4A). We then confirmed a direct interaction of miR-22 with the 3′UTR region of the *TET2* gene by luciferase reporter assay (Figure S4B **and Song *et al.*, in press)**. TET2 is a member of the TET methylcytosine dioxygenase family, which catalyzes the conversion of 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC). TET proteins have been implicated in epigenetic re-programming, embryonic stem cell maintenance and early development (Ficz et al., 2011; Ito et al., 2010). Importantly, recurrent *TET2* mutations have been identified in a variety of hematological malignancies including MDS, MPN (myeloproliferative neoplasm), CMMoL and AML (Abdel-Wahab et al., 2009; Delhommeau et al., 2009; Jankowska et al., 2009; Tefferi et al., 2009). It is noteworthy that the phenotypes elicited by miR-22 overexpression closely phenocopy the inactivation of *Tet2* in the hematopoietic system both *in vitro* and *in vivo* (Li et al., 2011; Moran-Crusio et al., 2011; Quivoron et al., 2011). It has also been documented that Tet2 expression is increased during hematopoietic differentiation, and conversely, that miR-22 is highly expressed in HSPCs while its expression declines in mature cells (Figure S4C and Ref. (Moran-Crusio et al., 2011)). We therefore tested whether miR-22 could act as an authentic *TET2*-targeting microRNA in the hematopoietic compartment by utilizing our conditional transgenic mouse model. To this end, we measured Tet2 expression in the peripheral blood and bone marrow of *miR-22$^{F/+}$;Mx1-Cre* or *Mx1-Cre* littermate mice at 2~3 weeks after pIpC administration. Importantly, real-time qPCR and Western blot analyses revealed a marked reduction in levels of Tet2 mRNA and protein, respectively, in *miR-22$^{F/+}$;Mx1-Cre* mice compared to littermate controls (Figures 5A, 5B, 5E–5G and Figure S4D).

On the basis of these observations, we then sought to determine whether miR-22–mediated TET2 inactivation is sufficient to impact the global epigenetic landscape in blood cells. We therefore analyzed 5-hmC and 5-mC levels in the genomic DNA of *miR-22$^{F/+}$;Mx1-Cre* mice. Immunofluorescence and dot blot analyses revealed a drastic (more than two-fold) reduction in 5-hmC levels and a concomitant increase in 5-mC levels in bone marrow of

these mice (Figures 5C and 5D), suggesting that changes in an important epigenetic mark (e.g. 5-hmC) may contribute to the ability of miR-22 to affect hematopoiesis and leukemogenesis.

To further understand the consequences of repression of TET2 by miR-22 in hematopoiesis, we next examined the effects of miR-22 overexpression on putative targets of TET2 (Kallin et al., 2012; Ko et al., 2010; Yamazaki et al., 2012). A comprehensive real-time qPCR analysis revealed that the expression levels of a substantial number of putative Tet2 target genes, including *Aim2*, *Hal*, *Igbt2* and *Sp140*, were markedly decreased in the hematopoietic stem cell compartment ($CD150^{pos}CD48^{neg}Flt3^{neg}CD34^{neg}$KSL cells) purified from *miR-22*$^{F/+}$;*Mx1-Cre* mice (Figure 5E and Figure S4E). Interestingly, AIM2 (absence in myeloma 2) has been reported to have a role in the reduction of cell proliferation by cell cycle arrest, and to also be highly methylated in MDS, especially in CMMoL patients with *TET2* mutations (Figueroa et al., 2010; Patsos et al., 2010; Yamazaki et al., 2012). Therefore we hypothesized that its silencing via methylation of the promoter might provide a growth advantage to cancer cells. Additionally, polymorphisms of *SP140* (SP140 nuclear body protein) have been found to correlate with chronic lymphoid leukemia, and its hypermethylation has been also demonstrated in CMMoL patients with *TET2* mutations, and hence its hypermethylation may influence the risk of leukemogenesis (Di Bernardo et al., 2008; Ko et al., 2010; Yamazaki et al., 2012). Indeed, we confirmed that the expression of Aim2 and Sp140 was drastically reduced at the levels of mRNA and protein in the bone marrow of *miR-22*$^{F/+}$;*Mx1-Cre* mice compared to littermate controls, and this reduction was associated with an increased promoter methylation (Figures 5F and 5G and Figure S4F). Taken together, these results suggest that miR-22 represses TET2 expression and thereby can remodel the epigenetic landscape, with global changes in 5-hmC levels and alteration in the expression of putative TET2 target genes including *AIM2* and *SP140*.

miR-22 enhances stem cell function and triggers hematological transformation through repression of TET2

Next, we explored the relevance of the cross talk between miR-22 and TET2 in hematopoiesis and hematological malignancies. To test whether TET2 directly contributes to the function of miR-22 in hematopoietic compartment, we first infected KSL cells isolated from wild-type mice with a retroviral vector encoding miR-22 and GFP. After the re-sorting, GFP^{+}KSL cells were additionally infected with a *TET2*-expressing lentiviral particle. *In vitro* colony replating and LTC-IC assays demonstrated that the phenotypes caused by miR-22 overexpression in hematopoietic compartment are potently suppressed by ectopic expression of TET2 (Figures S4G–S4J). Notably, an *in vivo* transplantation assay revealed that ectopic expression of TET2 leads to survival advantages for recipient mice transplanted with miR-22–expressing KSL cells (Figure S4K).

To further examine this interaction, we purified KSL cells from miR-22 transgenic mice and infected them with a retroviral vector encoding Cre recombinase and GFP. After the re-sorting of GFP^{+}KSL cells, cells were additionally infected with a TET2-expressing lentiviral particle and the resulting phenotypes were analyzed. *In vitro* colony replating and LTC-IC analyses demonstrated that the oncogenic function of miR-22 was antagonized by ectopic expression of TET2 (Figures 6A and 6B). Notably, when KSL cells of *miR-22*$^{F/+}$ mice were simultaneously co-infected with a *Cre* recombinase construct and a TET2-expressing construct, ectopic expression of TET2 more strikingly impaired the replating potential of GFP^{+}KSL cells (Figures S5A and S5B). In line with these observations, an *in vivo* transplantation assay revealed significant survival advantages in recipient mice transplanted with *miR-22*$^{F/+}$ KSL cells co-infected with Cre and TET2 compared to those with *miR-22*$^{F/+}$ KSL cells co-infected with Cre and control vector (Figure 6C). Importantly, the

positive survival effect drawn by ectopic expression of TET2 was more pronounced in secondary transplantation (Figure S5C).

