SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
STEM CELLS
DIFFERENTIATION
EPIGENETICS
MEDICAL RESEARCH

Received
6 September 2011

Accepted
16 January 2012

Published
17 February 2012

Correspondence and requests for materials should be addressed to H.N. (hnakajim@sc.itc.keio.ac.jp)

Tet2 disruption leads to enhanced self-renewal and altered differentiation of fetal liver hematopoietic stem cells

Hiroyoshi Kunimoto[1], Yumi Fukuchi[1], Masatoshi Sakurai[1], Ken Sadahira[1], Yasuo Ikeda[1,2], Shinichiro Okamoto[1] & Hideaki Nakajima[1]

[1]Division of Hematology, Department of Internal Medicine, Keio University School of Medicine, Tokyo 160-8582, Japan, [2]Department of Life Science and Medical Bioscience, Faculty of Science and Engineering, Waseda University, Tokyo 162-8480, Japan.

Somatic mutation of ten-eleven translocation 2 (*TET2*) gene is frequently found in human myeloid malignancies. Recent reports showed that loss of *Tet2* led to pleiotropic hematopoietic abnormalities including increased competitive repopulating capacity of bone marrow (BM) HSCs and myeloid transformation. However, precise impact of *Tet2* loss on the function of fetal liver (FL) HSCs has not been examined. Here we show that disruption of *Tet2* results in the expansion of $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) cells in FL. Furthermore, *Tet2* loss led to enhanced self-renewal and long-term repopulating capacity of FL-HSCs in *in vivo* serial transplantation assay. Disruption of *Tet2* in FL also led to altered differentiation of mature blood cells, expansion of common myeloid progenitors and increased resistance for hematopoietic progenitor cells (HPCs) to differentiation stimuli *in vitro*. These results demonstrate that *Tet2* plays a critical role in homeostasis of HSCs and HPCs not only in the BM, but also in FL.

Myelodysplastic syndrome (MDS) and myeloproliferative neoplasm (MPN) are two major hematopoietic malignancies, thought to arise from hematopoietic stem or progenitor cells. MDS is characterized by dysregulated hematopoietic differentiation with propensity to develop acute myeloid leukemia (AML)[1]. MPN is a spectrum of disease characterized by the expansion of maturing hematopoietic elements with minimal dysplasia. Some of the patients with MPN also progress to myeloid leukemia by acquiring additional mutations altering differentiation. Recent advances in high throughput genome-wide sequencing have made it possible to identify a number of recurrent somatic mutations in human MDS and MPN. One of the most frequently found mutations is a loss-of-function mutation of ten-eleven translocation 2 (*TET2*), which is located at chromosome 4q24 where uniparental disomy was frequently observed in human myeloid malignancies. Recurrent *TET2* mutation was identified in 10–20% of MDS and MPN[2,3], and subsequent studies reported high incidence of *TET2* mutations in secondary AML (20–40%) and chronic myelomonocytic leukemia (CMML) (40–50%)[4,5]. These results underscore the importance of *TET2* in maintaining homeostasis and malignant transformation of hematopoietic system.

DNA methylation is one of the most important epigenetic modifications, and aberrant DNA methylations are hallmark of cancers including AML[6]. It was recently reported that *TET* family proteins could convert 5-methylcytosine (5 mC) to 5-hydroxymethylcytosine (5 hmC)[7–9] and 5 hmC production by *Tet1* is critical for ES cell self-renewal and inner cell mass specification[8,10]. Moreover, *TET2* mutations associated with myeloid malignancies disrupt its catalytic activity, and BM cells from patients with *TET2* mutations contained lower levels of 5 hmC compared to normal controls[9]. On the other hand, mutations of isocitrate dehydrogenase (*IDH*) 1 and *IDH2*, enzymes involved in citrate metabolism, are seen in AML and brain tumors in mutually exclusive manner with *TET2*[11–14]. These mutations inhibit catalytic activity of *TET2* by 2-hydroxyglutarate (2-HG), an oncometabolite generated by mutant *IDH1/2*[14]. TET2 is dependent on alpha-keto-glutarate (α-KG) for its catalytic activity, and 2-HG has been shown to inhibit TET2 by competing with α-KG[15]. These results strongly suggest that impaired 5 hmC generation by mutant TET2 or by mutant IDH1/2 is one of the critical steps in myeloid transformation.

FL-HSCs and adult HSCs differ in several aspects of their phenotypes and functions. For example, FL-HSCs are different from adult HSCs in the expression of CD11b/ Mac-1 and CD34, while some markers such as CD150 and endomucin are commonly expressed in both populations[16,17]. FL-HSCs have higher, more robust capacity to reconstitute hematopoietic compartment in irradiated recipients as compared to adult HSCs[18,19]. Moreover,

regulation of self-renewal, one of the most critical features of HSCs, differs between FL and adult HSCs as marked by distinct dependence on polycomb group proteins such as Bmi-1, Rae28, and Mel-18[20–22]. These observations clearly indicate that FL-HSCs and adult HSCs are phenotypically and functionally distinct, and suggest that they may be regulated by distinct molecular machinery.

It was recently reported that inactivation of *Tet2* in mouse genome results in increased long-term repopulating capacity and competitive advantage of HSCs from adult BM, and eventually leads to myeloid transformation[23–25]. However, self-renewal capacity of HSCs was not precisely assessed by serial transplantation assay and effects of *Tet2* disruption on FL-HSCs have not been examined. Here we show that disruption of *Tet2* leads to the expansion of lineage negative (Lin^-), Sca-1^+, c-Kit^+ (LSK) multipotent progenitor (MPP) fraction and common myeloid progenitors (CMPs) in FL. In addition, self-renewal and long-term repopulating capacities were enhanced by *Tet2* disruption as evidenced by serial transplantation assay. These results clearly indicate critical roles of *Tet2* in homeostasis of HSCs and HPCs in FL.

Results

Characterization of *Tet2* gene-trap mice. *Tet2* gene was disrupted by inserting LacZ/ neomycin resistance (β-geo) cassette in intron 2, just before exon 3 (Figure 1A). mRNAs transcribed from the endogenous promoter are expected to terminate by being spliced to the trap cassette, which carries poly A signal at the end. Since exon 3 is the first coding exon, trapping the *Tet2* message before exon 3 should lead to complete ablation of *Tet2*. We first tested whether mRNA was efficiently terminated by the trap-cassette. Semi-quantitative and real-time quantitative RT-PCR using primers amplifying exon 1–3 revealed that mRNA reading through exon 3 in $Tet2^{gt/gt}$ mice was far less than 1% of that of $Tet2^{+/+}$ mice, indicating that almost all mRNAs were trapped by the cassette (Figure 1B and C). Of note, the level of mRNA in $Tet2^{+/gt}$ mice was approximately 20% of WT. We have also examined the catalytic activity of TET2 in gene-trap mice by dot blot assay. Quantification of the 5 mC and 5 hmC levels in DNA from FL cells confirmed a marked reduction of 5 hmC signals in $Tet2^{gt/gt}$ mice as compared to WT (Figure 1D). These data indicate that transcription of *Tet2* gene was efficiently disrupted in $Tet2^{gt/gt}$ mice, and therefore, $Tet2^{gt}$ allele can be regarded as a null allele.

As reported previously, intercross of heterozygous mice resulted in perinatal lethality of homozygous mice, and very few $Tet2^{gt/gt}$ mice survived to 1 week after birth[26]. Therefore, we used fetal liver (FL) cells for analyzing hematopoiesis for the following analysis.

