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TET2 Loss Dysregulates the Behavior of Bone Marrow Mesenchymal Stromal Cells and Accelerates $Tet2^{-/-}$-Driven Myeloid Malignancy Progression

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SUMMARY

TET2 is a methylcytosine dioxygenase that regulates cytosine hydroxymethylation. Although there are extensive data implicating a pivotal role of TET2 in hematopoietic stem/progenitor cells (HSPCs), the importance of TET2 in bone marrow mesenchymal stromal cells (BMSCs) remains unknown. In this study, we show that loss of TET2 in BMSCs increases cell proliferation and self-renewal and enhances osteoblast differentiation potential of BMSCs, which may in turn alter their behavior in supporting HSPC proliferation and differentiation. In addition, *Tet2* loss alters BMSCs in promoting *Tet2*-deficiency-mediated myeloid malignancy progression. *Tet2* loss in BMSCs also dysregulates hydroxylation of 5-methylcytosine (5mC) and the expression of genes that are key for BMSC proliferation and osteoblast differentiation, leading to alteration of biological characteristics *in vivo*. These results highlight the critical role of TET2 in the maintenance of BMSC functions and osteoblast differentiation and provide evidence that dysregulation of epigenetic modifiers in BMSCs contributes to the progression of myeloid malignancies.

INTRODUCTION

As a hallmark of epigenetic regulation, DNA methylation plays an important role in regulating gene expression (Gutierrez-Arcelus et al., 2013; Jones, 2012). Dynamic changes in the DNA methylation landscape of the genome are required for proper gene regulation and orchestration of various developmental processes. The ten-eleven translocation (TET) methylcytosine dioxygenase enzymes (TET1/2/3) catalyze the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC), and can further oxidize 5hmC to 5-formylcytosine and 5-carboxylcytosine (Gutierrez-Arcelus et al., 2013; He et al., 2011). Participating in the initial steps of active DNA demethylation, TETs are important regulators of cytosine methylation in the genome. TET1 and TET2 are highly expressed in embryonic stem cells (ESCs), while TET3 is active at early stages of embryogenesis and regulates post-fertilization paternal DNA re-programming through loss of DNA methylation. In addition, members of the TET family are playing an important role in ESC maintenance and inner cell mass specification. Recent studies have demonstrated that *TET2* is frequently mutated in hematological malignancies, and deletion of *Tet2* in mice leads to the development of myeloid malignancies (Delhommeau et al., 2009; Jankowska et al., 2009; Li et al., 2011; Moran-Crusio et al., 2011; Pan et al., 2017; Tefferi et al., 2009a, 2009b).

We, as well as others, have reported that the *Tet2*-deficient mice contained an increased hematopoietic stem/progenitor cell (HSPC) pool before the development of myeloid malignancies. Those HSPCs had an increased hematopoietic repopulating capacity with an altered cell differentiation skewing toward monocytic/granulocytic lineages (Li et al., 2011; Moran-Crusio et al., 2011; Pan et al., 2017). Moreover, *Tet2* mRNA is broadly expressed in hematopoietic cell subsets including stem/progenitor and mature cells, and 5hmC is present at clearly detectable levels in their genomes. Mutations or deletions of the *TET2* gene have been reported to frequently occur in myeloid malignancies (Delhommeau et al., 2009; Jankowska et al., 2009; Tefferi et al., 2009a, 2009b). *Tet2* regulates the function of HSPCs likely by modulating DNA methylation and subsequent epigenetic control of gene expression at the loci that are critical for the self-renewal, proliferation, and differentiation of HSPCs.

Mesenchymal stromal cells (MSCs) are multipotent progenitor cells with self-renewal capacities and osteogenic, adipogenic, and chondrogenic differentiation potential (Bianco et al., 2008; Pittenger et al., 1999). MSCs and their osteoblastic lineage progenies are cellular components of the bone marrow niche and have been shown to play an integral role in the maintenance of blood homeostasis and in balancing HSPC quiescence, proliferation, and differentiation. Previous studies indicate that MSCs support HSPCs

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through both direct and indirect interactions with HSPCs (Jing et al., 2010; Mendez-Ferrer et al., 2010; Mishima et al., 2010).

Although extensive genetic data implicate a critical role of TET2 in HSPCs, the importance of TET2 loss in bone marrow mesenchymal stromal cells (BMSCs) has not been delineated. In the present study, we elucidate the function of TET2 in BMSCs *in vivo* and *in vitro*. We show that TET2 loss in BMSCs alters BMSC self-renewal, proliferation, and osteoblast cell (OBC) differentiation potential. In addition, TET2 loss alters BMSC behavior and the ability to promote *Tet2*-deficiency-mediated myeloid malignancy progression. TET2 loss in BMSCs also dysregulated hydroxylation of 5mC and altered expression of key OBC-related genes. These findings indicate a pivotal role of TET2 in the regulation of BMSC functions and OBC development, and provide evidence that dysregulation of epigenetic modifiers in BMSCs contributes to the progression of myeloid malignancies.

