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

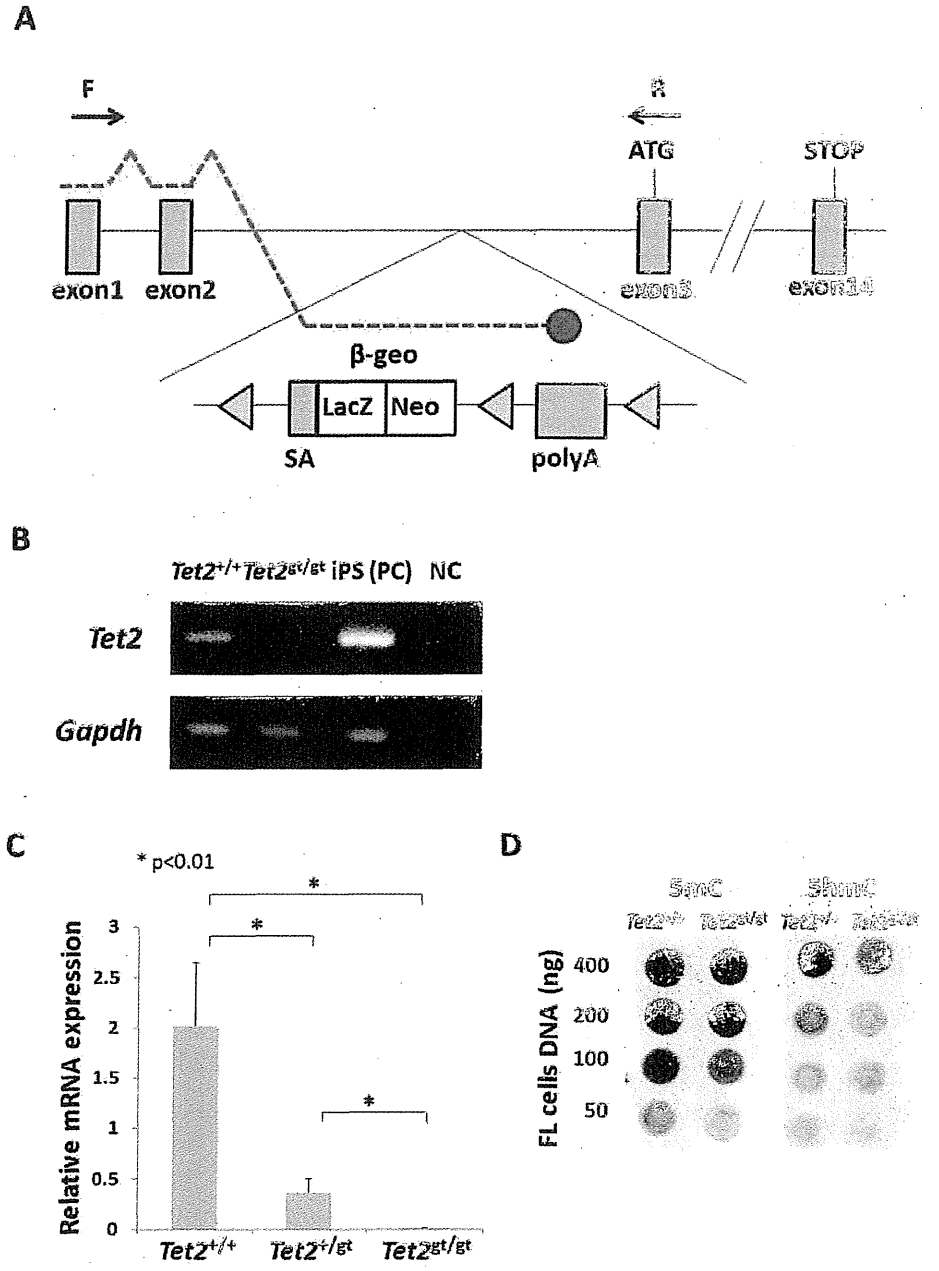

Figure 1 | Schematic illustration of *Tet2* gene-trap allele and validation of *Tet2* ablation. (A) Schematic illustration of *Tet2* gene-trap allele. SA; mouse En2 splicing acceptor site, β-geo; β-galactosidase/ neomycin-resistance fusion gene, LacZ; β-galactosidase, Neo; neomycin phosphotransferase, 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.