***Tet2* disruption leads to the expansion of multipotent progenitor cells and myeloid progenitors in fetal liver.** Initial analysis revealed that the numbers of whole FL cells or various hematopoietic progenitors in FLs were not significantly different between WT and *Tet2*-mutant embryos (Supplemental Figure S1 A and B). In addition, apoptotic status of FL cells as shown by the staining with annexin V and propidium iodide (PI) was indistinguishable between WT and *Tet2*-mutants (Supplemental Figure S2). Next we analyzed the frequency and numbers of hematopoietic stem and progenitor cells in *Tet2*-mutant FLs. Interestingly, percentage of lineage negative (Lin^-), Sca-1^+, c-Kit^+ (LSK) fraction that mainly consists of MPPs increased in $Tet2^{gt/gt}$ (1.45 +/− 0.62%) and $Tet2^{+/gt}$ FLs (1.21 +/− 0.43%) as compared to wild-type (WT) (0.85 +/− 0.34%) FLs (Figure 2A and B). Absolute number of LSK cells was also increased in *Tet2*-mutant FLs (Suppl. Figure S3A). However, percentages and absolute numbers of $CD150^+$LSK, $CD150^+CD48^-$LSK and $CD34^+$LSK cells, highly enriched fractions of FL-HSCs, were not statistically different between WT and *Tet2* mutants (Figure 2A and B, Suppl. Figure S3A). Analysis of myeloid-committed progenitor cells revealed that the frequency of common myeloid progenitors (CMPs) significantly increased in $Tet2^{gt/gt}$ FLs, whereas those of granulocyte-monocyte progenitors (GMPs) and megakaryocyte-erythroid progenitors (MEPs) did not (Figure 2A and C). The average cell number of $Tet2^{gt/gt}$ CMPs was higher than that of WT or $Tet2^{+/gt}$ CMPs, although the difference was not statistically significant (Suppl. Figure S3B). Taken together, disruption of *Tet2* leads to the expansion of LSK cells and CMPs, but not highly purified HSCs in FLs.

Self-renewal capacity of HSCs was dramatically enhanced by ablation of *Tet2*. Increased LSK fraction in $Tet2^{gt/gt}$ FLs with expansion of the MPP population could be due to enhanced self-renewal capacity of FL-HSCs upon disruption of *Tet2*. To address this issue, we performed serial transplantation assay using FL cells from WT and *Tet2*-mutant embryos (Figure 3A). Transplantation of 1 x 10^6 FL cells with 2 x 10^5 competitor cells into lethally irradiated recipients resulted in over 80% peripheral engraftment in all genotypes. Strikingly however, secondary and tertiary transplant led to dramatically increased peripheral blood (PB) chimerism of $Tet2^{gt/gt}$ cells compared to WT cells. Interestingly, chimerism of $Tet2^{+/gt}$ cells was intermediate between WT and $Tet2^{gt/gt}$ which was statistically higher compared to WT in the secondary and the tertiary transplants, while the difference to $Tet2^{gt/gt}$ cells was not statistically significant. We speculated that FL-HSCs might expand in the engrafted microenvironment, and therefore went on to examine the fraction of donor-derived HSCs in the recipients' bone marrow (BM). As expected, percentage of donor-derived HSCs ($CD34^-$LSK cells) in the recipient's marrow was significantly higher in $Tet2^{gt/gt}$ group (0.85 +/− 0.16%) compared to WT (0.49 +/− 0.18%) (Figure 3B). $Tet2^{+/gt}$ $CD34^-$LSK cells again showed intermediate expansion (0.74 +/− 0.11%), the difference of which was not statistically significant against WT or $Tet2^{gt/gt}$ cells. To investigate whether enhanced engraftment of *Tet2*-mutant FL cells was due to the increased number or enhanced long-term repopulating (LTR) and self-renewal capacity of FL-HSCs, we transplanted equal number of highly purified FL-HSCs ($CD34^+$LSK cells) from WT or $Tet2^{gt/gt}$ embryos with competitor cells and examined their engraftment in the recipients' PB (Suppl. Figure S4). Interestingly, $Tet2^{gt/gt}$ $CD34^+$LSK cells showed higher engraftment as compared to WT cells, indicating that LTR/ self-renewal capacity of FL-HSCs was enhanced by *Tet2*-loss.

Taken together, these results clearly indicate that LTR and self-renewal capacity of FL-HSCs is enhanced by disruption of *Tet2*, and they can expand in the BM microenvironment of transplanted recipients.

Disruption of *Tet2* impairs myeloid differentiation. In serial transplantation experiment, multilineage differentiation potential of *Tet2*-mutant FL cells was grossly maintained throughout transplants. However, close examination of peripheral blood (PB) by flow cytometry revealed some alteration of myeloid differentiation in *Tet2*-mutant cells. Interestingly, significant decrease of Gr-1^+CD11b^+ mature granulocytes was observed in both primary and secondary recipients of $Tet2^{gt/gt}$ cells (Figure 4A and B), while $F4/80^+CD11b^+$ monocytes were not affected (Figure 4A and C). Of note, we observed slight increase of mature B cells accompanied by slight decrease of mature T cells in $Tet2^{gt/gt}$ cells, whose significance must be substantiated by further investigation (Figure 4A). Taken together, these data suggest that disruption of *Tet2* not only affects HSC function, but also impose a significant defect on myeloid differentiation.

***Tet2* loss confers hematopoietic progenitor cells with resistance to differentiative stress.** Enhanced self-renewal capacity is often accompanied by resistance to differentiating stimuli. We tested this hypothesis by culturing FL cells from WT or *Tet2*-mutant embryos under a differentiative condition and examined their surface