RESULTS

Loss of *Tet2* Increases BMSC Self-Renewal and Proliferation Capacity

BMSCs were isolated from bone marrow of wild-type (WT) and $Tet2^{-/-}$ mice, and phenotypically validated by flow cytometry with a good viability (Figures S1A and S1B). BMSCs are able to form mesenspheres when plated at a low density due to their self-renewal ability (Mendez-Ferrer et al., 2010). We first examined the effect of *Tet2* deletion on BMSC self-renewal and proliferation by the non-adherent mesensphere assay. $Tet2^{-/-}$ mesenspheres were 728 ± 66.29 μm in diameter, which was significantly larger than that of WT (424 ± 40.06 μm) (Figure 1A). The numbers of mesensphere were also significantly higher in $Tet2^{-/-}$ mice bone marrow-derived MSC ($Tet2^{-/-}$ BMSC) cultures than that of WT mice bone marrow-derived MSC (WT BMSC) (Figure 1B). Colony-forming-unit fibroblast (CFU-F) assays revealed a significantly increased frequency of CFU-F in $Tet2^{-/-}$ BMSCs compared with WT BMSCs (Figure 1C). Meanwhile, the mRNA expression levels of *Sox2* and *Nanog* were also significantly higher in $Tet2^{-/-}$ BMSCs than in WT BMSCs, consistent with the increased self-renewal capacity and a higher frequency observed in $Tet2^{-/-}$ BMSCs (Figure 1D). To evaluate the cell proliferation of BMSCs *in vitro*, we counted the cell numbers every 3–4 days for 18 days. The growth curve revealed that deletion of *Tet2* in BMSCs acquired a more prominent proliferation capacity compared with WT BMSCs (Figure 1E), further verified by [^{3}H]thymidine incorporation assay (Figure 1F).

To confirm the impact of *TET2* loss on human BMSCs, lentiviral constructs carrying *TET2* small hairpin RNA were applied to knockdown *TET2* in BMSCs from healthy donors (*TET2*-KD BMSCs), and empty vector-infected BMSCs (EV-BMSCs) were used as the control. The knockdown efficiency was determined by qPCR analyses (Figure S1C). The levels of 5hmC in the *TET2*-KD BMSCs and EV-BMSCs were examined by flow cytometric analyses. Our results showed a significantly decreased 5hmC level in *TET2*-KD BMSCs compared with EV-BMSCs (Figures S1D and S1E). Sulforhodamine B assay showed that *TET2*-KD BMSCs had increased proliferation potential compared with EV-BMSCs (Figure 1G). There was a slight, while still significant, increase of the expression level of pluripotent-related genes in *TET2*-KD BMSCs, consistent with the significant increased self-renewal capacity and a higher frequency of CFU-F observed in $Tet2^{-/-}$ BMSCs (Figure S1F). Taken together, these results suggest that TET2 loss enhanced both human and murine BMSC self-renewal and proliferation potential.

Loss of *Tet2* Increases BMSC Osteoblast Differentiation and Hematopoietic Supportive Capacity

The osteoblast differentiation capacity of BMSCs has been shown to be important for the hematopoietic supportive activity. To evaluate the role of *Tet2* in osteoblast differentiation potential, we performed an osteoblast differentiation assay, followed by alkaline phosphatase (ALP) activity staining using $Tet2^{-/-}$ and WT BM cells. As a result, $Tet2^{-/-}$ mice had a significant increase in the number of CFU -osteoblasts compared with the WT mice, suggesting an effect of TET2 in murine osteoblast differentiation (Figure 2A). OBC frequencies from WT and $Tet2^{-/-}$ mice were analyzed as described previously (Schepers et al., 2013). Flow cytometry analysis showed that $Tet2^{-/-}$ mice obtained higher OBC frequency compared with WT mice (Figures S2A and S2B). Consistently, *TET2*-KD BMSCs exhibited a marked increase in alizarin red-positive calcium deposition in the osteogenic differentiation medium compared with EV-BMSCs, suggesting enhanced osteoblast differentiation of *TET2*-KD BMSCs *in vitro* (Figure 2B). The expression of two genes controlling osteoblast differentiation, *ALPL* and *SPP1*, was significantly higher in *TET2*-KD BMSCs (Figure 2C). Collectively, these data indicate that TET2 loss in either murine BMSCs or human BMSCs increases osteoblast differentiation.

Since TET2 loss enhanced self-renewal and osteoblast differentiation capacities in BMSCs, we sought to explore the effect of $Tet2^{-/-}$ BMSCs on their hematopoietic supportive activity. Cobblestone-area-forming cell (CAFC) assays were used to quantitatively evaluate the hematopoietic supportive activity of $Tet2^{-/-}$ BMSCs. $Tet2^{-/-}$ BMSCs supported 3- to 4-fold more CAFCs compared with WT BMSCs either cocultured with WT or $Tet2^{-/-}$ HSPCs (Figures S2C–S2E).

Figure 1. Loss of *Tet2* in BMSCs Leads to Pronounced Alterations of BMSC Cellular Phenotypes

(A) Self-renewal capacity of murine WT BMSCs and *Tet2*$^{-/-}$ BMSCs was assayed by clonal non-adherent sphere formation (n = 4 mice per genotype from three independent experiments). Pictures were obtained under 20× magnification. Scale bar represents 400 μm.

(B) The frequencies of the sphere from murine WT BMSCs and *Tet2*$^{-/-}$ BMSCs (n = 4 mice per genotype from three independent experiments).

(C) The frequencies of CFU-F per 1×10^6 bone marrow mononuclear cells (BMMNCs) from WT and *Tet2*$^{-/-}$ mice are shown (n = 5 mice per genotype from three independent experiments).

(D) qPCR analysis shows the expression levels of the pluripotent marker genes (*Sox2* and *Nanog*) of WT BMSCs and *Tet2*$^{-/-}$ BMSCs (n = 5 mice per genotype from three independent experiments).

(E) Murine WT and *Tet2*$^{-/-}$ BMSCs were cultured and expanded for 18 days and the growth curves were plotted (n = 5 mice per genotype from three independent experiments).

(F) Murine WT BMSC and *Tet2*$^{-/-}$ BMSC proliferation capacity was determined by [^{3}H]thymidine incorporation assay (n = 4 mice per genotype from three independent experiments).

(G) Sulforhodamine B assay shows the proliferative ability of human EV-BMSCs and *TET2*-KD BMSCs (n = 3 healthy donors per group from three independent experiments).

Data are presented as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S1.