Finally, we purified KSL cells from miR-22 transgenic mice after pIpC administration, infected them with a TET2-expressing lentiviral particle and evaluated the resulting phenotypes (Figure 6D). We found that all the phenotypes elicited by an elevation of miR-22 *in vitro* and *in vivo* in hematopoietic compartment, including repression of the expression of Aim2 and Sp140 in bone marrow, were also significantly suppressed by ectopic expression of TET2 (Figures 6E–6H and Figure S5D).

Importantly, when we purified KSL cells from primary miR-22 transgenic mice and infected them with TET2 followed by *in vivo* transplantation together with competitor cells, ectopic expression of TET2 delayed the onset of hematological disorder, accompanied with significant advantages in disease-free survival (Figure S5E). Taken together, these data suggest that TET2 is one of the major targets responsible for the miR-22 proto-oncogenic function in hematopoiesis and development of hematological malignancies.

miR-22 decoying offers novel therapeutic opportunities and its overexpression correlates with the silencing of TET2 protein in MDS and AML patients

Next, we investigated whether the miR-22/TET2 interaction may offer a therapeutic opportunity for the treatment of hematological malignancies. We therefore tested whether inhibition of miR-22 affects TET2 expression and activity, as well as the biology of leukemias. To this end, we inhibited miR-22 in human leukemia cell lines, K562 and U937, both of which express relatively high levels of miR-22 (Figure S5F and Ref. (Wang et al., 2012)), by using a miR-22 retroviral sponge or a custom-made LNA (locked nucleic acid (Petersen and Wengel, 2003)) miR-22 decoy as the miR-22 sponge. Critically, inhibition of miR-22 in human leukemic cells resulted in a significant reduction in cell proliferation as well as an elevation of the expression of TET2 and its targets, AIM2 and SP140 (Figures 6I and 6J and Figures S5G–S5I). It is worth noting that U937 cell line harbors *PTEN* (*p*hosphatase and *ten*sin homologue) mutant with premature termination of coding sequence (Liu et al., 2000). Hence in this setting the effects observed upon miR-22 decoying are not mediated by the ability of miR-22 to downregulate PTEN expression.

In full agreement with these observations, an *in vitro* colony replating assay revealed the inhibition of miR-22 by a miR-22 retroviral sponge in KSL cells purified from miR-22 transgenic mice led to a reduction in their colony forming capability (Figure S5J). Taken together, these data provide a rationale for the therapeutic potential of targeting miR-22 with LNA miR-22 inhibitors in the treatment of hematological malignancies.

The pathological roles of TET2 in myeloid malignancies have been previously highlighted as its mutations are widely observed in myeloid malignancies and MDS patients. Most of these earlier studies, however, were limited to mutation analysis, and found little relationship between the progression of MDS to AML and TET2 expression levels, although the phenotypes of *Tet2* knockout mice gave indications to the contrary (Moran-Crusio et al., 2011; Quivoron et al., 2011). Likewise, emerging evidence demonstrated no significant prognostic association between *TET2* mutations in MDS patients and WHO subtypes, the IPSS (International Prognostic Scoring System) score, cytogenetic status, or transformation to AML (Kosmider et al., 2009; Smith et al., 2010).

However, on the basis of our data we hypothesized that a miR-22–dependent reduction in the levels of TET2 might have been overlooked, and could play a key role in the development of myeloid malignancies. To test this hypothesis, we investigated a large cohort of patients with MDS by an immunohistochemical analysis. Strikingly, this analysis

revealed that ~60% of 107 MDS patients analyzed displayed a reduction in the levels of TET2 (36% of 107 MDS patients show Score 1 and 24% show Score 2), and that this reduction was directly correlated with poor patient survival rates of patients (Figures 7A and 7B and Figures S6A–S6D). Importantly, the percentage of blasts distributed in each WHO category of MDS stage was not directly associated with expression level of TET2, but a significant correlation was found between TET2 expression and poor survival rates within each WHO classification, as well as among MDS patients harboring a normal karyotype, suggesting that the poorer survival expectancy linked to TET2 downregulation does not simply reflect confounding factors, such as blast count and cytogenetic karyotype (Figure 7C and Figures S6E and S6F). Together, these results suggest that the contribution of TET2 to the pathogenesis of hematological malignancies extends far beyond the initial impact of its mutations.

Importantly, combined *in situ* hybridization and immunohistochemical analyses revealed that miR-22 expression was directly anti-correlated with the levels of TET2 in a large-cohort data set of MDS patients (n=107) (*Pearson's r* = −0.47, *P* = 2.4e-07): 28% of MDS patients that showed the reduced levels of TET2 (Score 1) also exhibited high miR-22 levels (Score 3) (Figure 7D and Figure S6G **and see also** Figure 1A).

Furthermore, as our *in vivo* analysis demonstrated that miR-22 triggers overt leukemia (Figure 4G), we investigated the miR-22/TET2 interactive relationship in AML with MLD (n=18). Our combined analyses of *in situ* hybridization and immunohistochemistry revealed a clear anti-correlation between miR-22 expression and the levels of TET2 (*Pearson's r* = −0.75, *P* = 2.9e-04); in addition, 22.2% of AML with MLD patients showing the reduced levels of TET2 (Score 1) displayed a high expression of miR-22 (Score 3) (Figure 7E). Western blot and real-time qPCR analyses also corroborated a relationship between miR-22 and TET2 in primary AML patients (Figure S6H). Finally, we confirmed these findings by analyzing the expression levels of miR-22 in a very large cohort of AML patients from a previously reported real-time qPCR database (Jongen-Lavrencic et al., 2008). Here miR-22 was found highly expressed in 58.1% of 214 AML patient samples compared to normal bone marrow from healthy donors (*P* = 0.028, Figure 7F). Taken together, these data strongly suggest that aberration of the miR-22-TET2 regulatory network is one of the common events in hematopoietic malignancies.

DISCUSSION

The findings presented in this study point to a number of important conclusions:

The first of these is that miR-22 is a powerful regulator of HSPC maintenance and self-renewal. These effects are so pronounced that miR-22–expressing HSPCs out-competed their wild-type counterparts in all the assays that we performed *in vivo* as well as *in vitro*. Importantly, in so doing miR-22 also skews hematopoietic differentiation, mainly toward myeloid compartment.

We have also identified miR-22 as a key determinant of the pathogenesis of both MDS and hematological malignancies. Remarkably, miR-22 plays a role in the initiation of MDS as well as full-blown leukemia; hence it exerts a *bona fide* proto-oncogenic activity. MDS is a genetically heterogeneous disease and we are only now beginning to fully appreciate its diversity. This study will therefore help in the reclassification of subtypes of MDS, and possibly AML, on the basis of miR-22 expression.