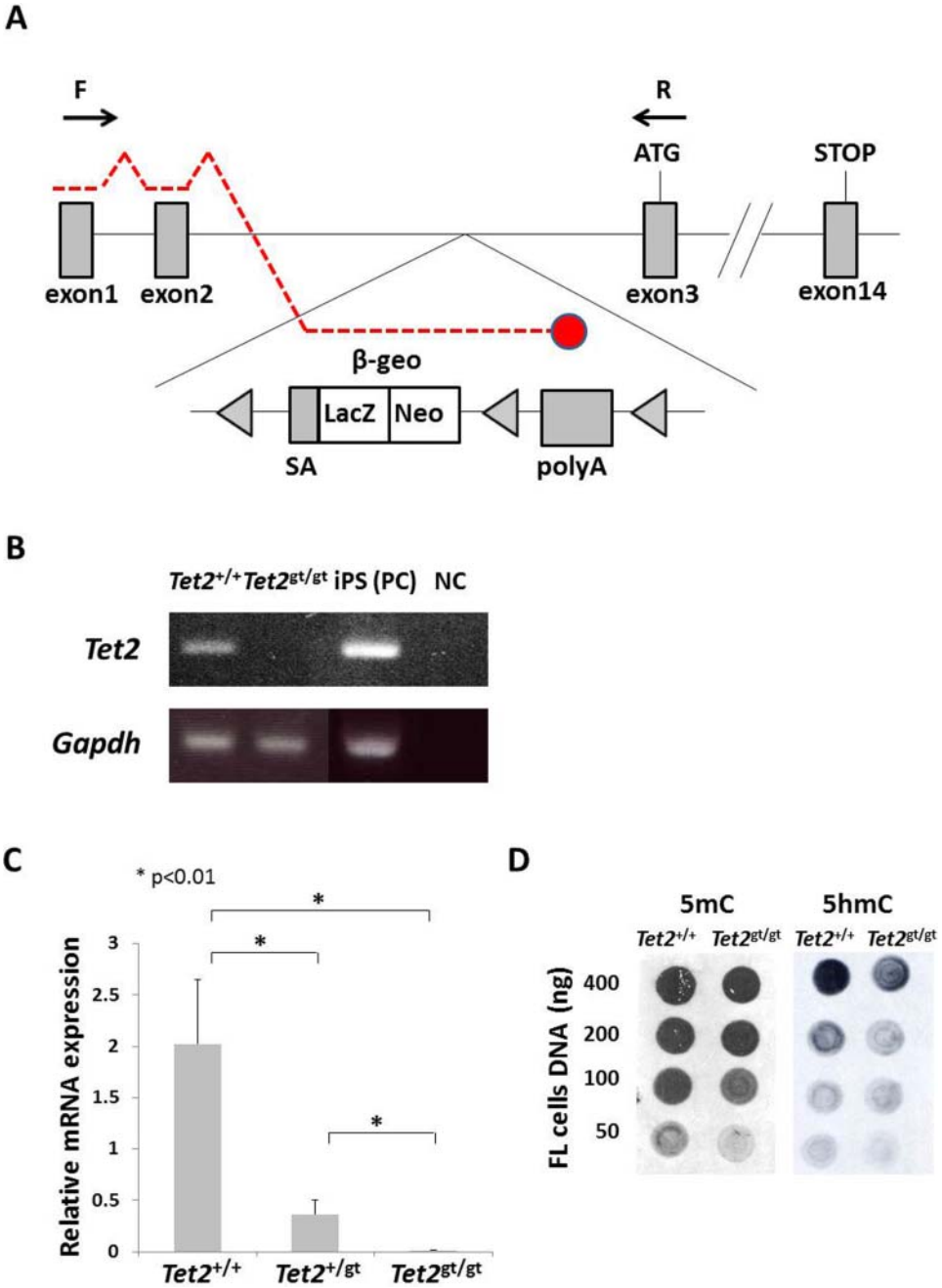

Figure 1 | Schematic illustration of *Tet2* gene-trap allele and validation of *Tet2* ablation. (A) Schematic illustration of *Tet2* gene-trap allele. SA; mouse En2 splicing acceptor site, β-geo; β-galactosidase/ neomycin-resistance fusion gene, LacZ; β-galactosidase, Neo; neomycin phosphotransferase, polyA; polyadenylation signal. Detailed structure and feature of gene-trap vector was described previously[26,31]. Arrows indicate the primers used for RT-PCR. Red broken lines are mRNA transcribed from the endogenous promoter. Red circle shows that mRNA is terminated by poly A signal in the trap cassette. (B) Efficiency of *Tet2* mRNA knockdown by RT-PCR. RT-PCR was performed as described in Methods using cDNAs derived from $Tet2^{+/+}$ and $Tet2^{gt/gt}$ fetal liver cells. PCR was run for 35 cycles. The positions of forward and reverse primers are shown in Figure 1A. cDNA from iPS cells was used for positive control. The electrophoretic gels for Gapdh are cropped. Uncropped images of the full-length gels are presented in Supplementary Figure 8. PC; positive control, NC; negative control. (C) Efficiency of *Tet2* mRNA knockdown by quantitative RT-PCR. qRT-PCR was performed as described in Methods using cDNAs derived from $Tet2^{+/+}$, $Tet2^{+/gt}$ and $Tet2^{gt/gt}$ fetal liver cells. The positions of forward and reverse primers are shown in figure 1A. Expression was normalized to the expression level of *Gapdh* in each fetal liver cells. The data represents the mean ± standard deviation (S.D.) (n=3 for each genotype). (D) Quantification of 5 mC and 5 hmC levels in DNA from FL cells by dot blot assay. Genomic DNA was spotted onto the membrane at the amount indicated on the left. DNA extraction and immunoblot were performed as described in Methods.

phenotype by flow cytometry (Suppl. Figure S5A). The rate of proliferation was comparable between each genotypes (Suppl. Figure S5B). As shown in Figure 5, $Tet2^{gt/gt}$ FL cells contained higher fraction of immature cells such as LSK (Figure 5A and B), Lin^- and c-Kit^+ cells (Figure 5A and C) as compared to WT after a liquid culture for 7-days with cocktails of cytokines. Surprisingly, percentage and absolute number of LSK fraction of $Tet2^{gt/gt}$ cells did not drop after 7-days of culture, while the latter of $Tet2^{+/+}$ and $Tet2^{+/gt}$ cells decreased approximately by half (Figure 5B). These results strongly suggest that *Tet2* loss confers immature hematopoietic cells in FL with resistance to differentiation in *in vitro* culture condition.

