A Zebrafish Model of Myelodysplastic Syndrome Produced through \textit{tet2} Genomic Editing

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The ten-eleven translocation 2 gene (\textit{TET2}) encodes a member of the TET family of DNA methylcytosine oxidases that converts 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) to initiate the demethylation of DNA within genomic CpG islands. Somatic loss-of-function mutations of \textit{TET2} are frequently observed in human myelodysplastic syndrome (MDS), which is a clonal malignancy characterized by dysplastic changes of developing blood cell progenitors, leading to ineffective hematopoiesis. Mutations in \textit{TET2} are frequently observed in human myelodysplastic syndrome, which is a clonal malignancy characterized by dysplastic changes of developing blood cell progenitors, leading to ineffective hematopoiesis.

\textit{TET2} belongs to the TET (ten-eleven translocation) family of methylcytosine oxidases, which require 2-oxoglutarate, oxygen, and Fe(II) for their activity. \textit{TET2}, like \textit{TET1} and \textit{TET3}, modifies the methylation status of the genome, regulating the transcription of specific genes by converting 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) and then to 5-formylcytosine (5fC) and finally to 5-carboxylcytosine (5caC). Each of the last 3 products is recognized and excised by thymine DNA glycosylase (TDG), completing the removal of the 5-methyl group and allowing the DNA repair machinery to proceed with replication. The resultant \textit{tet2} mutant zebrafish lines show decreased levels of 5hmC in hematopoietic cells of the kidney marrow but not in other cell types, most likely reflecting the ability of other \textit{Tet} family members to provide this enzymatic activity in nonhematopoietic tissues but not in hematopoietic cells. \textit{tet2} mutants are viable and fertile, providing an ideal model to dissect altered pathways in hematopoietic cells and, for small-molecule screens in embryos, to identify compounds with specific activity against \textit{tet2} mutant cells.

The role of \textit{TET2} mutations in myeloid malignancies has been studied in a number of mouse models (17–20). The hematopoietic stem cells (HSCs) in these models have low 5hmC content and exhibit increased self-renewal ability and a competitive advantage over wild-type HSCs for repopulating hematopoietic lineages. \textit{Tet2} knockout mice are viable and fertile and appear to develop normally. However, as they age, \textit{Tet2}-deficient mice are prone to develop myeloid malignancies, predominantly CMML, with 20 to 30% developing disease after 8 months of age, clearly suggesting that additional genetic lesions are needed to initiate myeloid malignancy.

Thus, the essential role of \textit{TET2} in maintaining the normal growth and development of myeloid lineage cells is evident from both studies in murine models of \textit{Tet2} loss and the demonstration...
of TET2 loss-of-function mutations in individuals with “clonal skewing” of hematopoietic cells from the blood and bone marrow. These findings suggest that the clonal dominance by TET2-mutated HSCs may represent a critical precursor event in the development of myeloid cell malignancies. Thus, the specific targeting of TET2 mutant HSCs might provide a means not only to treat patients with existing myeloid malignancies but also to prevent progression to myeloid malignancy in individuals with clonal skewing of hematopoiesis. Meeting this challenge will require animal models conducive to high-throughput analysis of hematopoietic cell phenotypes and to the discovery of genes and signaling pathways that expose targetable vulnerabilities in TET2 mutant cells.

The zebrafish has been shown to provide a faithful model of vertebrate hematopoiesis in which both small-molecule and genetic screens have proven particularly advantageous. For example, small-molecule screens conducted in zebrafish embryos have yielded insights directly relevant to pathways regulating both human HSCs and more differentiated hematopoietic cells, leading to the discovery of drugs that augment engraftment, as shown in clinical trials of cord blood cell transplantation in patients (21–30).

Here, we report the use of zinc finger nuclease technology to generate stable zebrafish lines with loss-of-function mutations in the tet2 gene that truncate the encoded protein and disrupt the catalytic activity of the hydroxylase. Homozygous tet2 mutant zebrafish are viable and fertile and have undetectable 5hmC content in blood cells of the kidney marrow but not in other tissues. This suggests that Tet2 is required for methylcytosine dioxygenation in hematopoietic cells but that this function can be supplanted by Tet1 or Tet3 in other tissues, providing an explanation for the observation that TET2 mutations are found only in hematologic malignancies. Moreover, the tet2 mutant zebrafish developed myelodysplasia of the kidney marrow at 11 month of age, with a clonal marrow cell population harboring decreased numbers of erythrocytes and increased numbers of myelomonocyte and progenitor cell populations. However, their peripheral blood counts at this age were normal, suggesting that the process had advanced to a stage similar to pre-MDS in patients. By 24 months of age, the tet2 mutant zebrafish had progressed to full-blown MDS, with a decrease in erythrocytes in the peripheral blood. Thus, the tet2<sup>m/m</sup> (homozygous for the mutation) zebrafish provides a reliable model for studying myeloid malignancies with TET2 loss, with a view to identifying specific small-molecule inhibitors and potential synthetic-lethal genetic interactions.