Furthermore, our comprehensive analysis of MDS patients has identified TET2 protein downregulation as a common event in hemopoietic malignancy (35.5% of total MDS patient

samples, and 22.2% of AML samples with MLD, with most of those patients exhibiting high expression of miR-22) as well as a novel factor in poor prognosis, findings which directly impact the management and therapeutic decisions for patients with myeloid malignancies. Our findings thus imply that the contribution of TET2 to the pathogenesis of myeloid malignancies could be related to more than its mutations, and identify the deregulation of the miR-22–TET2 pathway as one of the most frequent events in hematological malignancies. Importantly, they also provide a compelling rationale for targeted therapies that impact the mechanisms of regulation responsible for the control of TET2 levels (e.g. through miR-22 decoying).

We show that miR-22 negatively regulates the expression of TET2 and that its overexpression closely phenocopies, both *in vitro* and *in vivo*, many of the characteristics observed upon inactivation of TET2 (Figure 7G). Extensive mouse modeling efforts support these similarities in our current study as well as in other Tet2 related studies (Li et al., 2011; Moran-Crusio et al., 2011; Quivoron et al., 2011). Furthermore, our comprehensive analyses of a large cohort of MDS and AML patients likewise have demonstrated this antagonistic cross talk.

miR-22 also appears to exert a tumor-promoting role in other tissues, including mammary glands, through its ability to suppress the expression of other TET family members such as TET1 and TET3 (Figure S4D **and Song *et al.*, in press)**. Thus, miR-22 represents an alternative mechanism contributing to inactivation of TET2 in hematological malignancies, and more generally of TET family members, in addition to their mutations and deletion.

We previously demonstrated that miR-22 also targets the PTEN tumor suppressor (Poliseno et al., 2010). *Pten* loss has also been implicated in the pathogenesis of hematological malignancies (Yilmaz et al., 2006; Zhang et al., 2006). However, miR-22 through TET2 regulation can bestow to hemopoietic cells further competitive advantage as also supported by the fact that miR-22 decoying suppresses proliferation even in a *PTEN* mutant setting (Figure S5I). This may in turn explain the potent proto-oncogenic activity of miR-22. Indeed, there are several intriguing phenotypical differences between $Pten^{F/F}$;*Mx1-Cre* and $miR\text{-}22^{F/+}$;*Mx1-Cre* mouse models that could illuminate a unique and powerful role exerted by miR-22 in leukemogenesis. First, miR-22 increased the ability of HSCs to repopulate, which was then followed by leukemia development, whereas loss of *Pten* resulted in the exhaustion of normal HSCs. In addition, while *Pten*-loss induces short-term expansion in HSCs through excessive cell cycle entry followed by exhaustion, no significant alteration in stem cell compartment was observed at 2~3 weeks after the introduction of miR-22 (Figure S7). Additionally, while loss of *Pten* in HSCs is known to lead to MPN-like disease followed by leukemia development, hematological dysplasia with ineffective hematopoiesis is one of the main phenotypes caused by miR-22 expression, with bi- or pan-cytopenia observed in miR-22 transgenic mice. Furthermore, although HSCs from $Pten^{F/+}$;*Mx1-Cre* mice are known to be transplantable, the recipients do not develop hematopoietic syndromes (Yilmaz et al., 2006). In contrast, recipient mice transplanted with HSCs purified from $miR\text{-}22^{F/+}$;*Mx1-Cre* mice developed aberrant hematopoiesis followed by MDS and leukemia. Hence, the ability of miR-22 to concomitantly downregulate TET2 and PTEN provides it a unique mode of action, and confers on it the ability to overcome *PTEN*-loss driven stem cell exhaustion while favoring MDS development and leukemogenesis. This in turn raises the possibility that concomitant silencing of TET2 and PTEN by miR-22 could represent a powerful event underlying the pathogenesis of human cancer, as it may overcome the fail-safe mechanisms elicited by loss of *PTEN* (Berger et al., 2011).

Finally, since the technology to inhibit microRNAs for therapy is rapidly evolving (Elmen et al., 2008; Stenvang et al., 2008), this will impact on treatment options for a range of diseases

and disorders in the years to come. Indeed, LNA-based miRNA decoying has already come of age for the treatment of hepatitis-C (Lanford et al., 2010), and may prove very effective for the treatment of blood-borne diseases (Zhang et al., 2012). The data presented in this study thus support the notion that an LNA-based therapeutic targeting of miR-22 may represent an effective strategy for TET2 reactivation as a treatment modality for incurable diseases such as MDS and leukemia.

EXPERIMENTAL PROCEDURES

Mice

The details of generation of miR-22 transgenic mice are described in the Extended Experimental Procedures. C57BL/6 mice (B6-CD45.2) and C57BL/6 mice congenic for the CD45 locus (B6-CD45.1) were purchased from The Jackson Laboratory and used as recipients in transplantation assay.

Real-time quantitative PCR

The details of RNA isolation and real-time qPCR analysis are described in the Extended Experimental Procedures.

Immunofluorescence

Cells were fixed and stained as described (Ko et al., 2010). The details of staining of cells and the sources of antibodies used are also described in the Extended Experimental procedures.

Flow cytometry

The sources of antibodies used for flow cytometric analysis are described in the Extended Experimental procedures.

Long-term cultures and *in vitro* colony-forming assays

The details of colony replating assays are described in the Extended Experimental Procedures.

Competitive reconstitution assay

The details of competitive reconstitution assay are described in the Extended Experimental Procedures

Human sample analysis

Human patient samples were analyzed as described in detail in the Extended Experimental Procedures. This study was approved by the Institutional Review Board waiver and Human Tissue Utilization Committee, and clinical and hematological data were recorded after patients provided their informed consent in accordance with the Declaration of Helsinki.

microRNA *in situ* hybridization

The details of *in situ* hybridization analysis for miR-22 are described in the Extended Experimental Procedures.

Immunohistochemistry

The details of immunohistochemical analysis are described in the Extended Experimental Procedures.

Dynamic transcriptome analysis

The details of dynamic transcriptome analysis are described in the Extended Experimental Procedures.

Methylation specific PCR

DNA methylation status of *Sp140* in purified KSL cells (or Lin^{neg} cells) was examined by the methylation-specific PCR with genomic DNA treated with sodium bisulfite using the EZ DNA Methylation-Direct kit (Zymo Research). Primer sequences were previously described (Yamazaki et al., 2012). β-actin was used as internal control of genomic DNA.

Luciferase reporter assay

The details of luciferase reporter assay are described in the Extended Experimental Procedures.