When trypsinized cells from those cocultures were placed in semisolid medium to measure the number of myeloid progenitors (CFU-GM), a 3- to 5-fold increase in CFU-GM was observed from cocultures of WT or *Tet2*$^{-/-}$ HSPCs with *Tet2*$^{-/-}$ BMSCs compared with WT BMSCs (Figures 2D and 2E). In addition, *Tet2*$^{-/-}$ and WT HSPCs produced comparable numbers of CAFCs and CFU-GMs when cocultured with *Tet2*$^{-/-}$ BMSCs.

Figure 2. *Tet2*$^{-/-}$ BMSCs Exhibit Abnormal Hematopoietic Supportive Capacity

(A) The osteogenic differentiation capacity of murine WT BMSCs and *Tet2*$^{-/-}$ BMSCs are evaluated by ALP staining (n = 9 mice per genotype).

(B) The osteogenic differentiation potential of human EV-BMSCs and *TET2*-KD BMSCs are evaluated by alizarin red staining (n = 3 healthy donors per group from three independent experiments). Pictures were obtained under 4× magnification. Scale bar represents 400 μm.

(C) qRT-PCR show the expression of osteogenic differentiation-related genes (*ALPL* and *SPP1*) in human EV-BMSCs and *TET2*-KD BMSCs (n = 3 healthy donors per group from three independent experiments).

(D and E) After 4 weeks of coculture of murine WT Lin^{-}cKit^{+} cells (D) or *Tet2*$^{-/-}$ Lin^{-}cKit^{+} cells (E) with murine WT or *Tet2*$^{-/-}$ BMSCs, the cells were harvested, and CFU-GM assays were performed (n = 5 mice per genotype).

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To investigate the hematopoietic supportive potential of human *TET2*-KD BMSCs, equal numbers of cord blood (CB) CD34$^+$ cells were cocultured on EV-BMSC or *TET2*-KD BMSC feeder layers for 5 weeks. *TET2*-KD BMSCs supported 2- to 3-fold more hypocellular CAFCs than EV-BMSCs (Figures 2F, 2G, and S2F). The increased hematopoietic supportive potential of *TET2*-KD BMSCs is consistent with that found in *Tet2*$^{-/-}$ BMSC and HSPC coculture systems. Furthermore, long-term culture-initiating cell (LTC-IC) assay revealed that *TET2*-KD BMSCs promoted higher frequency of CFU-GM from CB-CD34$^+$ cells compared with EV-BMSCs (Figure 2H). Analysis of BMSC conditional medium cytokines by cytokine array revealed a significant aberrant secretion of several chemokines and cytokines in *Tet2*$^{-/-}$ BMSCs (Figure S2G), suggesting that TET2 loss in BMSCs may affect HSC self-renewal and differentiation by cytokines. Collectively, these data indicate that TET2 loss in BMSCs increases osteoblast differentiation and enhances the hematopoietic supportive capacity.

Tet2 Deletion in BMSCs Alters the Expression of Genes Critical for Osteoblast Differentiation and BMSC Proliferation

To determine whether loss of *Tet2* alters the gene expression profiling in BMSCs, we performed RNA sequencing (RNA-seq) on WT BMSCs and *Tet2*$^{-/-}$ BMSCs. Comparison of gene expression profiles of *Tet2*$^{-/-}$ BMSCs to those of WT BMSCs identified 795 differentially expressed genes (DEGs). Among these, 326 genes were upregulated and 469 genes were downregulated (Figure 3A). Gene ontology (GO) analyses of these upregulated genes identified enrichment of genes related to extracellular matrix, bone trabecular morphogenesis, and bone maturation in *Tet2*$^{-/-}$ BMSCs (Figure 3B). Furthermore, gene set enrichment analysis revealed that genes related to osteoblast differentiation were positively enriched in *Tet2*$^{-/-}$ BMSCs (Figure 3C). Taken together, a pool of dysregulated genes implicated in osteoblast development was identified in *Tet2*$^{-/-}$ BMSCs compared with WT BMSCs, consistent with the observation of enhanced osteoblast differentiation potential in *Tet2*$^{-/-}$ BMSCs (Figure 3D). Among these dysregulated genes, 63% (44 of 71) were downregulated and 37% (27 of 71) were upregulated. Then we confirmed several selected genes important for osteoblast differentiation that were dysregulated in *Tet2*$^{-/-}$ BMSCs by qPCR (downregulated genes: *Hes1*, *Wnt9a*; *Syk*, *Comp*, *Il-7,* and *Nox4*; upregulated genes: *Trp63*, *Fbn2*, *Sfrp2*, *Adamts12,* and *Eyal*) (Figures 3E and 3F). Moreover, GO analyses showed that upregulated genes in *Tet2*$^{-/-}$ BMSCs also fell into several categories, including cell growth, positive regulation of growth, and cell proliferation (Figure S3A). A panel of DEGs, which were important for the proliferation ability of BMSCs, were identified in *Tet2*$^{-/-}$ BMSCs compared with WT BMSCs. Among them, 44 were upregulated and 85 were downregulated (Figure S3B). Some representative dysregulated genes (*Ddit4*, *Pmaip1*, *Ifit3*, *Clmn,* and *Egln3*) implicated in BMSC proliferation in *Tet2*$^{-/-}$ BMSCs were confirmed by qPCR (Figure S3C). These data demonstrate that *Tet2* loss in BMSCs dysregulates the expression of genes critical for osteoblast differentiation and proliferation of BMSCs, which may contribute to the altered capacities of self-renewal and differentiation of *Tet2*$^{-/-}$ BMSCs.