**MATERIALS AND METHODS**

**Zebrafish maintenance and genetic manipulation.** Wild-type stocks of AB fish, transgenics, and mutant lines were maintained according to a previously reported protocol (31). Animal handling was approved by the Dana-Farber Institutional Animal Care and Use Committee. Tg(emybEGFP) and Tg(ela14EGFP) lines are described elsewhere (32, 33). Developmental staging was performed as described previously (34). For genome editing of the tet2 gene, we utilized protocols similar to those recently described and engineered zinc finger nucleases (ZFNs) made by the context-dependent assembly (CoDA) method (35, 36). PC52-based expression plasmids containing our CoDA ZFNs were linearized downstream of the simian virus 40 (SV40) polyadenylation signal and used as templates for in vitro transcription of ZFN mRNAs (Ambion). One-cell fertilized zebrafish embryos were injected with various amounts of mRNAs encoding ZFNs harboring DD/RR or EL/KK variant FokI nuclease domains. Site-specific ZFN function was verified by PCR on genomic DNA (gDNA) using tet2-specific primers and then sequencing.

**Whole-mount in situ hybridization.** Embryos at the desired time points were fixed overnight in 4% paraformaldehyde (PFA) at 4°C. Whole-mount in situ hybridization (WISH) was performed as previously described (31, 32).

**Genotyping.** For embryo stages and adult stages, fish were individually genotyped using one forward (5’ ATCTCAGGCTCTGGCAGCT 3’) and one reverse (5’ ATACAAGGCTCATCCACTGAT 3’) primer. The forward primer was located in the sixth exon of tet2 and the reverse primer in the eighth exon. PCR products 668 bp in length were sequenced with the forward primer.

**Immunohistochemistry.** Embryos were fixed for 1 h in 4% paraformaldehyde at room temperature and then washed in phosphate-buffered saline–Tween (PBST) and permeabilized in phosphate-buffered saline (PBS) plus 0.1% Triton for 30 min at room temperature. The embryos were blocked in 2% fetal bovine serum (FBS)-PBST for 1 h and then incubated in primary antibody overnight at 4°C. The anti-5hmC (Active Motif) antibody was used at a dilution of 1:100 and anti-enhanced green fluorescent protein (anti-EGFP) at 1:200. For 5hmC detection, an Alexa Fluor 647–goat anti-rabbit secondary antibody (1:1,000) was used, while for EGFP detection, an Alexa Fluor 488–goat anti-mouse secondary antibody (1:1,000) was used. Subsequently, embryos were washed in PBST and prepared for imaging.

**Cell suspension preparation and flow cytometry.** Wild-type, tet2<sup>wt/m</sup>, and tet2<sup>m/m</sup> fish were anesthetized with 0.02% tricaine before kidney removal. The kidney was dissected and placed in ice-cold 0.9× PBS containing 5% fetal calf serum (FCS). Single-cell suspensions were generated by aspiration, followed by mild teasing of the kidney on a 40-μm nylon mesh filter with a pipette tip. Blood cell populations were analyzed on a BD FACSARia with high forward scatter-side scatter (FSC/SSC), as previously described (38). Data analyses were performed using FlowJo software (TreeStar, Ashland, OR). Statistical analysis was done using Prism. Unpaired Student t tests were performed in Prism to determine the P value for each genotype group compared to controls.

**Peripheral blood cell counts.** Wild-type, tet2<sup>wt/m</sup>, and tet2<sup>m/m</sup> fish were anesthetized with 0.02% tricaine, and 1 μl blood was obtained by cardiac puncture. The 1-μl amount of peripheral blood was diluted in 499 μl of 0.9% PBS containing 5% FCS. Ten microliters of heparin sodium (1,000 units/ml) was added to prevent blood coagulation, and 20 μl of Flow-Check fluorescent spheres (10<sup>4</sup> fluorescent spheres/ml; Beckman Coulter, Inc.) was added to this solution. The number of cells corresponding to 1,000 counted fluorescent spheres was obtained using a BD FACSARia with high forward scatter/side scatter, as previously described. Data analyses were performed using FlowJo software (TreeStar, Ashland, OR).

**Quantitative-PCR analysis.** RNA from sorted cells was extracted with TRIzol reagent (Invitrogen). cDNA was synthesized with Superscript III (Invitrogen). Quantitative PCR (qPCR) was carried out on a Viia7 real-time PCR (RT-PCR) system using SYBR green. The fold change was calculated by the ΔΔC<sub>T</sub> method normalized to β-actin. The primer sequences are shown in Table 1.

**Kidney smears and MGG staining.** The kidney was dissected and smeared on glass slides. The slides were fixed and stained with May-Grünwald–Giemsa (MGG) stain (Sigma-Aldrich) according to the manufacturer’s instructions and visualized with an Olympus BX51 microscope (Olympus).
Zebrafish gene  | Primer$^a$  | Sequence
---|---|---
tet1  | Fw 5' GATCTGTAGGTCAGGAAGGTGAT 3', Rev 5' GTATGTAATGGGCTCTTCCTCTTCT 3'
tet2  | Fw 5' CAGTGTATAGAAAGAGGACCTG 3', Rev 5' CTCGGGATTATCCCTCCTTGTTACG 3'
tet3  | Fw 5' CTAGGACAAACTCCAGAGAAC 3', Rev 5' GTAACCATGTATGGCTCTCAATTG 3'
β-actin  | Fw 5' TACAATGAGCTCGCTGTTGC 3', Rev 5' ACATAAATGCGAGGGGTT 3'

$^a$ Fw, forward; Rev, reverse.