Statistical Analysis

Student's *t* test was utilized to determine statistical significance unless otherwise specified. *P*-values lower than 0.05 were considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank all the members of the Pandolfi laboratory for their comments and discussion. We are grateful to Thomas Garvey and Min Sup Song for critical editing of the manuscript. We are also especially thankful to Akinobu Matsumoto, Dina Stroopinsky and Mesruh Turkekul for technical support and help on *in situ* hybridization, to Lourdes Mendez for comments and help on hematological analysis, to Aviv Bergman for comments and support on statistical analysis of human patients data and to Miriam Fayad for support on cohort study of patients with hematological malignancies. K.I. was supported by NIH grants. LK was supported by an NHMRC Overseas Biomedical Fellowship. This work was supported by CA141457-03 NIH grants to P.P.P.

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Highlights

1. miR-22–TET2 regulatory network controls hematopoietic stem cell biology.
2. miR-22 triggers myelodysplastic syndrome and hematological malignancies.
3. Levels of miR-22 and TET2 are reliable prognostic factors in MDS patients.
4. miR-22 decoying offers novel therapeutic opportunities for MDS and leukemia patients.

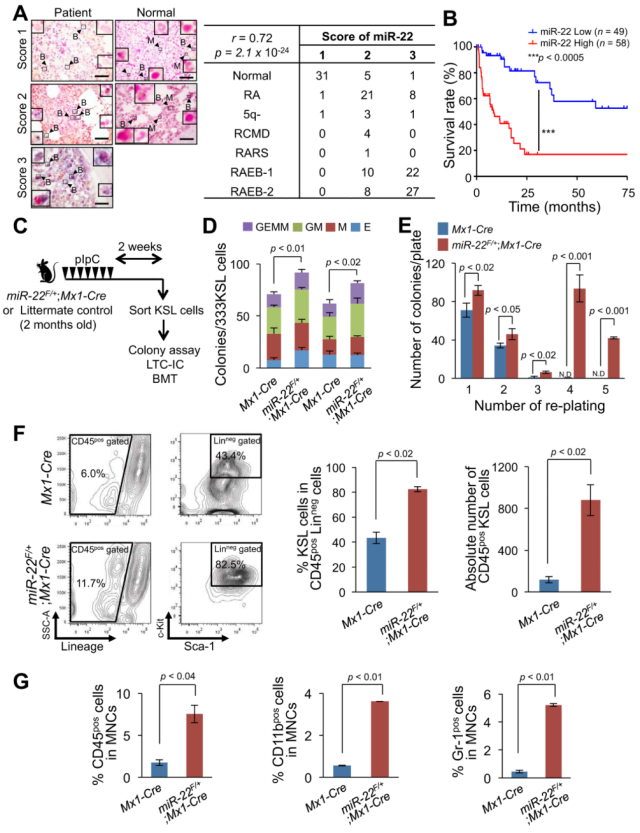

Figure 1. miR-22 is upregulated in MDS patients and leads to increased replating capacity *in vitro*

(A) miR-22 is highly expressed in MDS patient samples. Expression levels of miR-22 in blasts in subtypes of MDS were measured by *in situ* hybridization analysis. Normal (n=37), RA (n=30), 5q- (n=5), RCMD (n=4), RARS (n=1), RAEB-1 (n=32) and RAEB-2 (n=35). Representative images of *in situ* hybridization for miR-22 in MDS patients and normal bone marrow are shown (left). Insets of panels show miR-22 staining in blasts (arrow with B) and in differentiated myeloid cells (e.g. neutrophil, arrow with M) with a high magnification. The expression levels of miR-22 were also scored as described in *Extended Experimental Procedures* (right). The data were analyzed by Chi-square test. *r*, Pearson's *r*. Scale bars, 60 μm.

(B) miR-22 overexpression correlates with poor survival rates of human MDS patients. MDS patients were divided into two groups; low miR-22 expressing patients group (miR-22 Score 1 or 2, n=49, blue) and high miR-22 expressing patients group (miR-22 Score 3, n=58, red). Overall survival of these patients is demonstrated. *P*-value was generated by log-rank test.

(C) Overview of the experimental design for the conditional miR-22 expression in hematopoietic compartment. 2 months-old *miR-22*$^{F/+}$;*Mx1-Cre* mice or *Mx1-Cre* littermate controls were treated with pIpC for 2 weeks. 2 weeks after pIpC administration, KSL (c-Kit^{pos}Sca-1^{pos}Lin^{neg}) cells were sorted to evaluate the characteristics of miR-22 expressing hematopoietic stem/progenitor cells. LTC-IC, long-term culture initiating cells, BMT, bone marrow transplantation.

(D) Colony forming capacity of miR-22 expressing progenitor cells. 2 weeks after pIpC administration, sorted KSL cells from *miR-22*$^{F/+}$;*Mx1-Cre* mice or littermate controls were cultured in semi-solid medium. Counting and classification of colonies were performed in independent littermate pairs (n=3). GEMM, Colony-forming unit-granulocyte, erythroid,

macrophage, megakaryocyte, GM, Colony-forming unit-granulocyte, macrophage, M, Colony-forming unit-macrophage, E, Burst-forming unit-erythroid.

(E) miR-22 expression retains the ability to serially replate and generate colonies. *In vitro* colony replating assay was performed as shown in Figure S2D and colony counts in the indicated replatings were scored (n=3). N.D., non-detectable.

(F) Ectopic expression of miR-22 results in an increase of HSPCs in *in vitro* long-term culture with stromal cells. KSL cells isolated as described in (C) were co-cultured with OP-9 stromal cells for 2 weeks. Maintained Lin^{neg} cells and KSL cells were evaluated (n=3). Representative flow cytometry data of Lin^{neg} and the KSL cells (left) are shown. Mean percentage ± S.D. of KSL cells in Lin^{neg} cells (middle) and mean absolute numbers ± S.D. of $CD45^{pos}$KSL cells (right) are also shown.

(G) miR-22 increases the myeloid compartment. The percentages of $CD45^{pos}$ cells (left), $CD11b^{pos}$ cells (middle), or $Gr\text{-}1^{pos}$ cells (right) in total mononuclear cells (MNCs) were investigated 2 weeks after the co-culture with stromal cells from (F) (n=3). All error bars represent ± S.D.

“see also Figures S1, S2A–S2D and S7”.