Distinct DhMRs and Their Lack of Correlation with Gene Expression in *Tet2*$^{-/-}$ BMSCs

Given the role of TET proteins in 5mC hydroxylation, we employed a previously established chemical labeling and affinity purification method coupled with high-throughput sequencing (hMe-Seal) to profile the genome-wide distribution of 5hmC in *Tet2*$^{-/-}$ BMSCs and WT BMSCs. We found a global reduction in 5hmC across the whole genome of *Tet2*$^{-/-}$ BMSCs, indicating a hypohydroxymethylation in *Tet2*-deficient BMSCs (Figure 4A). Moreover, the majority of TET2-dependent 5hmC modifications in BMSCs are located within the gene body (Figure S3D). We have previously reported that the reduction of 5hmC upon *Tet2* loss was more prominent in lowly expressed genes than highly and intermediately expressed genes in mouse ESCs and HSPCs (Lin$^-$cKit$^+$ [LK] cells) (Zhao et al., 2015). However, significant reduction of 5hmC was restricted to the gene bodies of highly or intermediately expressed genes in BMSCs upon *Tet2* loss (Figure S3E), reflecting a cell lineage heterogeneity in the regulation of 5hmC marks by TET2.

To investigate the relationship between 5hmC and gene expression, we defined the differentially hydroxymethylated genomic regions (DhMRs) in *Tet2*$^{-/-}$ BMSCs compared with WT BMSCs. Integrational analysis of

(F) Representative photomicroscopy of cobblestone-forming areas (within the red dashed lines) after 5 weeks of coculture of cord blood (CB) CD34$^+$ cells with human EV-BMSCs or *TET2*-KD BMSCs (n = 3 healthy donors per group from three independent experiments). Pictures were obtained under 20× magnification. Scale bar represents 400 μm.
(G) Quantitative evaluation of the number of cobblestone colonies per 500 CB CD34$^+$ cells cocultured with human EV-BMSCs or *TET2*-KD BMSCs for 5 weeks (n = 3 healthy donors per group from three independent experiments).
(H) Quantitative evaluation of the number of CFU-GM colonies initiated from CAFC culture after 2 weeks of differentiation in H4435 methylcellulose (n = 3 healthy donors per group from three independent experiments).
Data are presented as the mean ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001. See also Figure S2.

Figure 3. Loss of *Tet2* in BMSCs Has an Altered Expression of Osteoblast Differentiation and Proliferation-Related Genes

(A) Heatmap of differentially expressed genes (DEGs) implicated in murine *Tet2*$^{-/-}$ BMSCs compared with WT BMSCs. The two lanes in each group represent heatmap results from two independent donors. $p < 0.05$, fold change >2, Log transformed data are presented.

(B) GO analysis of upregulated genes from RNA-seq data. Selected significant osteoblast- and bone-related ontology terms are shown.

(C) The gene set enrichment analysis plot analysis showed increased gene expression of the osteoblast and bone signature in *Tet2*$^{-/-}$ BMSCs compared with WT BMSCs. The normalized enrichment score = 1.99, $p < 0.01$ and false discovery rate < 0.25.

(D) Heatmap of differentially expressed osteoblast differentiation-related genes in *Tet2*$^{-/-}$ BMSCs compared with WT BMSCs. The two lanes in each group represent heatmap results from two independent donors. $p < 0.05$, log2 fold change >2, Log transformed data are presented.

(E and F) Relative expression levels of down- (E) or upregulated (F) osteoblast-related genes in *Tet2*$^{-/-}$ BMSCs were confirmed by qPCR. Data are presented as the mean ± SEM. ***$p < 0.001$. See also Figure S3.

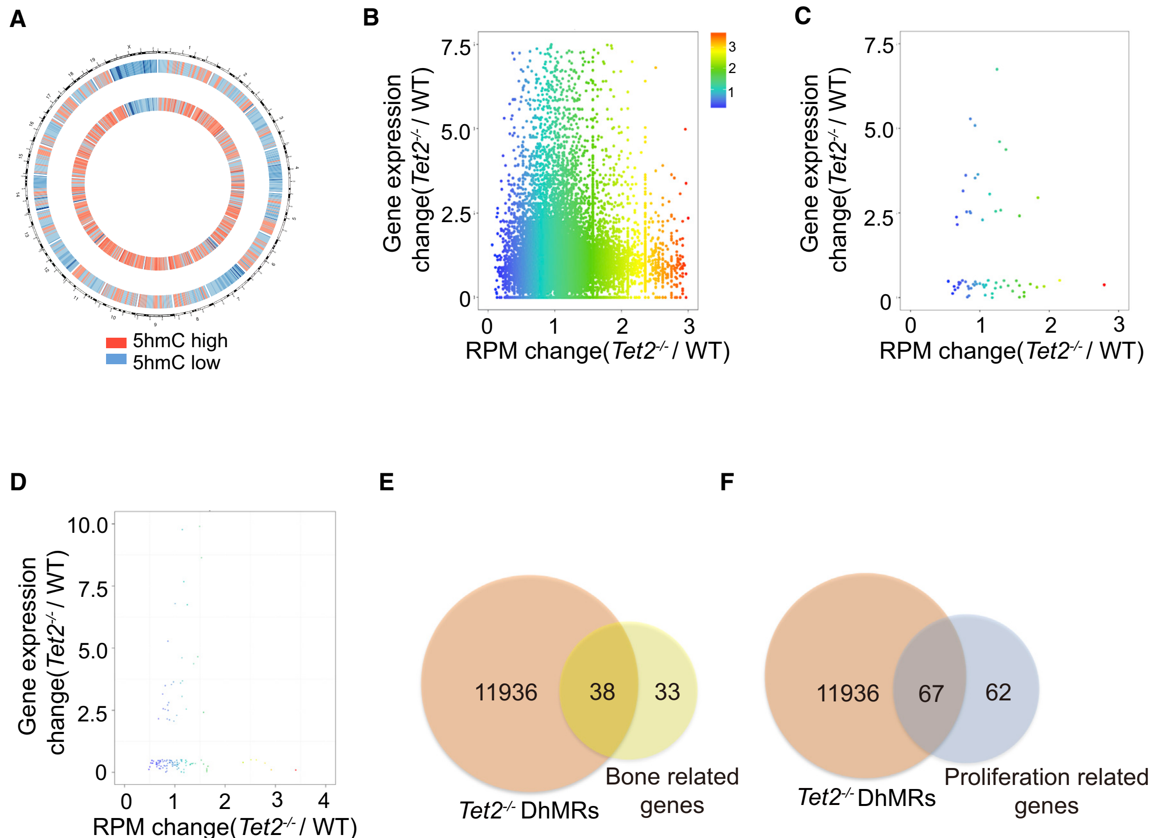

Figure 4. Distinct DhMRs and Their Lack of Correlation with Gene Expression in *Tet2*$^{-/-}$ BMSCs and WT BMSCs