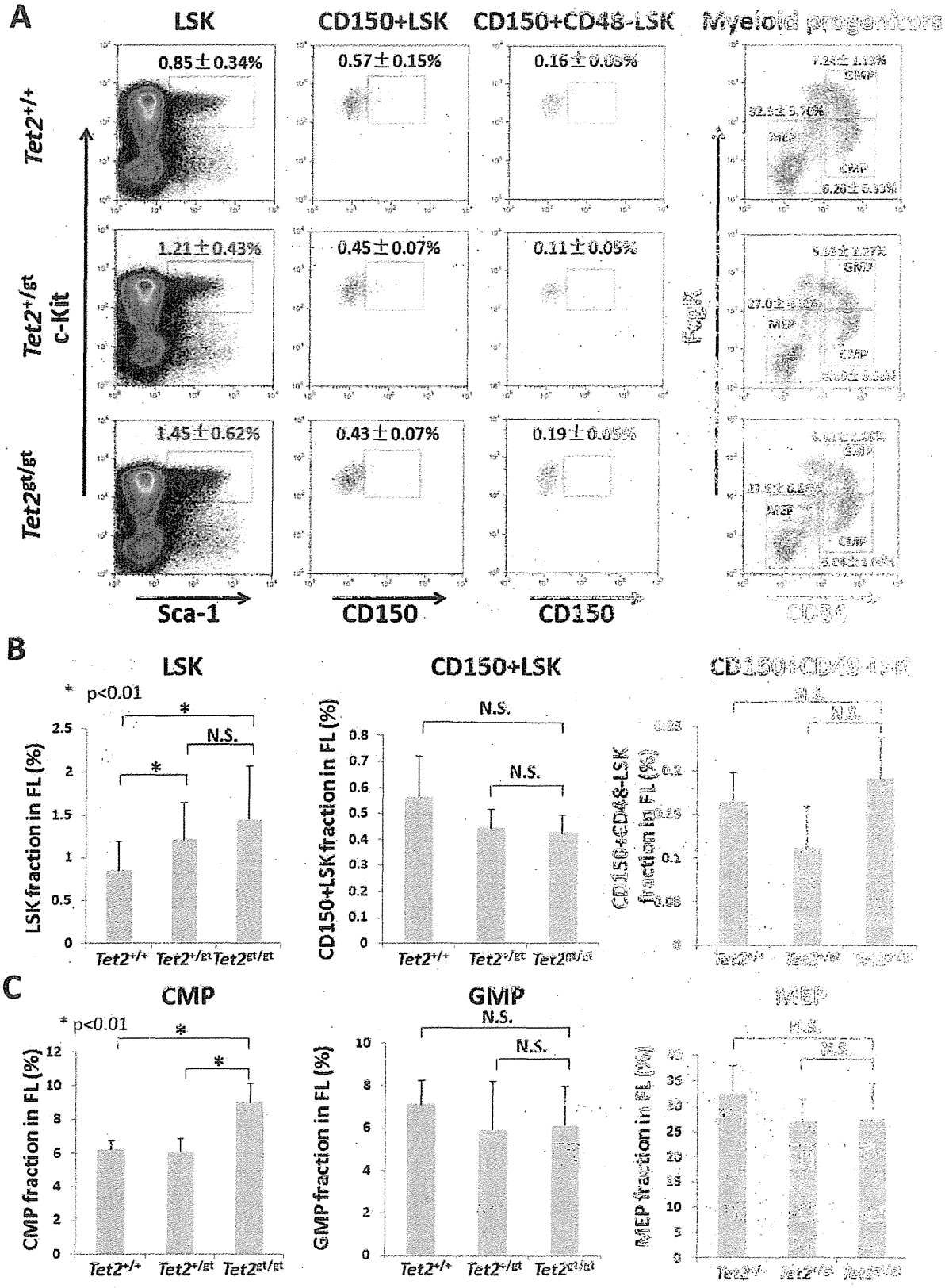

Figure 2 | Characterization of HSC, HPC and myeloid progenitor fractions in fetal livers of *Tet2* gene-trap mice. (A) Flow cytometric analysis of HSC/HPC fractions (LSK, $CD150^+LSK$, $CD150^+CD48^-LSK$) and myeloid progenitor fractions (CMP, GMP, MEP) in FLs was performed as described in Methods. Representative 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).