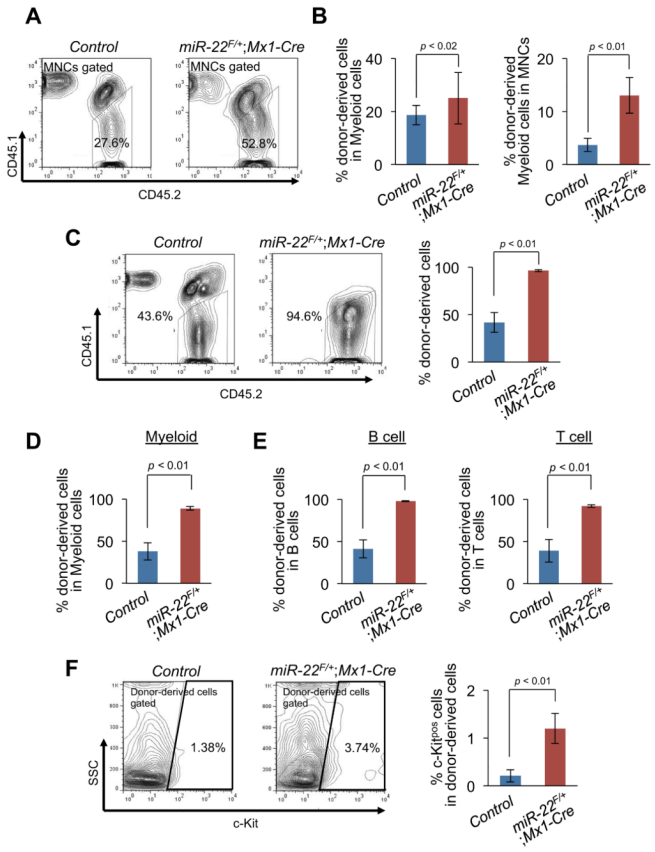

Figure 2. miR-22 leads to an enhanced repopulating capacity of hematopoietic stem progenitor cells *in vivo*

(A and B) Ly45.1 recipient mice were transplanted with the 1500 KSL cells from *miR-22*$^{F/+}$;*Mx1-Cre* mice (n=15) or littermate controls (n=10) after pIpC administration together with 4×10^5 Ly45.1/Ly45.2 competitor BM MNCs. Donor-derived chimerism in peripheral blood was analyzed 6 weeks after the transplantation. Representative flow data of the CD45.1/CD45.2 positivity (A) and the percentages of donor-derived cells in myeloid (CD11b^{pos} and/or Gr-1^{pos}) (B, left) and donor-derived myeloid cells in MNCs (B, right) of recipient mice are shown.

(C–E) Donor contribution in hematopoiesis of recipient mice 9 weeks after the transplantation. Representative flow data of the CD45.1/CD45.2 positivity with the percentages of donor-derived CD45.1^{neg}CD45.2^{pos} cells (C, left) are shown. Mean percentages ± S.D. of donor-derived cells in MNCs (C, right), myeloid cells (D), B cells (E, left) and T cells (E, right) are shown (n=10 for littermate controls and n=15 for *miR-22*$^{F/+}$;*Mx1-Cre*).

(F) c-Kit^{pos} cells are observed in peripheral blood of recipient mice transplanted with KSL cells from *miR-22*$^{F/+}$;*Mx1-Cre* mice. Representative flow cytometry data of c-Kit positivity in donor-derived cells (left) and the percentages of c-Kit^{pos} cells in donor-derived cells (right) are shown. (n=10 for littermate controls and n=15 for *miR-22*$^{F/+}$;*Mx1-Cre*). All error bars represent ± S.D.

“see also Figures S2E, S2F and S7”.