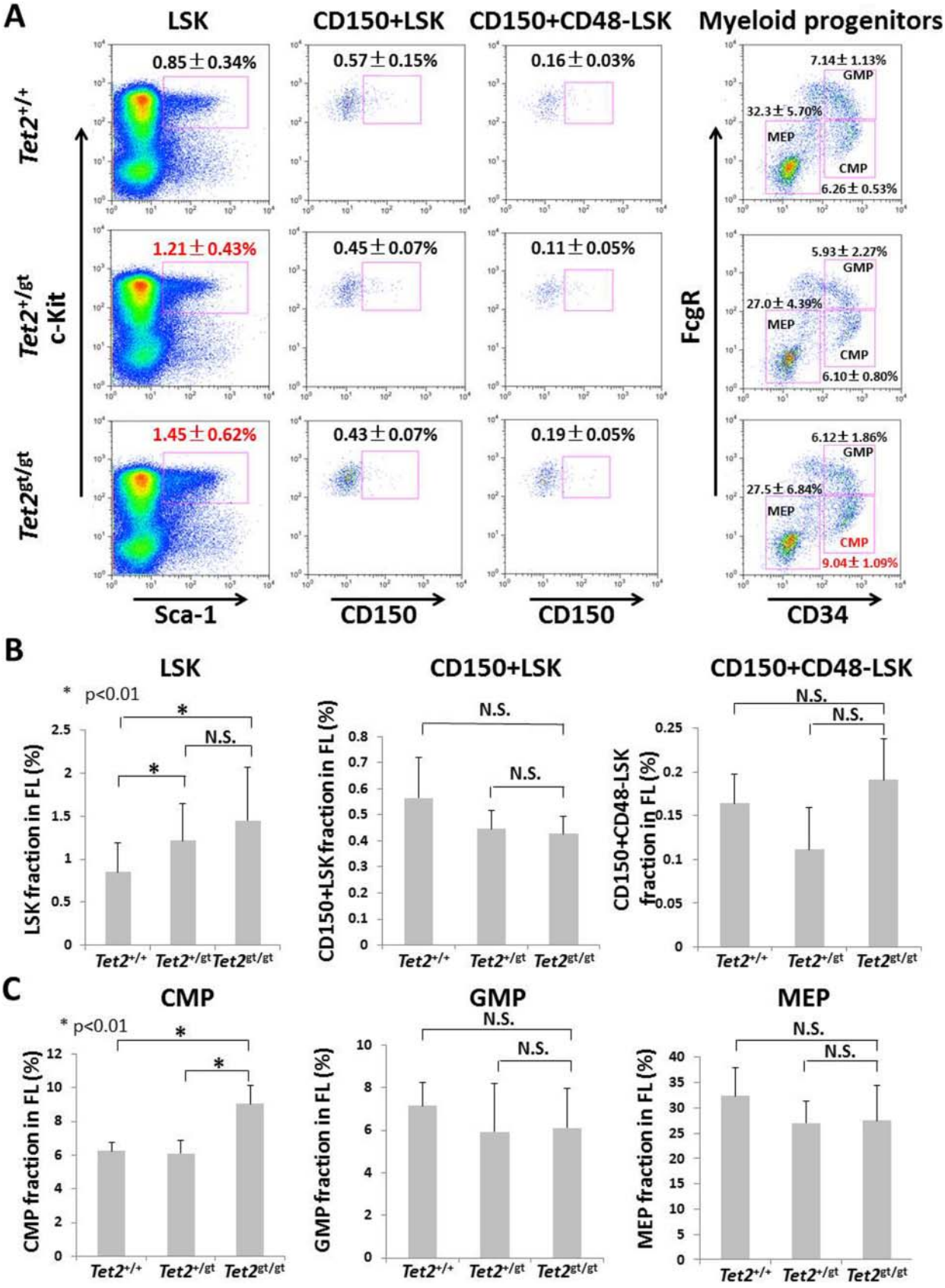

Figure 2 | Characterization of HSC, HPC and myeloid progenitor fractions in fetal livers of *Tet2* gene-trap mice. (A) Flow cytometric analysis of HSC/HPC fractions (LSK, $CD150^+$LSK, $CD150^+CD48^-$LSK) and myeloid progenitor fractions (CMP, GMP, MEP) in FLs was performed as described in Methods. Representative figures for each genotype are shown. The data represents the mean ± S.D. for each fraction (LSK; $Tet2^{+/+}$: n=14, $Tet2^{+/gt}$: n=23, $Tet2^{gt/gt}$: n=11, $CD150^+$LSK, $CD150^+CD48^-$LSK, and myeloid progenitors; n=3 for each genotype). (B and C) Percentages of each HSC/HPC fractions (B) or each myeloid progenitor fractions (C) within whole FL cells. The data represents the mean ± S.D. for each fraction (LSK; $Tet2^{+/+}$: n=14, $Tet2^{+/gt}$: n=23, $Tet2^{gt/gt}$: n=11, $CD150^+$LSK, $CD150^+CD48^-$LSK, and myeloid progenitors; n=3 for each genotype).

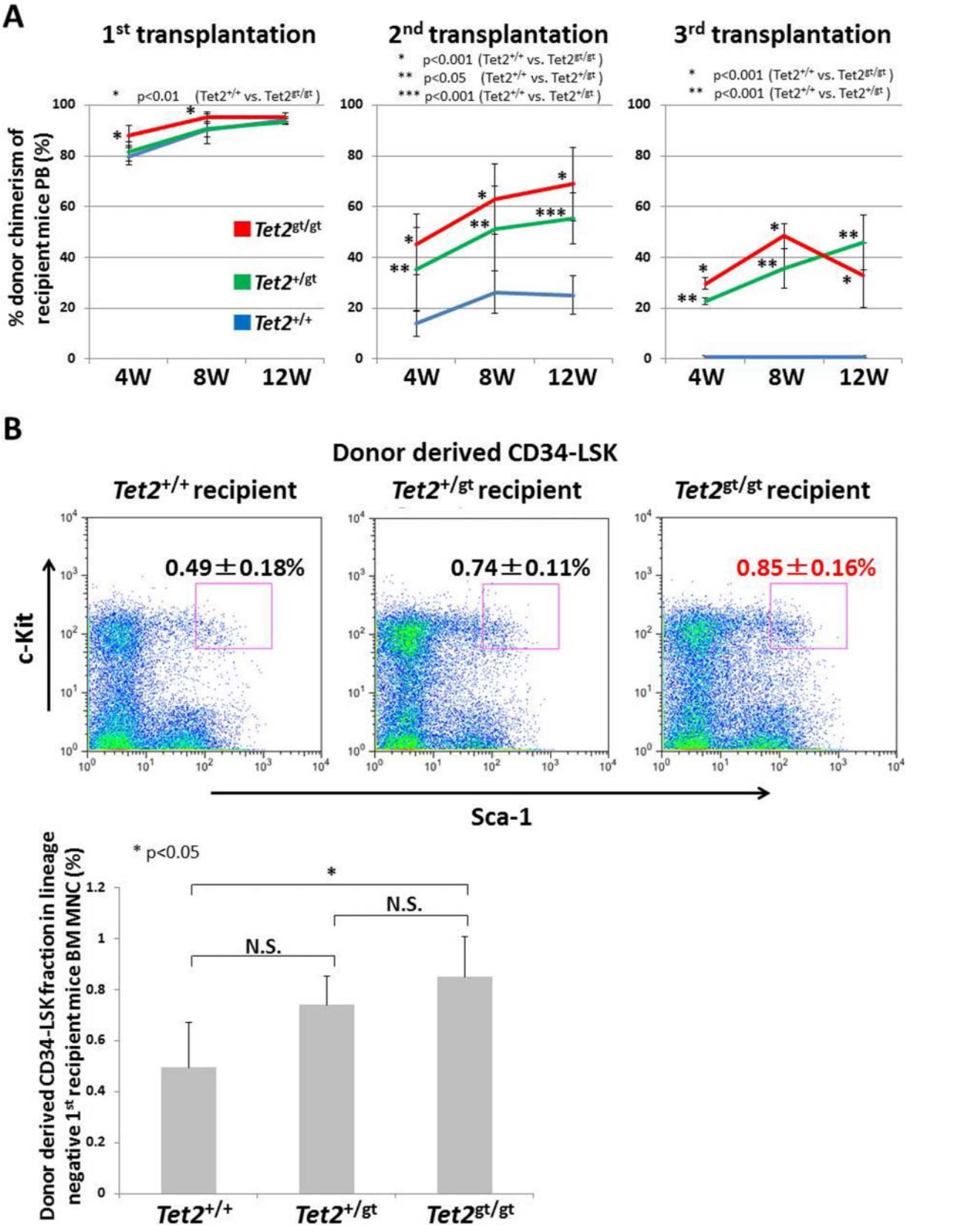

Figure 3 | Serial transplantation of *Tet2*-mutant fetal liver cells. (A) WT and *Tet2*-mutant FL cells (Ly5.2) were transplanted into lethally irradiated recipients (Ly5.1), and percentages of donor chimerism in recipient's PB were analyzed by flow cytometry at the indicated time points after transplantation. Serial transplantations were performed as described in Methods. The data represents the mean ± S.D. (1st transplantation; $Tet2^{+/+}$: n=4, $Tet2^{+/gt}$ and $Tet2^{gt/gt}$: n=5, 2nd and 3rd transplantation; $Tet2^{+/gt}$: n=4, $Tet2^{+/+}$ and $Tet2^{gt/gt}$: n=5). (B) (Upper panel) Flow cytometric analysis of donor derived HSC fraction ($CD34^{-}LSK$ cells) within Lin^{-} fraction of BM mononuclear cells in mice receiving the first transplants. Representative FACS pictures are shown. (Lower panel) Percentages of donor derived $CD34^{-}LSK$ cells within Lin^{-} fraction of BM mononuclear cells in mice receiving the first transplants. The data represents mean ± S.D. (n=3 for each recipient).