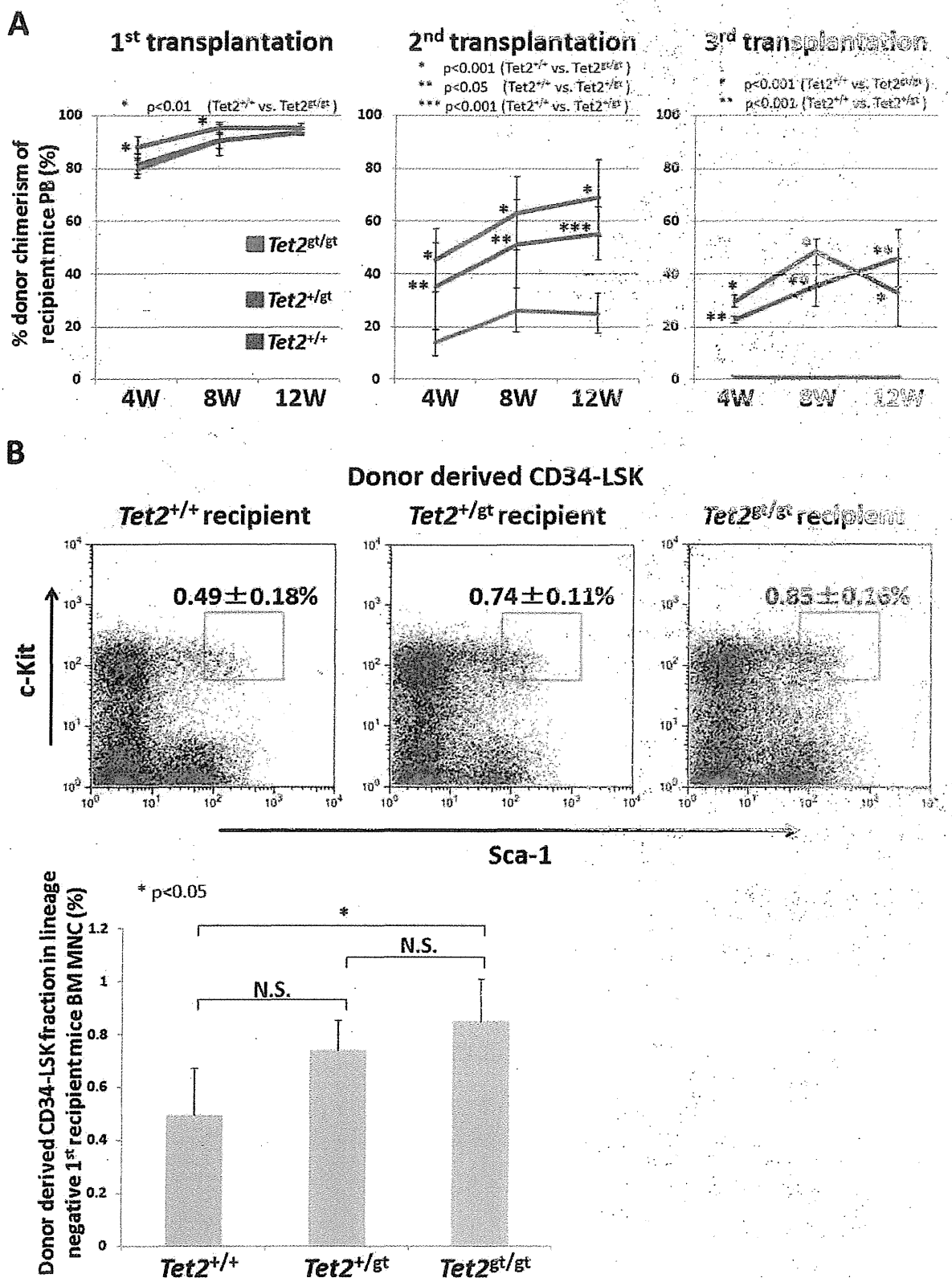

Figure 3 | Serial transplantation of *Tet2*-mutant fetal liver cells. (A) WT and *Tet2*-mutant FL cells (Ly5.2) were transplanted into lethally irradiated recipients (Ly5.1), and percentages of donor chimerism in recipient's PB were analyzed by flow cytometry at the indicated time points after transplantation. 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).