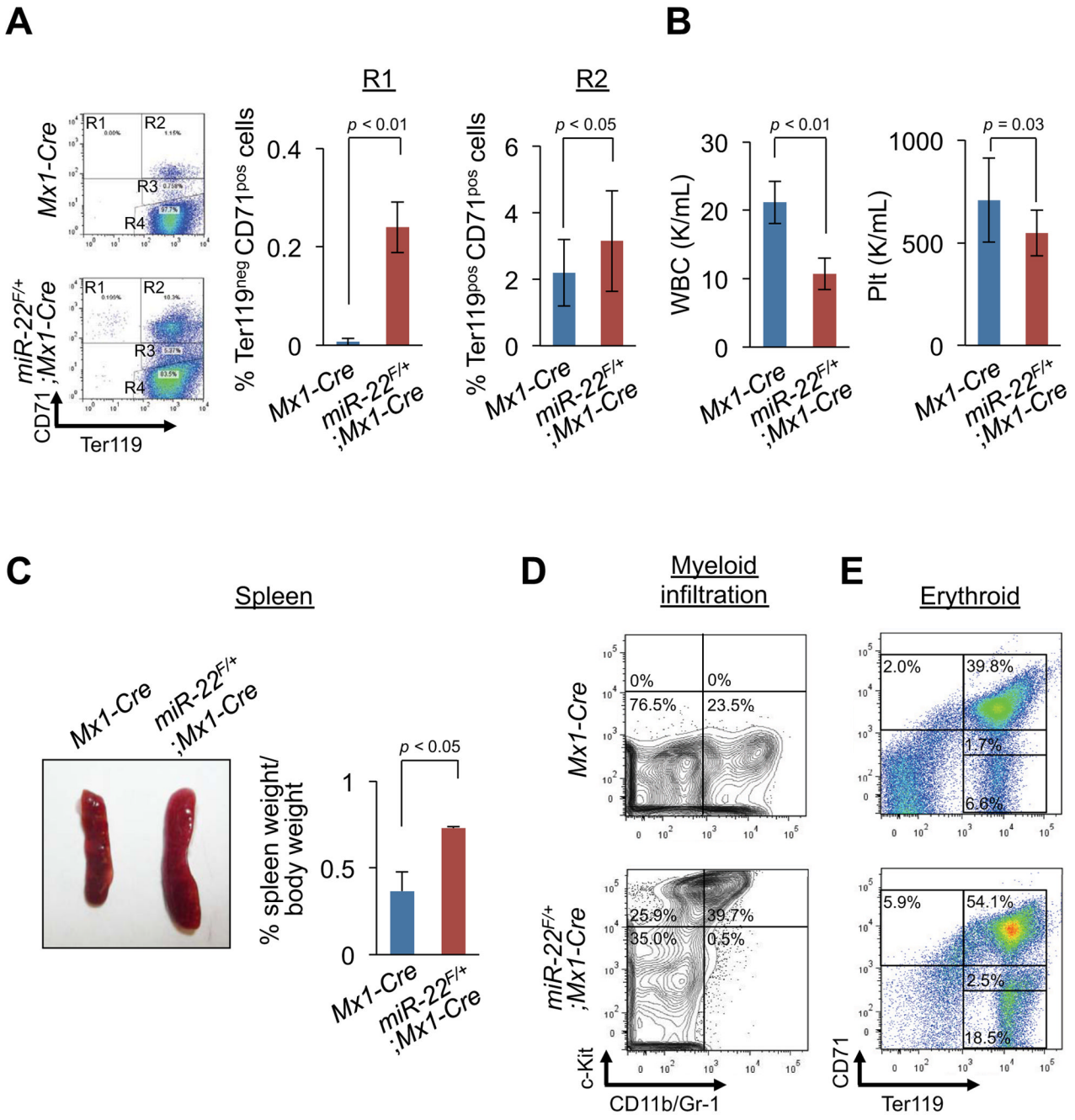

Figure 3. miR-22 overexpression develops hematological syndromes

(A and B) miR-22 overexpression leads to human MDS-like phenotypes characterized by defective erythropoiesis (A) and cytopenia (B) in miR-22 transgenic mice. After pIpC administration, 1500 KSL cells from *miR-22*$^{F/+}$;*Mx1-Cre* mice or littermate controls were transplanted into lethally irradiated Ly45.1 recipient mice with 4×10^5 Ly45.1/Ly45.2 competitor BM MNCs (n=10 for littermate controls and n=15 for *miR-22*$^{F/+}$;*Mx1-Cre*). 12 weeks after transplantation, peripheral blood of recipient mice was evaluated. Representative flow data of the Ter119/CD71 positivity (A, left), the percentages of R1 (Ter119^{neg}CD71^{pos}) (A, middle) and R2 (Ter119^{pos}CD71^{pos}) (A, right) are shown. White blood cell (WBC) (B, left) and platelet (Plt) counts (B, right) are also shown.

(C and D) SplenomegaIy and myeloid infiltration into spleen were observed in recipient mice transplanted with KSL cells from *miR-22*$^{F/+}$;*Mx1-Cre* mice. Representative images of spleens (C, left), spleen weight (n=3) (C, right), and representative flow data of the positivity of c-Kit and CD11b and/or Gr-1 in spleen (D) are shown.
(E) miR-22 expression leads to a differentiation defect in erythroid compartment. Representative flow data of the CD71/Ter119 positivity in spleen are shown. All error bars indicate ± S.D.
"see also Figures S2G–S2I".

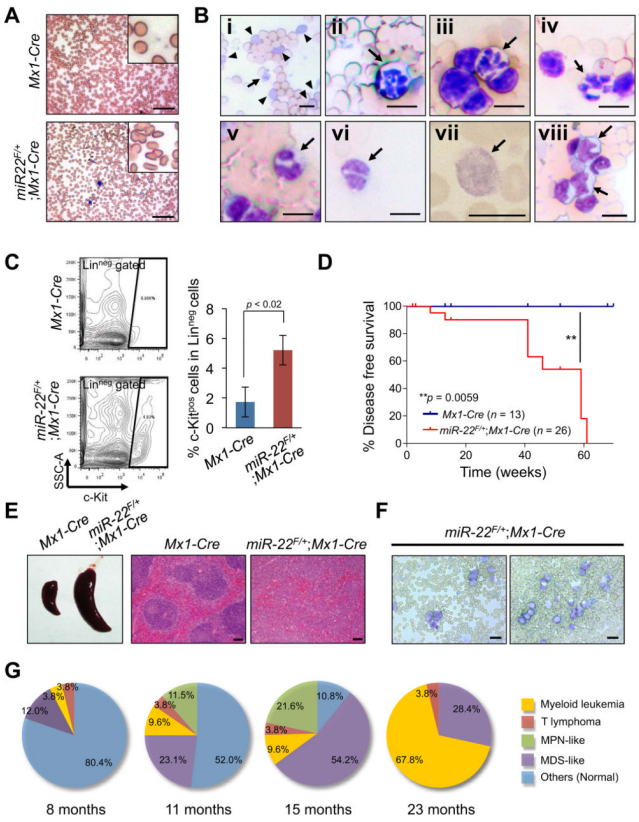

Figure 4. miR-22 transgenic mice develop primary hematological diseases

(A and B) miR-22 transgenic mice develop MDS-like hematological syndromes. Representative smears of peripheral blood of 8 months-old mice after pIpC administration are shown (A). Representative images of dysplastic erythroid cells (poikilocytosis, A; polychromasia, B-i arrowhead), dysplastic platelets (giant platelet, B-i arrow, B-vii), dysplastic neutrophils (hypersegmented neutrophils, B-ii-iv arrows; a pseudo-Pelger-Huet anomaly, B-v and vi) and dysplastic blasts (B-viii arrows) in miR-22 transgenic mice are also shown. Scale bars, 50 μm (A) and 10 μm (B).

(C) c-Kit^{pos} immature blasts are increased in miR-22 transgenic mice. Representative flow cytometry data (left) and mean percentages ± S.D. of c-Kit^{pos} cells in Lin^{neg} compartment (right) are shown (n=3).

(D) Disease free survival of *miR-22$^{F/+}$;Mx1-Cre* mice (n=26) and littermate controls (n=13).

(E and F) Representative lethal hematological syndromes observed in miR-22 transgenic mice. Representative images of spleens (E, left) and H&E staining (E, right) are shown. Scale bars, 100 μm. Representative smears of peripheral blood of miR-22 transgenic mice (6 months old) with increased myeloid blasts (F) are also shown. Scale bars, 20 μm.

(G) Pie charts representing the disease spectrum in *miR-22$^{F/+}$;Mx1-Cre* mice at the indicated ages (n=26). MPN, myeloproliferative neoplasm. All error bars indicate ± S.D. “see also Figure S3”.