(A) Dynamic change of normalized 5hmC read counts between murine *Tet2*$^{-/-}$ BMSCs and WT BMSCs in genome-wide differentially hydroxymethylated regions (DhMRs). The outer track represents the 5hmC read counts in genome-wide DhMRs in murine *Tet2*$^{-/-}$ BMSCs. The inner track represents 5hmC read counts in genome-wide DhMRs of WT BMSCs. The blue to orange bars represent the low to high 5hmC counts.

(B–D) Correlation between gene expression and 5hmC alteration. The vertical axis represents ratio of fragments per kilobase of exon per million fragments mapped of DEGs (B), bone-related DEGs (C), and proliferation-related DEGs (D) between murine *Tet2*$^{-/-}$ BMSCs and WT BMSCs. The horizontal axis represents the ratio of normalized 5hmC read counts between *Tet2*$^{-/-}$ and WT. The lack of linear regression suggests a lack of correlation between gene expression and 5hmC changes between murine *Tet2*$^{-/-}$ BMSCs and WT BMSCs.

(E and F) Venn diagram shows the overlap between DhMRs genes and osteoblast-related genes (E), or proliferation-related genes (F) identified in murine *Tet2*$^{-/-}$ BMSCs versus WT BMSCs. See also Figure S3.

DhMRs and gene expression in either *Tet2*$^{-/-}$ BMSCs or WT BMSCs did not show a clear correlation between DhMRs and gene expression change (Figure 4B). In addition, no strong correlation was seen between DhMRs with differential osteoblast-related genes and differential proliferation-related genes (Figures 4C and 4D). This is consistent with previous reports that 5hmC changes have no direct correlation with gene expression levels in mouse ESCs or LK cells (Ficz et al., 2011; Pastor et al., 2011; Zhao et al., 2015). Interestingly, through convergent analyses of DhMRs with the RNA-seq DEGs, we found that 38 of 71 osteoblast-related genes and 67 of 129 proliferation-related genes had altered 5hmC modifications in BMSCs upon *Tet2* loss (Figures 4E and 4F). These results indicate a role of cytosine modifications (5hmC) in marking specific genes and regulating gene expression.

Tet2 Loss in BMSCs Accelerated Progression of Myeloid Malignancies

As we reported previously (Li et al., 2011), *Tet2*$^{-/-}$ mice developed myeloid malignancies, including increased myeloid cells in bone marrow and massive hematopoietic cell infiltration in the liver and spleen (Figures S4A and S4B). Increased evidence suggests that aberrant functions of BMSCs may be associated with the pathophysiology and progression of hematopoietic malignancies. To elucidate the contribution of *Tet2* loss in alteration of BMSC function-associated pathogenesis of hematopoietic

Figure 5. *Tet2* Loss in Niche Cells, Especially BMSCs, May Accelerate Lethal Myeloid Malignancy

(A) Kaplan-Meier survival curve of non-transplanted *Tet2*$^{-/-}$ mice and WT or *Tet2*$^{-/-}$ recipient mice transplanted with WT or *Tet2*$^{-/-}$ BM cells (2×10^6 cells) up to 300 days (n = 6 per genotype for the transplanted group; n = 10 for *Tet2*$^{-/-}$ mice).

(B) Left panels: pie charts show the percentage of hematological malignancies in *Tet2*$^{-/-}$ or WT recipient mice that were transplanted with *Tet2*$^{-/-}$ BM cells. Middle panels: the gross morphologies of spleen from a representative WT or *Tet2*$^{-/-}$ recipient mice transplanted with *Tet2*$^{-/-}$ mice-derived BM cells, respectively. Right panels: representative H&E staining of liver and spleen from deceased/moribund WT or *Tet2*$^{-/-}$ recipient mice transplanted with *Tet2*$^{-/-}$ BM cells. The dashed lines show the area infiltrated with myeloid cells.

(C) Spleen weight of WT or *Tet2*$^{-/-}$ recipient mice transplanted with *Tet2*$^{-/-}$ mice cells.

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malignancies, we performed reciprocal transplantation experiments. BM cells from WT or *Tet2*$^{-/-}$ mice were transplanted into lethally irradiated WT or *Tet2*$^{-/-}$ recipients (Figure S4C). None of the *Tet2*$^{-/-}$ recipients transplanted with WT BM cells developed myeloid malignancies. A fraction of WT and *Tet2*$^{-/-}$ recipients receiving *Tet2*$^{-/-}$ BM cells developed myeloid malignancies with similar characteristics as in *Tet2*$^{-/-}$ mice, including higher WBC counts, more bone marrow cellularity, and disrupted splenic and hepatic architecture by massive hematopoietic cell infiltration and splenomegaly (Figures S4D and S4E). However, *Tet2*$^{-/-}$ recipient mice transplanted with *Tet2*$^{-/-}$ BM cells demonstrated a lower survival rate and a higher incidence of myeloid malignancy compared with WT recipient mice transplanted with *Tet2*$^{-/-}$ BM cells (Figures 5A–5C). To further examine the role of *Tet2* loss in niche cells in the development of myeloid malignancies, Lin^{-} MLL-AF9 cells were transplanted into lethally irradiated WT and *Tet2*$^{-/-}$ recipients. Intriguingly, all *Tet2*$^{-/-}$ recipients (n = 6) died within 70 days, whereas six of seven WT recipients that receiving Lin^{-} MLL-AF9 cells died by 100 days after the transplantation (Figure 5D), suggesting a role of *Tet2*$^{-/-}$ niche cells in promoting MLL-AF9-mediated myeloid malignancies. These data collectively support the notion that *Tet2* loss in the niche cells accelerates the progression of myeloid malignancies in mice.