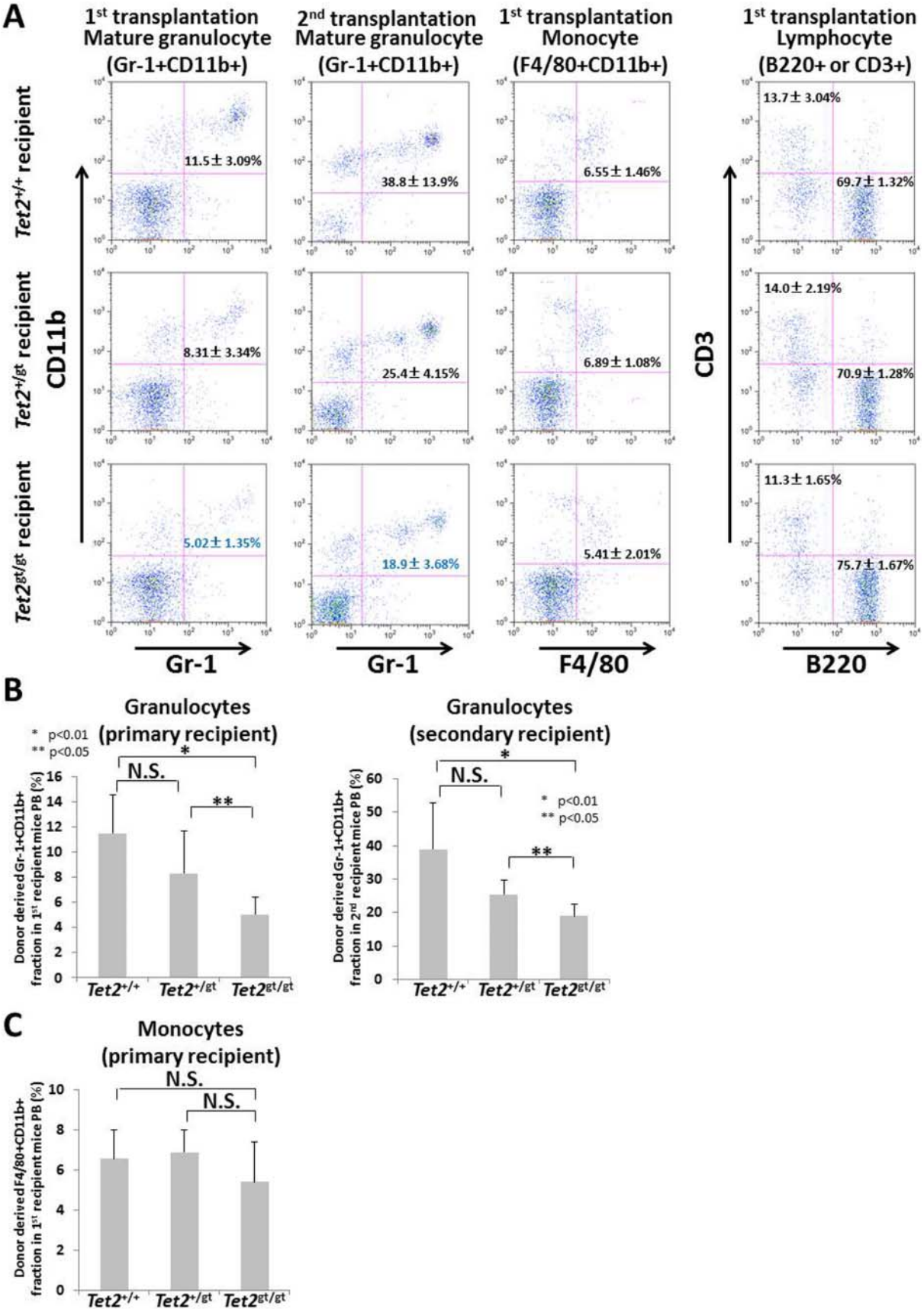

Figure 4 | Differentiation of *Tet2*-mutant FL cells in transplanted recipient mice. Fractions of mature granulocytes (Gr-1$^+$CD11b$^+$), monocytes (F4/80$^+$CD11b$^+$) and lymphocytes (B220$^+$ or CD3$^+$) in mice transplanted with WT or *Tet2*-mutant FL cells were analyzed at 12 weeks after the 1st or 2nd transplantation by flow cytometry. The data represents the mean ± S.D. (1st transplantation; *Tet2*$^{+/+}$ recipient: n=4, *Tet2*$^{+/gt}$ and *Tet2*$^{gt/gt}$ recipient : n=5, 2nd transplantation; *Tet2*$^{+/gt}$ recipient : n=4, *Tet2*$^{+/+}$ and *Tet2*$^{gt/gt}$ recipient : n=5). (A) Representative FACS pictures are shown. (B, C, D) Percentages of granulocytes (B), monocytes (C), and B or T cells (D) are shown on the graphs. The data are mean ± S.D.

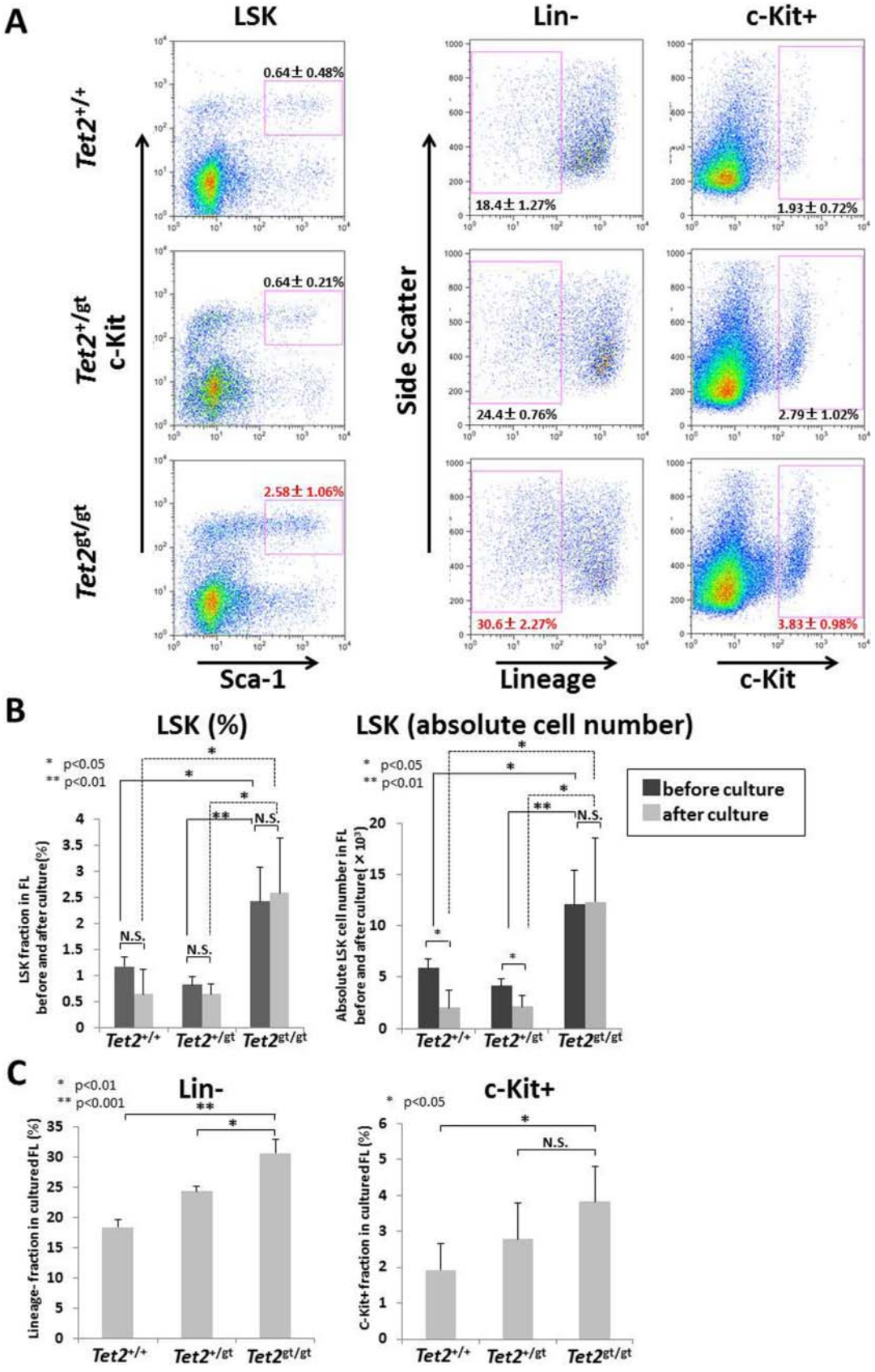

Figure 5 | ***Tet2*-mutant FL cells are resistant to differentiative stress in liquid culture.** WT or *Tet2*-mutant FL cells were cultured for 7-days with various cytokines (rmSCF, rmIL-6, rhFLT3L, rhTPO, and rmIL-3), and LSK, Lin^- and c-Kit^+ cells were analyzed by flow cytometry as described in the Methods. (A) Representative FACS pictures of the cells after the cultures are shown. The data are mean ± S.D. (n=3 for each genotype). (B) Percentages and absolute cell numbers of LSK cells before and after liquid culture for 7-days with various cytokines (rmSCF, rmIL-6, rhFLT3L, rhTPO, and rmIL-3). The data are mean ± S.D. (n=3 for each genotype). (C) Percentages of Lin^- or c-Kit^+ cells after liquid culture for 7-days. The data are mean ± S.D. (n=3 for each genotype).