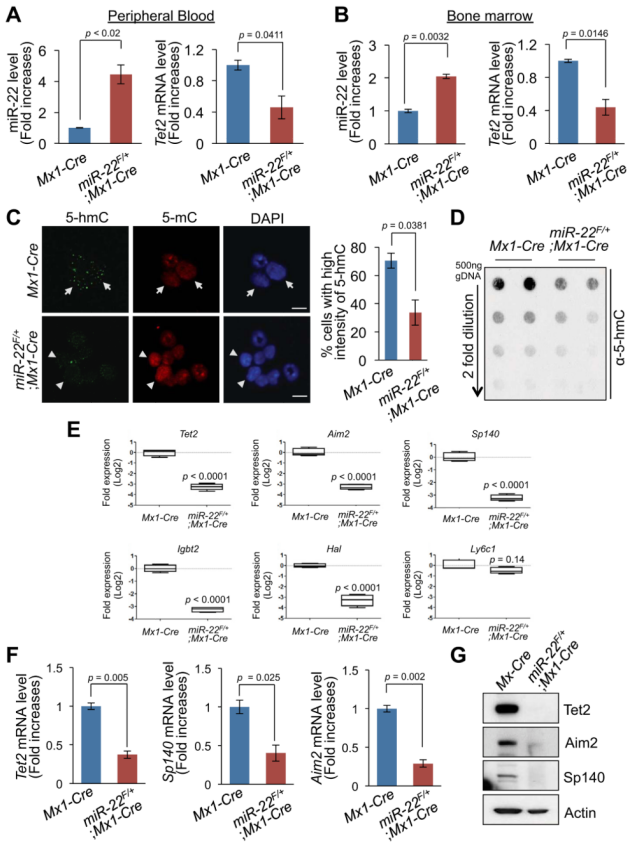

Figure 5. miR-22 acts as an epigenetic modifier by directly targeting TET2

(A and B) A reduction in the levels of *Tet2* mRNA in miR-22 transgenic mice. Expression levels of miR-22 (left) and *Tet2* mRNA (right) in mononuclear cells from peripheral blood (A) and bone marrow (BM) (B) of *Mx1-Cre* control or *miR-22*$^{F/+}$;*Mx1-Cre* mice 2–3 weeks after pIpC administration were determined by real-time qPCR (n=6).

(C) miR-22 expression results in a significant reduction in the levels of 5-hmC in hematopoietic compartment. 5-hmC and 5-mC were evaluated by immunofluorescence analysis in BM cells isolated from *miR-22*$^{F/+}$;*Mx1-Cre* mice and littermate controls 3 weeks after pIpC administration (left). Cells expressing high levels of 5-hmC show low levels of 5-mC (arrow) and conversely, cells expressing low levels of 5-hmC exhibit high levels of 5-mC (arrowhead). Bar graph depicts mean percentages ± S.D. of cells expressing high levels of 5-hmC (n=3) (right). Scale bars, 10 μm.

(D) miR-22 expression leads to a significant reduction in global 5-hmC expression levels in the genome in BM cells. 5-hmC levels were analyzed by quantitative dot blot assay with genomic DNA purified from BM cells of *miR-22*$^{F/+}$;*Mx1-Cre* or littermate control mice.

(E) Plots depict relative expression levels of putative Tet2 target genes (*Aim2*, *Sp140*, *Igbt2* and *Hal*) in CD150^{pos}CD48^{neg}Flt-3^{neg}CD34^{neg}KSL cells of *miR-22*$^{F/+}$;*Mx1-Cre* mice and littermate controls 2~3 weeks after pIpC administration (n=4). *Ly6c1* and *Actb* were used as a Tet2 unrelated gene control and an internal control, respectively.

(F and G) Repression of putative downstream genes of Tet2 in miR-22 transgenic mice. Expression levels of Tet2, Sp140 and Aim2 mRNAs (F) or proteins (G) in BM of *miR-22*$^{F/+}$;*Mx1-Cre* mice or littermate controls 2–3 weeks after pIpC administration were evaluated (n=3). All error bars indicate ± S.D.

“see also Figures S4A–S4F”.

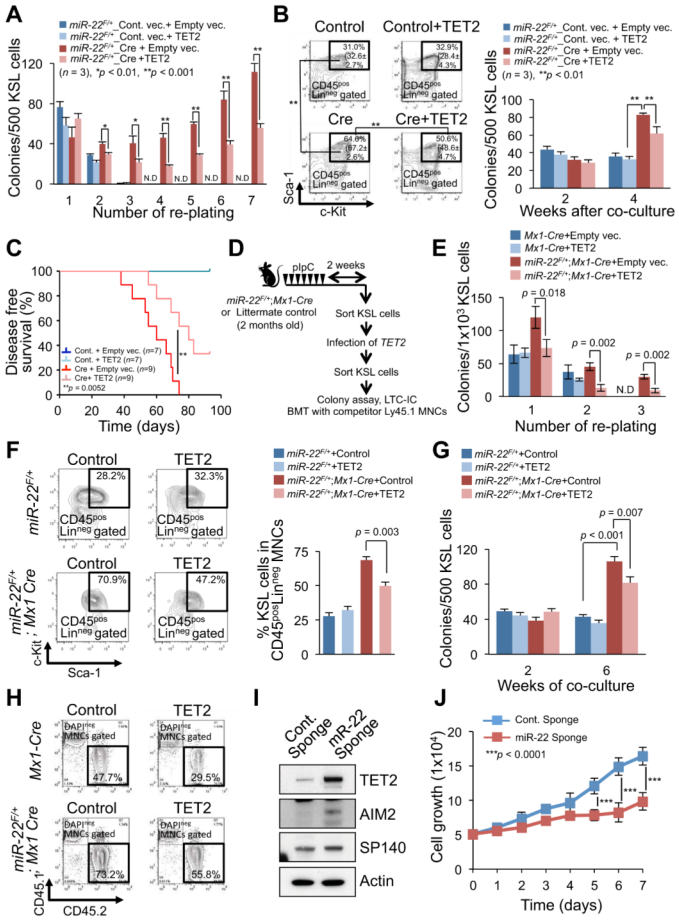

Figure 6. miR-22–TET2 regulatory network affects hematopoietic stem cell function and hematological transformation

(A and B) Ectopic expression of TET2 reduces the colony forming capacity of miR-22 expressing hematopoietic stem/progenitor cells. The sorted KSL cells from *miR-22*$^{F/+}$ mice were infected with Cre-GFP or GFP control vector. GFP^{+}KSL cells were resorted and infected with empty vector or TET2 expressing lentiviral particles. Cells were then incubated in semi-solid medium. Colony replating assay was performed and the resulting colonies were counted at the indicated replatings (n=3) (A). Some of TET2 infected cells were also subjected to LTC-IC assay. Representative flow cytometry data of the positivity of c-Kit/Sca-1 in Lin^{neg}CD45^{pos} gated cells 2 weeks after co-culture with stromal cells and mean percentages ± S.D. of KSL cells in Lin^{neg} CD45^{pos} (in brackets) are shown (n=3) (B, left). Colony forming capacities were also determined at the indicated weeks after co-culture with stromal cells (n=3) (B, right).

(C) TET2 attenuates the hematopoietic malignancies induced by miR-22 overexpression. KSL cells sorted from *miR-22*$^{F/+}$ mice were infected with Cre-GFP or GFP control vector followed by TET2 infection, and 1500 GFP^{+}KSL cells were transplanted into recipient mice with 2.0×10^{5} Ly45.1 competitor bone marrow mononuclear cells (BM MNCs). Disease free survival of recipient mice was examined by Kaplan-Meier survival curves. Log-rank test was used to generate *P*-value.