To explore which *Tet2*$^{-/-}$ niche cell component contributes most to the progression of myeloid malignant *in vivo*, three strains of conditional knockout mice were generated by intercrossing *Tet2*$^{f/f}$ mice with transgenic mice carrying *Prx1Cre* (knockout *Tet2* in BMSCs) (Greenbaum et al., 2013), *Col2.3Cre* (knockout *Tet2* in osteoblasts) (Colaianni et al., 2012; Henneicke et al., 2011), or *Ve-CadherinCre* (knockout *Tet2* in endothelial cells) (Li et al., 2012; Zovein et al., 2008), respectively (Figure S5A). *Tet2*$^{-/-}$ BM cells were then transplanted into lethally irradiated conditional *Tet2* knockout strains of recipients (Figure S5B). Deletion of *Tet2* in osteoblasts (*Col2.3Cre*) and endothelial cells (*Ve-CadherinCre*) had moderate effects on the initiation/progression of *Tet2*$^{-/-}$-driven myeloid malignancies. Strikingly, deletion of *Tet2* in BMSCs (*Prx1Cre*) was associated with a significantly accelerated malignant progression, shortened survival, massive hematopoietic cell infiltration in liver and spleen, and more pronounced splenomegaly (Figures 5E and S5C–S5E). In addition, the incidence of myeloid malignancies was the highest in *Tet2*$^{f/f}$*;Prx1Cre* recipient mice (60%), compared with 44.4% in *Tet2*$^{f/f}$*;Col2.3Cre* and *Tet2*$^{f/f}$*;Ve-CadherinCre*, and 35.7% in *Tet2*$^{f/f}$ recipient control mice (Figure 5F). These data suggest that *Tet2* loss in BMSCs contributes most in the bone marrow niche to the progression of *Tet2*$^{-/-}$-driven myeloid malignancies.

DISCUSSION

Epigenetic mechanisms play an important role in the regulation of stem cell fate. DNA methylation is one of the most important epigenetic modifications. However, whether DNA methylation underlies the physiology/pathophysiology of the bone marrow microenvironment is not well established. The discovery of TET family dioxygenases that oxidize 5mC to 5hmC has led to profound progress in understanding the mechanism underlying DNA demethylation and disease progression. Here, we demonstrate that the altered DNA demethylation by TET2 loss leads to dysregulation of BMSC fate, promoting myeloid malignancy progression.

The existence of MSCs was first demonstrated by Till and McCulloch in the bone marrow (McCulloch and Till, 1960; Till and McCulloch, 2011). As a vital component of bone marrow microenvironment, MSCs have the capacity of self-renewal and differentiation into osteoblasts, adipocytes, and chrondrocytes, which make them unique in comparison with other niche cells. In this study, we show that *Tet2* loss in BMSCs led to significantly enhanced mesensphere formation and upregulation of genes critical for stem cell pluripotency. These data indicate that *Tet2* loss increases MSC self-renewal capacity. In addition, *Tet2* loss also increased the frequencies of CFU-F in the bone marrow. Moreover, TET2 loss in both murine and human BMSCs led to an accelerated proliferation of BMSCs. These data imply that TET2 may play an important role in the maintenance of the balance between BMSC self-renewal and differentiation.

MSCs can differentiate into osteoblasts (Bianco et al., 2008; Pittenger et al., 1999). It has been reported that normal frequency of osteoblasts in the bone marrow

(D) Kaplan-Meier survival curve of WT or *Tet2*$^{-/-}$ recipient mice (n = 7 for WT mice, n = 6 for *Tet2*$^{-/-}$ mice) that were transplanted with 2 × 10^5 Lin^{-} MA9 cells up to 100 days. The Lin^{-} MA9 cells were hematopoietic progenitor cell cotransduced with MSCVneo-MLL-AF9+pGFP-V-RS-shNC (i.e., MA9) through spinoculation.

(E) Kaplan-Meier survival analysis of *Tet2*$^{f/f}$ (n = 11) and three kinds of *Tet2* conditional knockout recipient mice, *Tet2*$^{f/f}$*;Col2.3Cre* (n = 8), *Tet2*$^{f/f}$*;Ve-CadherinCre* (n = 9), and *Tet2*$^{f/f}$*;Prx1Cre* (n = 18), which were transplanted with *Tet2*$^{-/-}$ BM cells up to 250 days.

(F) Pie charts show the percentage of hematological malignancies in different kinds of *Tet2* conditional knockout recipient mice that were transplanted with *Tet2*$^{-/-}$ BM cells.