Leukocytosis and HSC/ HPC expansion in adult heterozygous gene-trap mice. It was reported that myeloproliferation and extramedullary hematopoiesis occurred with age in *Tet2*$^{-/-}$ and *Tet2*$^{+/-}$ mice[23,24]. Since *Tet2*$^{gt/gt}$ mice did not survive to adulthood, we examined *Tet2*$^{+/gt}$ mice for evidence of myeloproliferation. Consistent with the previous reports, white blood cell (WBC) count in PB was significantly increased in *Tet2*$^{+/gt}$ mice at the age of 38-weeks (Suppl. Figure S6A). In addition, percentage of LSK fraction in *Tet2*$^{+/gt}$ BM was significantly higher compared to that of WT, while CD150^{+}LSK cells and myeloid progenitors were not statistically different between *Tet2*$^{+/gt}$ and WT (Suppl. Figure S6B and C). In contrast however, signs of extramedullary hematopoiesis such as splenomegaly or expansion of HPCs in spleen were not evident in *Tet2*$^{+/gt}$ mice, and they did not develop fatal myeloproliferative disorder during an observation over 40-weeks. It is also interesting to note that there was no sign of myeloproliferation such as increased WBC count or expansion of mature myeloid cells in the *Tet2*$^{gt/gt}$ FL cell recipients with more than 80% donor chimerism at least until 12-weeks after transplantation (Suppl. Figure S7). Taken together, these data indicate that extensive myeloproliferation is not a frequent phenomenon in *Tet2*$^{+/gt}$ mice or mice transplanted with *Tet2*$^{gt/gt}$ FL cells, and suggest that additional factors must cooperate with *Tet2* to develop myeloid transformation.

Discussion

Accumulating evidence suggests that altered regulation of cytosine hydroxymethylation is a critical pathogenic event in myeloid malignancies, such as MDS, MPN and AML. *TET* family proteins are reported to convert 5 mC to 5 hmC, and *TET2* mutations found in myeloid malignancies disrupt this enzymatic functions. Moreover, it was recently reported that *TET2* catalytic activity was inhibited by 2-hydroxyglutarate, an abnormal catalytic product generated by mutant *IDH1* or *IDH2* proteins that are frequently found in myeloid malignancies. These findings strongly suggest that dysregulation of 5 mC to 5 hmC conversion can be a critical step in myeloid transformation.

We showed that disruption of *Tet2* in FL led to increased self-renewal and LTR capacity of FL-HSCs. Furthermore, LSK fraction that mainly consists of MPPs clearly increased in *Tet2*$^{gt/gt}$ FL, whereas both percentages and numbers of highly enriched FL-HSC fractions (CD150^{+}LSK, CD150^{+}CD48^{-}LSK and CD34^{+}LSK cells) were not significantly different between WT and *Tet2*$^{gt/gt}$ mice. These findings are consistent with the ones reported recently for BM cells, showing increased *in vitro* serial replating capacity and competitive advantage of *Tet2*$^{-/-}$ HSCs over WT cells[23–25]. Taken together with our data, it is suggested that *Tet2* is critical for HSC/ HPC homeostasis in both FL and adult BM. It should be noted, however, that the previous studies have only examined competitive repopulating capacity of *Tet2*$^{-/-}$ HSCs in a single round of transplantation, and did not precisely addressed self-renewal capacity of HSCs by 'serial' transplantation. In contrast, we performed serial transplantation assays and showed that *Tet2*$^{gt/gt}$ FL-HSCs presented dramatically increased PB chimerism over WT cells in secondary and tertiary recipients. Furthermore, CD34^{-}LSK HSC fraction derived from *Tet2*$^{gt/gt}$ FL was significantly increased in the recipient's BM as compared to the one from WT, indicating that expansion of *Tet2*$^{gt/gt}$ HSCs is cell autonomous phenomenon and can occur in the BM microenvironment. Importantly, *Tet2*$^{gt/gt}$ cells showed only a mild impairment in myeloid differentiation. Taken together, these data clearly indicate that self-renewal capacity of FL-HSC is enhanced by inactivation of *Tet2* without major defects on multilineage differentiation capacity.

Enhanced self-renewal capacity of HSCs by *Tet2* inactivation is compatible with high incidence of *TET2* mutation in MDS. MDS is characterized by an expansion of self-renewing malignant clone, which ultimately overrides normal hematopoiesis in the BM. Loss-of–function mutation of *TET2* clearly endows HSCs with such fundamental feature of MDS, setting a molecular basis for acquiring additional mutations and disease progression. Since *Tet2* mutation causes only a mild impairment in myeloid differentiation, it seems that clonal evolution of MDS to overt leukemia must include a step acquiring mutation that blocks differentiation.

We have also shown that CMP fraction, but not GMP and MEP, was significantly increased to 9.04 +/− 1.09% in *Tet2*$^{gt/gt}$ FL, as compared to 6.26 +/− 0.53% in WT. Two recent studies have described increased percentage of CMP and GMP, or increased absolute number (but not percentage) of CMP and MEP in the *Tet2*$^{-/-}$ BM[23–25]. Despite some differences in amplifying cell types, CMP amplification is commonly observed either in FL or in the BM. This is in fact consistent with high incidence of TET2 mutation in human myeloid tumors such as chronic myelomonocytic leukemia (CMML), which is characterized by extensive myeloproliferation and myelodysplasia. Recent studies actually reported extramedullary hematopoiesis and the following myeloid transformation in *Tet2*$^{-/-}$ mice that was reminiscent of human CMML[23–25]. They showed peripheral leukocytosis and splenomegaly with proliferation of myeloid elements occurring in aged mice. Although we could not examine adult *Tet2*$^{gt/gt}$ mice due to their perinatal lethality, analysis of *Tet2*$^{+/gt}$ mice over 30-weeks of age showed a significant increase of WBC counts in PB. However, in contrast to their results, expansion of HSCs/ HPCs and myeloid cells in spleen could not be documented in *Tet2*$^{+/gt}$ mice. Phenotypic discrepancies between the studies also exist in differentiation of myeloid cells in PB. We observed impaired differentiation of *Tet2*$^{gt/gt}$ FL cells to Gr-1^{+}CD11b^{+} mature granulocytes in the transplanted recipients (Figure 4), whereas previous studies have shown the increase of mature granulocytes in PB of *Tet2*$^{-/-}$ mice[23–25]. On the other hand, shRNA-mediated knockdown of *Tet2* or introduction of mutant IDH2 into murine BM cells resulted in decreased differentiation to granulocytes[14], which is consistent with our data. These data suggest that the effect of *Tet2* loss on myeloid differentiation can be affected by various experimental factors including the strategies for *Tet2* targeting, the cell source (BM vs. FL) and levels of *Tet2* expression. It is clear that disruption of *Tet2* critically affects early and late stages of myeloid differentiation, however, revealing the precise molecular mechanism of myeloid regulation by *Tet2* awaits future investigations.