(D) Overview of the experimental design for introduction of TET2 into KSL cells from miR-22 transgenic mice. KSL cells purified from miR-22 transgenic mice were subjected to infection with TET2 expressing vector, and their characteristics were evaluated *in vivo* and *in vitro*.

(E) Colony forming capacity of miR-22 transgenic progenitor cells is attenuated by ectopic expression of TET2. 2 weeks after pIpC administration, sorted KSL cells from *miR-22*$^{F/+}$;*Mx1-Cre* mice were infected with TET2 expressing vector and resorted KSL cells were then subjected to the incubation in semi-solid medium. The resulting colony numbers were scored in three independent littermate pairs in the indicated platings (n=3). N.D., non-detectable.

(F and G) Ectopic expression of TET2 causes a reduction in LTC-IC capacity of miR-22 transgenic progenitor cells. TET2 infected KSL cells from *miR-22*$^{F/+}$;*Mx1-Cre* mice or littermate controls, as shown in Figure 6D, were co-cultured with stromal cells for the indicated weeks. Maintained KSL cells were evaluated 2 weeks after co-culture (n=3) (F). At the indicated times after co-culture, the capacity of colony formation was also determined in semi-solid medium (n=3) (G).

(H) Increased reconstitution capacity of KSL cells of miR-22 transgenic mice is reduced by ectopic expression of TET2. The sorted KSL cells from *miR-22*$^{F/+}$;*Mx1-Cre* mice or littermate controls were infected with TET2 expressing vector. 1×10^4 KSL cells were resorted and subjected to the bone marrow transplantation with 5×10^5 competitor BM MNCs. Representative flow cytometry data of the CD45.1/CD45.2 positivity at 6 weeks after transplantation are shown.

(I) Inhibition of miR-22 increases the expression of TET2 and its putative target genes in human leukemic cells. K562 cells were infected with the vector encoding miR-22 sponge or control sponge, and 48 hrs after the infection cell lysates were subjected to immunoblot analysis for the indicated proteins.

(J) Inhibition of miR-22 suppresses the proliferation of human leukemic cells. K562 cells were infected with the vector encoding miR-22 sponge or control sponge. 48 hrs after puromycin selection, 5×10^4 cells were incubated for 7 days. Optical densities of the cells were determined at the indicated times (n=3). All error bars indicate ± S.D. “see also Figures S4G–S4K and S5”.

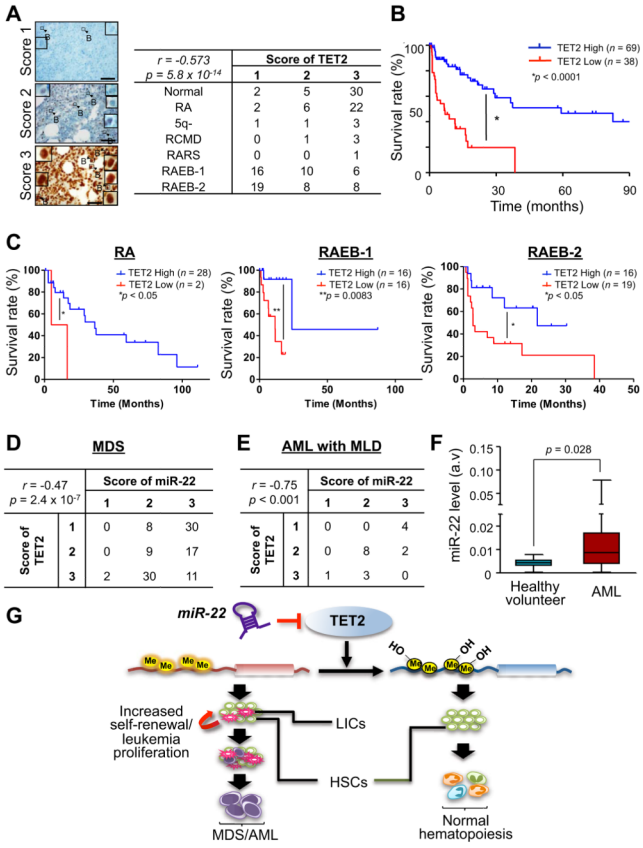

Figure 7. miR-22 overexpression directly correlates with the silencing of TET2 in human MDS and AML patients

(A) TET2 expression is downregulated in human MDS patients. Expression levels of TET2 protein in $CD34^{pos}$ blasts in subtypes of MDS were evaluated by immunohistochemical analysis. Representative images of immunohistochemical analysis for TET2 protein in patients with subtypes of MDS are shown (left). Insets represent TET2 staining in blasts (arrow with B) with a high magnification. Scoring the expression levels of TET2 in blasts was performed as described in *Extended Experimental Procedures* (right). The data were analyzed by Chi-square test. *r*, Pearson's *r*. Scale bars, 60 μm.

(B) Downregulation of TET2 levels correlates with poor survival rates of human MDS patients. Overall survival of patients that express low TET2 (TET2 Score 1, n=38, red) or high TET2 (TET2 Score 2 or 3, n=69, blue) is shown. *P*-value is generated by log-rank test.

(C) Survival rates of low TET2 (Score 1) and high TET2 (Score 2 or 3) patients with RA (left), RAEB-1 (middle) and RAEB-2 (right). Survival rate of *P*-value is generated by log-rank test.

(D) miR-22 overexpression correlates with downregulation of TET2 in MDS patients. 107 MDS patient specimen were subjected to *in situ* hybridization and immunohistochemical analyses, and the expression levels of miR-22 and TET2 were evaluated. The data were analyzed by Chi-square test. *r*, Pearson's *r*.

(E) The expression levels of miR-22 and TET2 in 18 AML with MLD patient samples were evaluated. The data were analyzed by Chi-square test. *r*, Pearson's *r*. AML, acute myeloid leukemia; MLD, multiple lineage dysplasia.

(F) miR-22 is highly expressed in human AML patients. Expression levels of miR-22 in $CD34^{pos}$ bone marrow cells from healthy donors (n=9) and AML patients (n=215) are shown.

(G) Proposed model of oncogenic role of miR-22 in hematopoiesis. miR-22 negatively regulates TET2 tumor suppressor, leading to a reduction of 5-hmC and a potentiation of global gene methylation. This genetic remodeling in turn enhances hematopoietic stem cell function and promotes hematological transformation. HSCs, hematopoietic stem cells, LIC, leukemia-initiating cells.
“see also Figure S6”.