Data are presented as mean ± SEM. *p < 0.05, ns, not significant. See also Figures S4 and S5.

correlates with the hematopoietic supportive activity of the niche *in vivo*. In particular, increased trabecular bone and osteoblast numbers are accompanied by increased HSPCs in the bone marrow (Calvi et al., 2003; Zhang et al., 2003). There was also a dramatic reduction in HSPCs in the event of bone or osteoblast deficiency (Corral et al., 1998; Visnjic et al., 2004). In the current study, we found that higher osteoblast differentiation potential in TET2-deficient BMSCs is accompanied by a significantly increased hematopoietic supportive activity *in vivo*. As previously reported (Li et al., 2011; Moran-Crusio et al., 2011; Pan et al., 2017), the global deletion of *Tet2* in mice led to increased HSPC proliferative capacity and skewed differentiation toward the monocytic/granulocytic lineages. Since BMSCs have been demonstrated to possess the ability to support hematopoiesis, we attempted to illustrate the impact of TET2-loss-mediated alteration of BMSC functions on their hematopoietic supportive activity and the progression of myeloid malignancy. We show that TET2 deficiency in BMSCs increases their hematopoietic supportive activity, promotes both normal and *Tet2*-deficient HSPC proliferation, and accelerates myeloid malignancies *in vivo*.

MSCs may affect HSCs by altered secreted cytokine and/or altered cell-cell contact signaling pathway (Mendez-Ferrer et al., 2010; Mishima et al., 2010). Here we detected multiple cytokine levels in the conditional medium of WT and *Tet2*$^{-/-}$ BMSCs by cytokine array. Several cytokines were significantly altered in *Tet2*$^{-/-}$ BMSC, such as GM-CSF, CCL3, and CCL5, which can facilitate HSC differentiation toward myeloid cells (Ergen et al., 2012; Metcalf, 1989; Mukaida et al., 2017). These results indicate that *Tet2*-deficient BMSCs likely promote HSPC proliferation by altered cytokine secretion. However, the precise mechanism underlying TET2 alteration in BMSC-mediated myeloid malignancies remains to be elucidated in depth.

TET2 is one of the demethylation enzymes that catalyze the conversion of 5mC to 5hmC, which therefore could alter methylation-driven gene expression (Gutierrez-Arcelus et al., 2013; He et al., 2011; Jones, 2012). Previous studies demonstrated that the phenotype and fate of stem cells rely on the precise control of gene expression by complex transcriptional and epigenetic networks (Huang et al., 2014). In this study, we demonstrated that *Tet2*$^{-/-}$ BMSCs had dramatically decreased hydroxymethylation signatures and dysregulated gene expression profiling. The dysregulated genes were enriched in genes related to osteoblast and proliferation pathways, which are in accordance with the increased osteoblast differentiation and proliferation of TET2-deficient BMSCs. There is no correlation between gene expression and 5hmC changes in *Tet2*$^{-/-}$ BMSCs, which is rather noteworthy. This finding is similar to the previous studies using either mouse ESCs or LK cells (Ficz et al., 2011; Pastor et al., 2011; Zhao et al., 2015). These observations suggest that distinct cytosine modifications (particularly 5hmC) can mark specific genes in *Tet2*-deficient BMSCs without altering their expression, and additional mechanism(s) may be involved in gene regulation.

It is likely that *Tet2* deficiency in BMSCs dysregulates osteoblast and proliferation-related genes and leads to aberrant behavior (Brady et al., 2015; Dieudonne et al., 2013; Zhang et al., 2016). We confirmed 11 genes that are related to osteoblast differentiation and bone development by qPCR. The upregulated genes (*Trp63*, *Fbn2*, *Sfrp2*, *Adamts12,* and *Eya1*) are all positive regulators of osteoblast differentiation (Cho et al., 2008; Ji et al., 2016; Nistala et al., 2010; Sworder et al., 2015; Yang et al., 1999), and the downregulated genes (*Hes1*, *Wnt9a*, *Syk, Comp, IL-7,* and *Nox4*) are negatively impacted in osteoblast differentiation (Guo et al., 2014; Jian et al., 2016; Mandal et al., 2011; Yoshida et al., 2011; Zhang et al., 2009; Zhou et al., 2009). These altered genes may cooperate to contribute the enhanced osteoblast differentiation potential in *Tet2*$^{-/-}$ mice. In addition, we identified five downregulated genes in *Tet2*$^{-/-}$ BMSCs, including *Ddit4*, *Pmaip*, *Ifit3, Clmn,* and *Egln*, which are negatively related to cell proliferation (Henze et al., 2014; Ishida et al., 2008; Kazezian et al., 2015; Marzinke and Clagett-Dame, 2012; Wang et al., 2015). These results indicate that loss of *Tet2* dysregulates hydroxylation of 5mC and gene expression in BMSCs, which may in turn alter their proliferation and differentiation potential involved in dysregulated hematopoietic supportive activity.

There is increasing evidence that aberrant functions of MSCs can be associated with the pathophysiology and progression of hematopoietic malignancies. Medyouf et al. (2014) reported that myelodysplastic syndrome patient-derived MSCs were more efficient than healthy age-matched MSCs, in promoting the development and expansion of diseased HSPCs. Our transplantation data indicate that, although *Tet2* deficiency in niche cells can accelerate disease progression, *Tet2* deficiency alone is insufficient to cause myeloid malignancy. This is supported by the fact that deletion of *Tet2* in BMSCs mediated by *Prx1-cre* is associated with a severe myeloid malignancy progression and reduced survival rate, suggesting an important regulatory role of DNA methylation in the niche in the progression of hematopoietic myeloid malignancies. In fact, myeloid malignancies are capable of altering the architecture of the bone marrow microenvironment, causing an expansion of OBCs, which, in turn, contribute to disease progression (Schepers et al., 2013). Collectively, our data indicate that, as one of the most important niche cell components, *Tet2*-deficient BMSCs could potentially contribute to the progression of myeloid malignancy by altering osteoblast and proliferation potencies.