Dot blot analysis. Dot blot analysis was performed as previously described (15).

Whole-genome sequencing and analysis. Genomic DNA from the kidney and the fin were prepared using the DNeasy blood and tissue kit (Qiagen). Sequencer-ready DNA fragment libraries were prepared from genomic DNA using PCR-free and Nano kits (Illumina). The sequences were run in a 100-bp paired-end run in the high-capacity run mode using HiSeq 2000 (Illumina).

Before mapping, the raw reads were trimmed and the low-quality bases were removed using the FASTX tool kit (http://hannonlab.cshl.edu/fastx_toolkit/) with quality thresholds at a quality number corresponding to a PHRED score (Q) of $\geq 20$ and a length of $\geq 75$. The trimmed reads were mapped using the BWA MEM algorithm (http://bio-bwa.sourceforge.net/), and duplicated reads were marked using the Picard tool kit (http://sourceforge.net/projects/picard/). The GATK package was used to make single-nucleotide polymorphism (SNP) and insertion-deletion mutation (indel) calls between fin and kidney samples (see the description of GATK best practices [http://www.broadinstitute.org/gatk/guide/best-practices]). The SNPs at locations with more than 20 reads from each from kidney and fin samples are listed in Table S1 in the supplemental material.

RESULTS

Generation of tet2 mutant zebrafish lines by genome editing.

Zinc finger nuclease technology (35, 36) was used to induce double-strand DNA breaks within the coding sequences in exon 7 of the tet2 gene in the zebrafish to produce lines with premature stop codons within the catalytic domain of the protein (Fig. 1A). Single-cell embryos injected with mRNAs encoding the zinc finger FokI fusion proteins targeting the site were grown to maturity and outcrossed to identify F1 progeny in which infidelity in repair by nonhomologous end joining introduced indels at the cleavage site that can disrupt the tet2 reading frame. We generated two independent tet2 mutant alleles, encoding Tet2Δ4 and Tet2Δ5, that possess 4- and 5-bp deletions, respectively, in exon 7 (Fig. 1B). The Tet2Δ4 and Tet2Δ5 lines were derived independently from separate injected embryos with genome editing after cleavage with the Zn finger nuclease. Although the data shown here are from experiments performed using the Tet2Δ4 line, we performed each experiment with both the Tet2Δ4 and Tet2Δ5 lines. Indels introducing premature stop codons within exons 7 and 8 are observed in human patients with myeloid malignancies (39). Thus, our mutant animals are faithful to mutations found in human myeloid malignancies. The 4-bp deletion in Tet2Δ4 (ACAT) and the 5-bp deletion in Tet2Δ5 (CAAGG) led to stop codons in amino acid 1162 (AA1162) and AA1168, respectively (Fig. 1C), before the third iron-binding site, AA1573, and the 2-oxoglutarate binding site, AA1588, which are found in the double-stranded beta-helix (DSBH) region of the catalytic domain in the Tet2 protein. These binding sites within the catalytic domain are crucial for the activity of the enzyme in converting 5mC to 5hmC (Fig. 1D). There is only one orthologue of TET2 in the zebrafish genome, and alignment of the predicted zebrafish and human proteins showed a high level of conservation for the three iron-binding sites (zebrafish
AA1126, AA1129, and AA1573; human AA1382, AA1384, and AA1881) and the 2-oxoglutarate binding site (zebrafish AA1588; human AA1896) (see Fig. S1 in the supplemental material). We observed the same results in studies with each of the independently derived lines. Quantitative-RT-PCR (qRT-PCR) analysis of the tet2 RNA sequences in heterozygous fish showed that RNAs containing the mutations were expressed and apparently were not subject to nonsense-mediated decay, even though they encode a truncated protein (see Fig. S2A in the supplemental material). The two heterozygous tet2 mutant lines, Tet2Δ4 and Tet2Δ5, were inbred to generate homozygous tet2Δm/Δm zebrafish lines, which were viable and fertile and lacked any detectable developmental or morphological defects. Thus, we conclude that tet2 loss is dispensable for the development and viability of the zebrafish.