Epigenetic modification is a fundamental process for stem cells to maintain pluripotency and capacity to self-renew. Although *TET* family protein is a major player in this process, the way in which they regulate self-renewal seems different between cell types or among family members. *Tet1* is essential for self-renewal and maintenance of ES cells (ESCs) as shown by shRNA-mediated knockdown studies, and therefore it has a 'positive' regulatory role in this process[8]. Interestingly however, *Tet2* is clearly a 'negative' regulator for self-renewal of HSCs as revealed by the present study and others. Therefore, while both *Tet1* and *Tet2* are critical for stemness, they work in opposite manner in regulating self-renewal. This may be the reflection of different cellular environment (such as epigenetic status) between ESCs and HSCs, or due to the different inherent function of *Tet1* and *Tet2*. Revealing the molecular targets of these genes is absolutely essential for answering these questions.

In summary, we showed that *Tet2* inactivation in FL resulted in enhanced LTR and self-renewal capacity of FL-HSCs and altered differentiation in myeloid lineage. Current data indicate that conversion of 5 mC to 5 hmC as well as 5-formylcytosine and 5-carboxylcytosine[27] is a key enzymatic function of *Tet2* in HSC regulation. However, there still might be unknown features of *Tet2* that are essential for these processes. Moreover, recent study suggests that the role of TET proteins is not a mere transcriptional de-repressor, but they fine-tunes transcription either positively or negatively, acting as global regulators of transcription[28]. Further studies are definitely required to elucidate precise molecular mechanism by which *TET2* regulates HSC stemness and hematopoietic differentiation.

Methods

Mice. C57BL/6 (B6) mice were from Japan CLEA Inc. (Tokyo, Japan), and B6-Ly5.1 mice were from Sankyo Lab Service Co. (Tsukuba, Japan). *Tet2* gene trap mice were described previously[26]. All mice were housed and maintained under specific pathogen-free (SPF) condition. E14.5 embryos were used in all FL experiments. All animal experiments were reviewed and approved by the Internal Review Board of Keio University School of Medicine.

RT-PCR. Total RNA was extracted from FL cells using a TRIZOL Reagent (Invitrogen) according to the manufacturer's protocol. RNA was treated with RNase-free DNase I (Invitrogen) to remove contaminating genomic DNA. cDNA was reverse-transcribed using SuperScript II reverse transcriptase (Invitrogen). The quantity of cDNA was normalized according to the expression of GAPDH measured by real-time RT-PCR using a THUNDERBIRD SYBRqPCR Mix kit (TOYOBO) and StepOnePlus™ real-time PCR system (Applied Biosystems). Real-time PCR was peformed according to the manufacturer's protocol. Semi-quantitative RT-PCR was performed using Ex Taq–HS polymerase (TaKaRa Bio) as described previously[29].

Dot blot assay. Genomic DNA was extracted from FL cells using a DNeasy Blood & Tissue Kit (QIAGEN) according to the manufacturer's protocol. DNA amount was measured using SmartSpec 3000 (BIO-RAD). DNA was manually spotted onto PROTRAN BA85 nitrocellulose membranes (Schleicher & Schuell). Membranes were first probed with anti-5-methylcytidine antibody (Eurogentec) or anti-5-hydroxymethylcytidine antibody (Active Motif), which were then probed with horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin (Ig) polyclonal antibody. Bound antibodies were visualized by enhanced chemiluminescence (ECL; Amersham).

Analysis of fetal liver cells by flow cytometry. For the analysis of hematopoietic stem/progenitor cells and myeloid progenitor cells in FLs, FLs were dissected from E14.5 embryos and single cell suspension was made in phosphate-buffered saline (PBS). After lysing red blood cells in ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA) at 37°C for 5 minutes, cells were spun and suspended in PBS supplemented with 5% fetal bovine serum (FBS). For HSC/ HPC (LSK, $CD150^+$LSK, $CD150^+CD48^-$LSK) analysis, cells were stained with biotin anti-B220 (RAE-6B2, e-Bioscience), biotin anti-CD19 (6D5, BioLegend), biotin anti-Ter119 (TER-119, e-Bioscience), biotin anti-Gr-1 (RB6-8C5, BioLegend), phycoerythrin (PE)-Sca-1 (D7, e-Bioscience), allophycocyanin (APC)-c-Kit (2B8, e-Bioscience), Alexa488-CD150 (TC15-12F12.2, BioLegend), and biotin anti-CD48 (HM48-1, BioLegend), followed by staining with streptavidin-PE-Cy7 (BioLegend). For the analysis of myeloid progenitor cells (CMP, GMP, MEP), cells were stained with fluorescein isothyocyanate (FITC)-CD34 (RAM34, BD Pharmingen), PE-FcγRII/III (2.4G2, BD Pharmingen), APC-c-Kit, biotin anti-B220, biotin anti-CD19, biotin anti-Ter119, biotin anti-Gr-1, biotin anti-IL7Rα (A7R34, e-Bioscience) and biotin anti-Sca-1 (D7, BioLegend), followed by staining with streptavidin-PE-Cy7. Stained cells were analyzed by MoFlo (Beckman Coulter) or FACS Calibur (BD Bioscience).

Analysis of donor-derived mature blood cells and BM HSCs after transplantation. Percentages of donor chimerism together with differentiation to multiple lineages in recipient's PB were analyzed by flow cytometry at 4, 8, and 12 weeks after transplantation. After lysis of red blood cells, total white blood cells were stained with peridinin-chlorophyll proteins-cyanin 5.5 (PerCp-Cy5.5)-CD45.2 (104, BioLegend), or combination of the following monoclonal antibodies: FITC-Gr-1 (RB6-8C5, BD Pharmingen), PE-CD11b (M1/70, BD Pharmingen), FITC-F4/80 (BM8, BioLegend), FITC-B220 (RA3-6B2, BioLegend), and PE-CD3 (145-2C11, e-Bioscience). For analyzing donor-derived HSCs in the BM, cells were collected from bilateral femurs and tibias of the recipient mice 20 weeks after transplantation. Mononuclear cells were separated by density-gradient centrifugation using Lymphoprep (Axis-Shield Poc AS), and lineage-positive cells were depleted using Lineage Cell Depletion Kit (Miltenyi Biotec) according to the manufacturer's protocol. $CD34^-$LSK cells were analyzed as described previously[30]. Briefly, lineage-negative cells were stained with FITC-CD34 (RAM34, BD Pharmingen), APC-Cy7-CD45.1 (A20, BioLegend), PE-Sca-1 (D7, e-Bioscience), APC-c-Kit (2B8, e-Bioscience), and an anti-lineage antibody cocktail in the Lineage Cell Depletion Kit, followed by staining with streptavidin-PE-Cy7. Cells were analyzed by FACS Calibur and MoFlow cytometer.

Serial transplantation assay. FL cells were separated from E14.5 embryos (Ly5.2) and suspended in IMDM supplemented with 10% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 50 μg/ml gentamicin and 2 mM L-glutamine. Competitor cells were collected from the BM of 7-week-old Ly5.1 mice. 1×10^6 whole fetal liver cells (Ly5.2) with 2×10^5 competitor BM cells (Ly5.1) were intravenously injected into lethally irradiated recipient mice (Ly5.1) through tail veins. For secondary or tertiary transplantation, 2×10^6 whole BM cells taken from the first or secondary recipient mice 12 weeks after transplantation were transplanted into lethally irradiated recipient mice (Ly5.1).