To conclude, we demonstrated that TET2 plays an important role in regulating the behavior of BMSCs in addition to its intrinsic role in HSPCs: participating in aberrant hematopoiesis. TET2 loss in BMSCs increases cell proliferation and self-renewal, enhances osteoblast differentiation potential of BMSCs, which may in turn alter their behavior and ability to support HSPC proliferation and differentiation. Moreover, we demonstrated that BMSCs are the most important niche cell components in *Tet2*$^{-/-}$ mice that contribute to the progression of *Tet2* deletion-driven myeloid malignancy. Our study provides proof of concept for the identification of potential therapeutic targets in BMSCs for patients with myeloid malignancies.

EXPERIMENTAL PROCEDURES

Generation of *Tet2*$^{f/f}$ and *Tet2*-Conditional Knockout Mice

Mice harboring the *Tet2* allele with exon 11 flanked by two *loxP* sites were generated by the Xu laboratory (Zhao et al., 2015). *Tet2*$^{f/f}$ mice were crossed with mice carrying tissue-specific Cre-recombinase transgenes (Jackson Laboratory) to generate the deleted *Tet2* (null) allele lacking exon 11: (1) Col2.3-cre, which targets recombination in mineralizing osteoblasts; (2) Ve-Cadherin-cre, which targets endothelial cells; and (3) Prx1-Cre, which targets MSCs. *Tet2* knockout (*Tet2*$^{-/-}$) mice were generated as reported previously (Li et al., 2011). Animal care was in accordance with institutional guidelines and approved by the Institutional Animal Care and Use Committee, University of Miami Miller School of Medicine and Department of Comparative Medicine, and Chinese Academy of Medical Sciences and Peking Union Medical College.

Bone Marrow Transplantation Assay

The bone marrow transplantation (BMT) assays shown in Figures 5A and 5D were performed by transplanting BM cells (2×10^6) from WT, *Tet2*$^{-/-}$, *Tet2*$^{f/f}$;*Col2.3Cre*, *Tet2*$^{f/f}$;*Prx1Cre*, or *Tet2*$^{f/f}$;*Ve-CadherinCre* mice into lethally irradiated (700 + 400 cGy) WT or *Tet2*$^{-/-}$ mice by tail vein injection. For the BMT assay shown in Figure 5C, donor bone marrow progenitor (Lin^{-}) cells were prepared from B6.SJL (CD45.1) mice, and then cotransduced with MSCVneo-MA9+pGFP-V-RS-shNC (i.e., MA9) through spinoculation. Colony cells were then collected and washed with PBS twice, and then transplanted via tail vein injection into lethally irradiated (960 cGy) WT or *Tet2*$^{-/-}$ (CD45.2) recipient mice. For each recipient mouse, a total of 0.25×10^6 donor cells, together with an additional radioprotective dose of whole BM cells (1×10^6) freshly harvested from a WT mouse, were transplanted as described previously (Somervaille et al., 2009; Zhao et al., 2016). Mice were monitored daily for signs of pathology.

Human Samples

BMSCs derived from five healthy donors were included in this study. Human CB CD34$^+$ cells were isolated from the CB of ten healthy donors after obtaining informed consent. The study was approved by the Ethics Committee of Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences according to guidelines of the 1975 Helsinki Declaration, and informed consent was received according to the institute's guidelines on the use of human subjects.

BMSC Osteoblast Differentiation Assays

To induce human BMSC osteogenic differentiation, 5×10^4/well BMSCs were cultured in osteogenic differentiation medium, containing DMEM/F12 supplemented with 10% fetal bovine serum (HyClone), 10^{-7} mol/L dexamethasone (Sigma), 10 mmol/L β-glycerophosphoric acid (Sigma), and 200 μmol/L ascorbic acid (Sigma) for 21 days. Cells were stained with alizarin red (Sigma), according to the manufacturer's instruction.

For mice BMSC osteoblast differentiation, 1.6×10^6 bone marrow mononuclear cells were cultured for 7 days using osteogenic differentiation medium (MesenCult medium supplemented with 10^{-7} M dexamethasone, 50 μg/mL ascorbic acid, and 10 mM β-glycerophosphate), cells were stained with ALP activity (Sigma), according to the manufacturer's instruction.

Mesensphere Assays

For mesensphere formation, BMSCs were plated at a density of 1,000 cells/cm^2 in ultralow adherent 96-well plates (Corning) as described previously (Mendez-Ferrer et al., 2010). See Supplemental Experimental Procedures for details.

CFU-F Assay

The CFU-F assay was performed to determine the frequency of BMSCs in mice. See Supplemental Experimental Procedures for details.

Cell Proliferation Assay

Manual counting and thymidine incorporation assay were used to evaluate the proliferative ability of mice BMSCs. See Supplemental Experimental Procedures for details.

Long-Term Coculture of HSPCs with BMSCs

The function of mice- or human-derived BMSCs to support HSPCs, LK cells, or CB CD34$^+$ cells were measured by CAFC assay and LTCIC assay. See Supplemental Experimental Procedures for details.

Statistical Analysis

All statistical data are presented as the mean ± SEM of at least three independent experiments. Unpaired two-tailed Student's t tests were used to assess statistical significance; p values less than 0.05 were considered significant. Statistical analyses were performed with Prism 5.0 software.

ACCESSION NUMBERS

The accession number for the RNA-seq data and hME-seal data reported in this paper is NCBI GEO: GSE100073.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and three tables and can be found

with this article online at https://doi.org/10.1016/j.stemcr.2017.11.019.

AUTHOR CONTRIBUTIONS

F.-C.Y., Z.Z., M.X., W.Y., and T.C. supervised the study. R.L., Y.Z., and Z.C. performed the experiments. R.L., Y.Z., W.X., and S.C. analyzed RNA-seq and ChIP-seq data. F.-C.Y., Z.Z., M.X, Z.C., W.X., S.C., L.L., and J.W. participated in interpretation of data. Z.C. and J.B. acquired healthy donor specimens. J.B., W.Y., T.C., and M.X. participated in designing the study and revised the manuscript. All authors reviewed, edited, and approved the manuscript.

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