**tet2Δm/Δm zebrafish embryos undergo normal hematopoiesis.** To assess the effect of tet2 loss on embryonic hematopoiesis, we performed WISH on the progeny of a cross of two tet2Δm/Δm adult fish expressing α-globin (Fig. 2A1 to A3) and cmyb (Fig. 2B1 to B3) at 32 and 36 h postfertilization (p.f.). Analysis of RNA expression of the two genes showed no differences in cells expressing α-globin (erythroid cells) or cmyb (hematopoietic stem cells) in tet2Δm/Δm (homozygous wild type), tet2Δm/+, and tet2Δm/Δm embryos, in contrast to recent findings by Ge et al. using morpholinos (40). Moreover, cells expressing mpox (myeloid cells) were not impaired in tet2Δm/Δm zebrafish embryos in comparison with those in wild-type and heterozygous embryos (see Fig. S2B in the supplemental material). Whole-mount o-dianisidine staining for heme was also unchanged in 4-day-old tet2Δm/Δm, tet2Δm/+, and tet2Δm/Δm fish at 5 days of age was performed by cell counts in the Tg(cmyb-GFP) background (Fig. 2D1 to D3). We did not observe a significant difference in the number of cd41+ cells in tet2Δm/Δm and tet2Δm/Δm embryos compared with tet2Δm/+ fish embryos (Fig. 2D4). Similarly, the numbers of myeloperoxidase-positive (mpx+) cells in the CHT regions of 7-day-old tet2Δm/Δm, tet2Δm/+, and tet2Δm/Δm embryos were assessed by histochemistry (Fig. 2E1 to E6). This analysis did not reveal any differences between the three groups (Fig. 2E7). To investigate Tet2 loss in zebrafish hematopoietic stem and progenitor cells (HSPCs), we determined the relative numbers of cmyb+ cells in 5-day-old tet2Δm/Δm, tet2Δm/+, and tet2Δm/Δm zebrafish embryos using the Tg(cmyb-GFP) zebrafish transgenic reporter line (32) (see Fig. S2C in the supplemental material). We did not observe a difference in the relative numbers of cmyb-EGFP+ HSPCs per embryo in tet2Δm/Δm, tet2Δm/+, and tet2Δm/Δm embryos (see Fig. S2D in the supplemental material). These results show that loss of Tet2 function does not detectably impair embryonic or early definitive hematopoiesis.

**Analysis of 5hmC expression in zebrafish embryonic hematopoietic stem cells.** To investigate Tet2 activity in zebrafish hematopoietic stem cells budding from the ventral wall dorsal aorta at 36 h p.f., we used the Tg(cmyb-GFP) zebrafish transgenic reporter line and performed double labeling for GFP and 5hmC (36). Interestingly, wild-type zebrafish cmyb-GFP-positive HSPCs did not express 5hmC at this stage of zebrafish hematopoiesis (Fig. 3A2 to A4, white arrows) in the aorta-gonad-mesonephros (AGM) region (Fig. 3A1), but 5hmC expression was apparent in cmyb-negative cells in the intermediate cell mass (ICM) region of these embryos (yellow arrows). In the cmyb-GFP-positive background, tet2Δm/Δm embryos were indistinguishable from wild-type embryos, including the finding that cmyb-GFP-positive tet2Δm/Δm HSPCs did not express 5hmC (Fig. 3A5 to A7).

At 48 h p.f. in the Tg(cmyb-GFP) background, the 5hmC expression in tet2Δm/Δm embryos colocalized with GFP expression in approximately 10 to 15% of the cells expressing cmyb in the CHT (Fig. 3B1 to B4). Thus, although 5hmC expression is not present when HSPCs bud from the dorsal aorta, 5mc modification into 5hmC is initiated when the HSPCs populate the CHT region. tet2Δm/Δm embryos at 48 h were indistinguishable from tet2Δm/+ embryos, with 5mc modification progressing to 5hmC in HSPCs at this stage of development, despite the absence of functional Tet2 (Fig. 3B5 to B7).

**tet2 loss in zebrafish leads to decreased 5hmC levels in kidney marrow blood cells.** To investigate whether tet2 loss results in decreased levels or loss of 5hmC in the nuclear DNA of cells in the tissues of adult zebrafish, we performed immunohistochemistry on tissue sections of 4-month-old tet2Δm/Δm, tet2Δm/+, and tet2Δm/Δm fish using an anti-5mc antibody (Fig. 4). Interestingly, the 5hmC modification was undetectable in most of the blood cells of the kidney marrow of the tet2Δm/Δm fish (Fig. 4A3) and markedly different from the 5hmC expression in the tet2Δm/Δm zebrafish kidney marrow (Fig. 4A1). Kidney tubule cells in the same fields showed high levels of 5hmC staining in tet2Δm/Δm fish (Fig. 4A3), providing an internal nonhematopoietic control cell population for the analysis. Similarly, 5hmC modification was readily detected in all the cellular nuclei of other organs, such as brain (Fig. 4B1 and B3), intestine (Fig. 4C1 and C3), and muscle (Fig. 4D1 and D3), in both genotypes. In tet2Δm/Δm fish, 5hmC modification was reduced in the kidney marrow compared with the 5hmC expression in the tet2Δm/Δm zebrafish kidney marrow, but in a more patchy and less striking pattern than the 5hmC expression in the whole kidney marrow of the tet2Δm/Δm zebrafish. Similarly, 5hmC was detected in all the cellular nuclei of other organs, such as brain, intestine, and muscle (Fig. 4B2, C2, and D2). These results indicate that tet2 loss prevents 5hmC modification in hematopoietic cells of the kidney marrow by 4 months of age but not in the nuclei of renal tubule cells or the cells of other tissues and organs. Thus, wild-type Tet1 and Tet3 appear to be able to perform this modification in most tissues.
tissues of adult zebrafish when Tet2 function is lost, but not in hematopoietic cells.