In vitro liquid culture assay. Whole FL cells were collected from E14.5 embryos and suspended in IMDM supplemented with 15% FBS, 100 U/ml penicillin G, 100 μg/ml streptomycin, 50 μg/ml gentamicin and 2 mM L-glutamine and cytokines (rmSCF 50 ng/mL, rmIL-6 50 ng/mL, rhFLT3L 50 ng/mL, rhTPO 50 ng/mL, rmIL-3 20 ng/mL). Cells were cultured in humidified atmosphere with 5% CO_2 at 37°C, and split on day 2, 4, and 6 to keep cell density between 5 x10^5/ ml and 1 x10^6/ ml. Cell numbers were enumerated on day 2, 4, and 6. Cells were collected on day 7 for the analysis of LSK, lineage negative, and c-Kit positive cells.

Statistical analysis. All statistical analyses were performed using unpaired Student's t-test. P values$<$0.05 were considered statistically significant.

1. Corey, S. J. *et al.* Myelodysplastic syndromes: the complexity of stem-cell diseases. *Nat Rev Cancer* **7**, 118–129 (2007).
2. Delhommeau, F. *et al.* Mutation in TET2 in myeloid cancers. *N Engl J Med* **360**, 2289–2301 (2009).
3. Langemeijer, S. M. *et al.* Acquired mutations in TET2 are common in myelodysplastic syndromes. *Nat Genet* **41**, 838–842 (2009).
4. Abdel-Wahab, O. *et al.* Genetic characterization of TET1, TET2, and TET3 alterations in myeloid malignancies. *Blood* **114**, 144–147 (2009).
5. Jankowska, A. M. *et al.* Loss of heterozygosity 4q24 and TET2 mutations associated with myelodysplastic/myeloproliferative neoplasms. *Blood* **113**, 6403–6410 (2009).
6. Figueroa, M. E. *et al.* DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. *Cancer Cell* **17**, 13–27 (2010).
7. Tahiliani, M. *et al.* Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* **324**, 930–935 (2009).
8. Ito, S. *et al.* Role of Tet proteins in 5 mC to 5 hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature* **466**, 1129–1133 (2010).
9. Ko, M. *et al.* Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2. *Nature* **468**, 839–843 (2010).
10. Koh, K. P. *et al.* Tet1 and Tet2 regulate 5-hydroxymethylcytosine production and cell lineage specification in mouse embryonic stem cells. *Cell Stem Cell* **8**, 200–213 (2011).
11. Mardis, E. R. *et al.* Recurring mutations found by sequencing an acute myeloid leukemia genome. *N Engl J Med* **361**, 1058–1066 (2009).
12. Parsons, D. W. *et al.* An integrated genomic analysis of human glioblastoma multiforme. *Science* **321**, 1807–1812 (2008).
13. Ward, P. S. *et al.* The common feature of leukemia-associated IDH1 and IDH2 mutations is a neomorphic enzyme activity converting alpha-ketoglutarate to 2-hydroxyglutarate. *Cancer Cell* **17**, 225–234 (2010).
14. Figueroa, M. E. *et al.* Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell* **18**, 553–567 (2010).
15. Xu, W. *et al.* Oncometabolite 2-hydroxyglutarate is a competitive inhibitor of alpha-ketoglutarate-dependent dioxygenases. *Cancer Cell* **19**, 17–30 (2011).
16. Kim, I., He, S., Yilmaz, O. H., Kiel, M. J. & Morrison, S. J. Enhanced purification of fetal liver hematopoietic stem cells using SLAM family receptors. *Blood* **108**, 737–744 (2006).
17. Matsubara, A. *et al.* Endomucin, a CD34-like sialomucin, marks hematopoietic stem cells throughout development. *J Exp Med* **202**, 1483–1492 (2005).
18. Morrison, S. J., Hemmati, H. D., Wandycz, A. M. & Weissman, I. L. The purification and characterization of fetal liver hematopoietic stem cells. *Proc Natl Acad Sci U S A* **92**, 10302–10306 (1995).
19. Harrison, D. E., Zhong, R. K., Jordan, C. T., Lemischka, I. R. & Astle, C. M. Relative to adult marrow, fetal liver repopulates nearly five times more effectively long-term than short-term. *Exp Hematol* **25**, 293–297 (1997).
20. Park, I. K. *et al.* Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. *Nature* **423**, 302–305 (2003).
21. Kajiume, T., Ninomiya, Y., Ishihara, H., Kanno, R. & Kanno, M. Polycomb group gene mel-18 modulates the self-renewal activity and cell cycle status of hematopoietic stem cells. *Exp Hematol* **32**, 571–578 (2004).
22. Ohta, H. *et al.* Polycomb group gene rae28 is required for sustaining activity of hematopoietic stem cells. *J Exp Med* **195**, 759–770 (2002).
23. Moran-Crusio, K. *et al.* Tet2 Loss Leads to Increased Hematopoietic Stem Cell Self-Renewal and Myeloid Transformation. *Cancer Cell* **20**, 11–24 (2011).
24. Quivoron, C. *et al.* TET2 Inactivation Results in Pleiotropic Hematopoietic Abnormalities in Mouse and Is a Recurrent Event during Human Lymphomagenesis. *Cancer Cell* **20**, 25–38 (2011).
25. Li, Z. *et al.* Deletion of Tet2 in mice leads to dysregulated hematopoietic stem cells and subsequent development of myeloid malignancies. *Blood*, in press.
26. Tang, H., Araki, K., Li, Z. & Yamamura, K. Characterization of Ayu17-449 gene expression and resultant kidney pathology in a knockout mouse model. *Transgenic Res* **17**, 599–608 (2008).
27. Ito, S. *et al.* Tet Proteins Can Convert 5-Methylcytosine to 5-Formylcytosine and 5-Carboxylcytosine. *Science* **333**, 1300–1303 (2011)
28. Williams, K. *et al.* TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. *Nature* **473**, 343–348.
29. Fukuchi, Y. *et al.* Comprehensive analysis of myeloid lineage conversion using mice expressing an inducible form of C/EBP alpha. *EMBO J* **25**, 3398–3410 (2006).
30. Shibata, F. *et al.* Roundabout 4 is expressed on hematopoietic stem cells and potentially involved in the niche-mediated regulation of the side population phenotype. *Stem Cells* **27**, 183–190 (2009).
31. Taniwaki, T. *et al.* Characterization of an exchangeable gene trap using pU-17 carrying a stop codon-beta geo cassette. *Dev Growth Differ* **47**, 163–172 (2005).

Acknowledgments

We thank excellent technical assistance by Akiko Ito and Junko Kawakita. We also thank S. Suzuki (FACS Core Laboratory, Keio University School of Medicine) for FACS sorting and K. Takubo (Department of Cell Differentiation, The Sakaguchi Laboratory of Developmental Biology, Keio University School of Medicine) for FACS sorting and valuable advice. This work was supported in part by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan and Keio University Special Grant-in-Aid for Innovative Collaborative Research Projects.

Authorship contributions

HK performed research, analyzed the data, and wrote a part of the paper. YF, MS and KS performed research. YI and SO supervised the study. HN designed research, analyzed the data, provided financial and administrative support, and wrote the paper.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: Yasuo Ikeda is a member of Board of Directors of Chugai Pharmaceutical Co., Ltd.

How to cite this article: Kunimoto, H. *et al*. *Tet2* disruption leads to enhanced self-renewal and altered differentiation of fetal liver hematopoietic stem cells. *Sci. Rep.* **2**, 273; DOI:10.1038/srep00273 (2012).