To investigate the levels of expression of each tet family member in hematopoietic cells, we sorted zebrafish kidney marrow cells into four subpopulations: (i) erythrocytes, (ii) lymphocytes, (iii) progenitor cells, and (iv) myelomonocytes (Fig. 5). Analysis of wild-type cells showed that each of the tet family members was expressed in all four subpopulations. tet1 was expressed at higher levels than tet2 and tet3 in each cell population except progenitor cells, which expressed equal levels of tet1 and tet2. Interestingly, in the tet2<sup>m/m</sup> animals, the levels of tet1, tet2, and tet3 expression were dramatically upregulated in each subpopulation except the myelomonocytes. This suggests the TET2 protein normally mediates a feedback loop regulating transcription of both itself and the other TET family members. Thus, the nonredundancy of Tet1 and Tet3 in hematopoietic cells is not due to a lack of mRNA expres-
Loss of Tet2 Leads to MDS in Zebrafish

Figure 4: tet2 loss leads to reduced 5hmC expression in the kidney marrow in tet2<sup>m/m</sup> adult fish. (A1 to A3) Immunohistochemistry for 5hmC expression on paraffin sections from adult fish shows loss of 5hmC expression in the blood cell progenitors of the kidney marrow in the tet2<sup>m/m</sup> (A3) not the tet2<sup>wt/wt</sup> fish. However, the renal glomerular cells (red outlines) in the same field of tet2<sup>m/m</sup> adult fish express 5hmC. tet2 loss does not affect 5hmC expression levels in cell nuclei in brain (B1 to B3), intestine (C1 to C3), and muscle (D1 to D3).

Zebrafish homozygous for mutant tet2 develop premalignant dysplasia at 11 months of age. FSC/SSC flow cytometry was performed on whole-kidney marrow from tet2<sup>m/m</sup> zebrafish and their siblings. The relative percentages of four different populations (erythrocytes, myelomonocytes, lymphocytes, and progenitor cells) were determined individually in each fish (n = 10 per group) at the ages of 4, 6, 8, and 11 months. At 4 and 6 months, the
percentages of erythrocyte, myelomonocyte, progenitor cell, and lymphocyte populations in the kidneys of the wild-type, heterozygous, and homozygous fish were similar (see Fig. S4A and B in the supplemental material). However, at 8 months of age, there was a clear decrease in the erythrocyte population and an increase in progenitor cells in \textit{tet2}^{m/m} compared with wild-type fish (see Fig. S4C in the supplemental material).

Analysis of the different cell populations from the whole-kidney marrow of 11-month-old \textit{tet2}^{m/m} fish and their siblings, using FSC/SSC, revealed a decreased number of erythrocytes and expanded numbers of myeloid and progenitor cells in the kidney marrow of \textit{tet2}^{m/m} compared to wild-type fish (Fig. 6). Eleven-month-old heterozygous \textit{tet2}^{wt/m} fish showed a significant decrease in the erythrocyte population and a significant increase in the myelomonocyte population in the kidney marrow, but there was no change in the progenitor cell populations of these animals (Fig. 6).

**FIG 5** Analysis of \textit{tet1}, \textit{tet2}, and \textit{tet3} expression in different blood cell types. qRT-PCR in cDNA from the erythrocytes, lymphocytes, progenitor cells, and myelomonocytes isolated from the kidney marrow was performed to examine the expression of each \textit{tet} family member in \textit{tet2}^{wt/wt}, \textit{tet2}^{wt/m}, and \textit{tet2}^{m/m} adult zebrafish. The expression levels are shown relative to \( \beta \)-actin. For this experiment, kidney marrow from 9 \textit{tet2}^{wt/wt}, \textit{tet2}^{wt/m}, and \textit{tet2}^{m/m} adult zebrafish was drawn in 3 groups, each consisting of 3 animals. The bars represent the averages of triplicate runs, and the error bars represent standard errors of the mean (SEM).
MGG staining of the kidney marrow smears from tet2wt/wt, tet2wt/m, and tet2m/m fish at 11 months of age revealed differences in the morphology of the erythrocyte population and the appearance of dysplastic cells of the myeloid lineage in a subset of heterozygous fish (Fig. 7B1 to B3) and in all homozygous fish (Fig. 7C1 to C3). The erythrocytes in a subset of heterozygous fish (6 of 11) and in all 10 of the tet2m/m fish showed deep basophilic cytoplasm compared to the light-pink coloration of erythrocytes in the tet2wt/wt fish (Fig. 7A1 to A3). Analysis of total RNA amounts from 300,000 red blood cells sorted from the kidney marrow of tet2wt/wt and tet2m/m adult fish showed a 1.5- to 1.8-fold increase in rRNA concentrations in red blood cells with Tet2 loss, potentially explaining the dark basophilic cytoplasm observed in tet2m/m and tet2m/m red blood cells (see Fig. S5 in the supplemental material).

Analysis of the peripheral blood showed that the total blood cell counts of 11-month-old tet2m/m fish were similar to those in tet2wt/wt and tet2wt/m fish (Fig. 8A and B). In addition, profiling of the peripheral blood cells by FSC/SSC analysis showed no changes in the different populations of the blood cells in the tet2wt/wt, tet2wt/m, and tet2m/m adult fish (Fig. 8C). These data indicate that loss of tet2 in zebrafish leads to the development of myelodysplasia of the kidney marrow cells, or what might be called pre-MDS, since the blood counts are still compensated for to normal at this age.

Assessment of clonality in zebrafish premyelodysplasia. To assess the clonal evolution of hematopoietic cells in the kidney marrow of the tet2m/m adult fish, we performed whole-genome sequencing of genomic DNA isolated from the kidney marrow and genomic DNA isolated from the fin of an 11-month-old zebrafish whose kidney marrow smears showed a high percentage of dysplastic myeloid cells. Our data, summarized in Table S1 in the supplemental material, show that the genomic DNA kidney marrow contains new somatically acquired point mutations and indels compared with the genomic DNA sequence obtained from the fin. These mutations do not affect coding sequences and thus do not likely represent drivers of the expansion of myeloid progenitor cells and the myelodysplastic phenotype observed at 11 months of age. Rather, they likely reflect passenger alterations that become apparent only after clonal expansion of tet2 mutant hematopoietic cells.

Zebrafish homozygous for mutant tet2 develop MDS at 24 months of age. Flow cytometry analysis of the different cell populations from the whole-kidney marrow of 24-month-old tet2m/m fish and their siblings revealed a decreased percentage of erythrocytes and increased percentages of myeloid and progenitor cells in the kidney marrow of tet2m/m compared to wild-type fish (Fig. 9A). Twenty-four-month-old heterozygous tet2wt/m fish showed a significant decrease in the erythrocyte population and a significant increase in the myelomonocyte population in the kidney marrow, but there was no change in the progenitor cell populations of these animals (Fig. 9A). Aberrant morphology of the erythrocyte population and the appearance of dysplastic cells of the myeloid lineage, similar to those observed at 11 months of age (Fig. 7), were present in tet2wt/m (6 out of 8) and tet2m/m (12 out of 12) fish but not in the tet2wt/wt fish (12 out of 12) (Fig. 9B to D). Analysis of the peripheral blood showed a decrease in the absolute number of erythrocytes in the peripheral blood in the tet2m/m adult fish compared with tet2wt/wt and tet2mt/wt fish (Fig. 10A). The presence of anemia, together with the dysplastic changes in the kidney marrow progenitor cells, indicates progression to MDS in the tet2m/m
zebrafish animal model. Furthermore, MGG staining of peripheral blood smears from 24-month-old tet2m/m fish showed that the erythrocytes in the tet2m/m fish exhibited an aberrant rounded shape compared with the characteristic flattened elliptical shape observed in the tet2wt/wt fish (Fig. 10E to G). This phenotype was accompanied by a significant increase in the cytoplasm-to-nucleus ratio, which indicates an arrest in maturation within circulating erythrocytes in tet2m/m fish (Fig. 10E), consistent with the development of MDS.

DISCUSSION

In patients with hematopoietic malignancies such as MDS, TET2 disruption affects global genomic methylation due to a deficiency in 5-methylcytosine dioxygenation in hematopoietic cells that modifies the epigenetic landscape of the tumor cells. In the last few years, several mouse models with targeted disruption of Tet2 in the hematopoietic system have been reported, and they have been used to study the role of Tet2-inactivating mutations in normal hematopoietic cell development, the evolution of clonal dominance, and the development of hematopoietic malignancies. Tet2-deficient mice predominantly developed CMML, followed by hematopoietic malignancies, such as MDS and MPN (23–26). Similar to the results reported in murine models (23–26), the tet2m/m zebrafish we studied did not exhibit defects in embryonic hematopoiesis, remained viable and fertile, and developed clonal myelodysplasia of the kidney marrow as they aged, culminating in true MDS with anemia. At 24 months of age, tet2 zebrafish mutants develop increased percentages of progenitor cells and myelomonocytes and decreased percentages of erythrocyte lineage cells in the kidney marrow. In the peripheral blood, the 24-month-old tet2 zebrafish mutants have anemia with erythroid cell dysplasia, indicating progression to MDS. This is consistent with clonal dominance with a bias toward myelomonocytic differentiation at the expense of erythroid differentiation in patients with TET2 mutations observed by Itzykson and coworkers (44).

Interestingly, in fish with heterozygous or homozygous loss of tet2, the nuclear 5hmC modification was selectively lost in the hematopoietic cells of the kidney marrow. Other tissues of the tet2 mutant fish, such as brain, muscle, and intestine, exhibited normal 5hmC levels, presumably due to compensation by Tet1 or Tet3, a finding that may not have been apparent in some of the murine models because of the use of vav-CRE to delete Tet2 spe-

FIG 7 Morphological analysis of blood cell types in the kidneys of tet2wt/wt, tet2wt/m, and tet2m/m 11-month-old fish. Analysis of kidney smears revealed the presence of all hematopoietic lineages in tet2wt/wt, tet2wt/m, and tet2m/m 11-month-old fish. (A1 to A3) May-Grunwald–Giemsa staining of kidney marrow smears for tet2wt/wt fish shows no defects in maturation or the morphology of the blood cells. (B1 to B3) May-Grunwald–Giemsa staining of tet2m/m kidney marrow smears shows normal morphology of mature erythrocytes and mature myeloid and hematopoietic progenitor cells (6 of 10) (B1 and B2), as well as dysplastic myeloid and progenitor cells (4 of 10) (B2 and B3), in a subset of tet2m/m fish. The erythrocyte lineage in the tet2m/m mutants (4 of 10) (B2 and B3) also shows morphological differences compared with tet2wt/wt erythrocytes. (C1 to C3) May-Grunwald–Giemsa staining of kidney marrow smears for tet2m/m erythrocytes (10 of 10) shows dysplasia in the myeloid and progenitor cell lineages; also, the erythrocytes have a dark basophilic cytoplasm compared with tet2wt/wt erythrocytes. Red arrows, mature erythrocytes; orange arrows, progenitor cells; black arrows, mature myeloid cells; green arrow, eosinophil; asterisks, dysplastic myeloid cells.
specifically in hematopoietic cells. We interpret our result to suggest that Tet1 and Tet3 can replace the activity of Tet2 in nonhematopoietic tissues so that 5hmC levels appear normal in most tissues of tet2 mutant animals. In contrast, Tet2 appears to play a unique role in 5hmC formation in hematopoietic cells that cannot be compensated for by normal, or even increased, levels of expression of tet1 and tet3. We believe this finding explains why TET2-inactivating mutations are selected only in hematopoietic malignancies and not in other tumor types. Recent studies have indicated that gain-of-function mutations in IDH1 and IDH2 enable the mutant proteins to disrupt the normal function of TET proteins (45). Both hematopoietic malignancies and gliomas can acquire IDH1-IDH2 gain-of-function mutations, but gliomas do not exhibit TET2 loss-of-function mutations. Presumably this is because other TET proteins are able to generate 5hmC in TET2 mutant glial cells but not TET2 mutant hematopoietic cells, while IDH1-IDH2 mutant proteins would be expected to interfere with the activities of all three TET proteins. Thus, our findings also explain the lack of TET2 mutations in gliomas, even though IDH1-IDH2 mutants appear to act by interfering with TET function (46).

Recently, the effect of tet2 loss on embryonic zebrafish hematopoiesis was reported, based on morpholino-mediated tet2 knockdown in embryos, which led to inhibition of erythropoiesis,

FIG 8 Analysis of total blood counts in tet2<sup>wt/wt</sup>, tet2<sup>wt/m</sup>, and tet2<sup>m/m</sup> 11-month-old fish. (A) Representative schematic of the forward versus side scatter analysis plots for the peripheral blood populations in 11-month-old tet2<sup>wt/wt</sup>, tet2<sup>wt/m</sup>, and tet2<sup>m/m</sup> fish. (B) Analysis of the total peripheral blood counts for 11-month-old tet2<sup>wt/wt</sup>, tet2<sup>wt/m</sup>, and tet2<sup>m/m</sup> fish shows no significant differences among the three different genotypes. (C) Comparison of absolute cell numbers for erythrocytes, progenitor cells, myelomonocytes, and lymphocytes in the peripheral blood of 11-month-old tet2<sup>wt/wt</sup>, tet2<sup>wt/m</sup>, and tet2<sup>m/m</sup> fish reveals no differences among the three genotypes. The short horizontal lines represent the error bars, and the long horizontal lines represent the mean values.
apparently due to loss of hematopoietic cells expressing the \textit{scl}, \textit{gata-1}, and \textit{cmyb} genes (40). In contrast, in our homozygous \textit{tet2} loss-of-function zebrafish mutant embryos, we did not observe any changes in HSPC numbers, as reflected by \textit{cmyb} and CD41 expression, or any defects in embryonic erythropoiesis or in myeloperoxidase-expressing myeloid cells. Our results are consistent with those in most murine models, in which hematopoiesis is normal until 8 months of age, and in fact, homozygous \textit{Tet2} mutant HSCs exhibit an advantage in competitive repopulation after stem cell transplantation (23–26). An exception is the work in murine models of Kunimoto and colleagues (47), who report that \textit{Tet2} disruption leads to enhanced self-renewal and altered differentiation of fetal liver hematopoietic stem cells in mice harboring a LacZ gene trap cassette within exon 2 of the murine \textit{Tet2} gene. In this mouse line, the homozygous disruption of \textit{Tet2} also results in perinatal lethality. These results are quite unique, in that most murine knockout lines and our \textit{tet2m/m} zebrafish are viable and fertile and lack evidence of defects in embryonic and larval hematopoiesis.

We suspect that abnormalities of primitive erythropoiesis observed in the study of Ge and colleagues (40) after morpholino knockdown of \textit{tet2} may reflect, at least in part, the nonspecific activation of \textit{p53} by the morpholino (48). It is well known that morpholinos can produce spurious phenotypes through this mechanism, and most investigators now confirm the specificity of phenotypes in morphants by either repeating the knockdown in \textit{p53 m/m} embryos or simultaneously knocking down \textit{p53} (31, 49). This does not appear to have been done in the study by Ge et al. (40), providing an additional example of morphant phenotypes that may not accurately reflect the specific role of the gene under investigation.

It is possible that residual truncated protein expressed in our zebrafish mutant retains scaffolding or other activity that restores a normal embryonic hematopoiesis phenotype, but we strongly advise that interested investigators test additional \textit{tet2} zebrafish mutants generated by genome editing before performing additional studies based on morpholino knockdown of \textit{Tet2} (50).

It is interesting that both wild-type and \textit{tet2m/m} fish lacked 5hmC expression in myb-GFP-positive cells in the AGM at 36 h.p.f. (Fig. 3A2 to A7) and that 5hmC was expressed in corresponding MYB-negative cells in the ICM (Fig. 3A3 and A6). The 5hmC$^+$ cells in the ICM correspond to GATA$^+$ cells in the same region in an RFP line, although we have not been able to locate a suitable antibody to precisely demonstrate colabeling of these cells with both GATA and 5hmC. We think that this finding is consistent with the interpretation that the 5hmC modification becomes necessary to upregulate the expression of differentiation-specific genes as HSPCs begin to give rise to different blood cell lineages during hematopoiesis. In early hematopoietic cells of the ICM and CHT, however, it appears that \textit{Tet1} or \textit{Tet3} can replace the activity of \textit{Tet2}, and apparently this redundancy is lost as the cells migrate to the kidney marrow (Fig. 3A1, A2, and A3). We have shown that
FIG 10 Analysis of total blood count in tet2wt/wt, tet2wt/m, and tet2m/m 24-month-old fish. (A to D) Comparison of absolute cell numbers for erythrocytes (A), progenitor cells (B), myelomonocytes (C), and lymphocytes (D) in the peripheral blood of 24-month-old tet2wt/wt, tet2wt/m, and tet2m/m fish reveals a decrease in the total number of erythrocytes but not the other blood cell types in the tet2m/m fish compared with tet2wt/wt fish. Statistical analysis was performed using an unpaired Student t test. The long horizontal lines denote the mean values. (E) Scatter plot showing the cytoplasm/nucleus ratio of the circulating erythrocytes from 24-month-old tet2wt/wt and tet2m/m fish. Statistical analysis was performed using an unpaired Student t test. The long horizontal lines denote the mean values. (F and G) Analysis of the peripheral blood smears by MGG staining reveals a difference in the morphology of the erythrocytes between 24-month-old tet2wt/wt (F) and tet2m/m (G) fish.
the tet1 and tet3 mRNAs are expressed in kidney marrow hematopoietic cells, and thus, further work is needed to identify the underlying mechanisms that make Tet1 and Tet3 unable to function in place of Tet2 in the kidney marrow cells of juvenile and adult fish.

It has been known for years that about 10% of people over 50 years of age and 20% over 80 years develop clonal skewing of hematopoiesis, particularly within the myeloid lineages. Very recent studies have shown that in ~6% of these individuals, this clonal advantage is imparted by somatically acquired inactivating mutations of TET2, leading to enhanced self-renewal and clonal dominance of the HSGs that acquire TET2 mutations (16). Even though these individuals have clonal hematopoiesis, their circulating blood cell counts may remain normal for prolonged periods. An attractive possibility is that nontoxic drugs could be identified that are able to selectively suppress TET2 mutant hematopoietic stem cells and allow the return of normal polyclonal hematopoiesis in these individuals. It appears likely that TET2 loss-of-function mutations could represent the initial genetic hit in some cases of myeloid malignancies and that suppression of their self-renewal advantage might prevent the acquisition of additional mutations leading to myeloid malignancies, as proposed recently for DNMT3a mutations by Shlush and coworkers (51).

Our tet2m/m zebrafish develop premyleodysplasia at 11 months of age and MDS at 24 months of age. This model may prove particularly useful for identifying suppressors of tet2 mutant hematopoietic cells, because the adult fish develop MDS. Embryos from the tet2m/m line are well suited for phenotype-based small-molecule drug screens and genetic screens to identify synthetic-lethal interactions with Tet2 loss in HSPCs. It is reassuring in this regard that our tet2 mutant zebrafish provide a faithful model of progressive myelodysplasia with age that occurs in patients. It recapitulates important aspects of human myeloid disease, such as loss of 5hmC specifically in the blood cells, myeloid skewing, and clonal hematopoiesis culminating in MDS. Thus, this mutant line affords a powerful tool for analyzing the therapeutic effects of candidate small molecules and genes that are synthetic lethal with tet2 loss in hematopoiesis and in the onset and progression of myelodysplasia.

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E.G. designed, performed, and analyzed the experiments. M.R.M., A.T.L., and M.K. performed experiments. Y.-Y.K. and S.Y. analyzed data. J.D.S. and J.K.J. designed and assembled the engineered zinc finger nucleases. E.G. and A.T.L. designed the experiments and revised the manuscript.

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