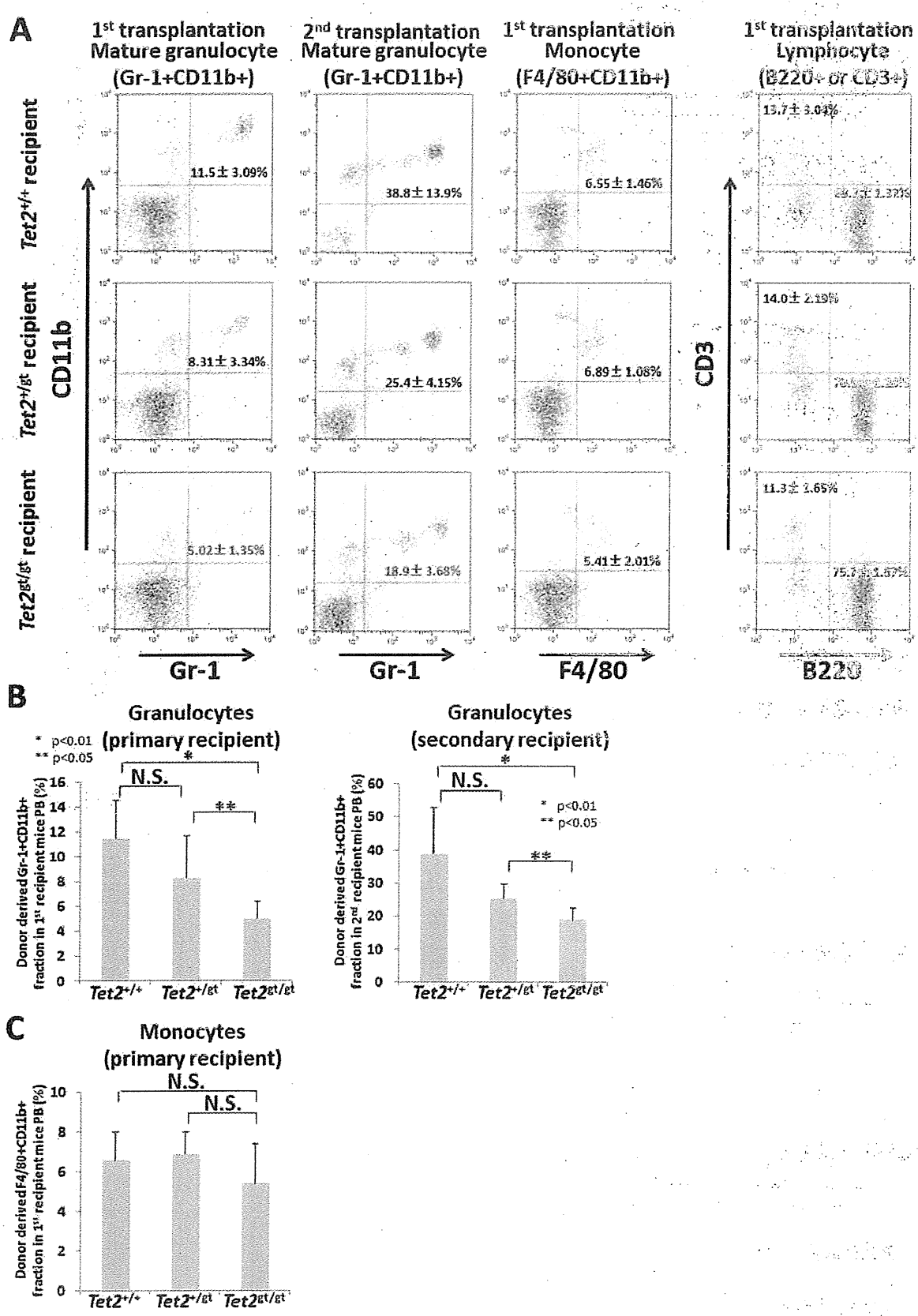

Figure 4 | Differentiation of *Tet2*-mutant FL cells in transplanted recipient mice. Fractions of mature granulocytes (Gr-1$^+$CD11b$^+$), monocytes (F4/80$^+$CD11b$^+$) and lymphocytes (B220$^+$ or CD3$^+$) in mice transplanted with WT or *Tet2*-mutant FL cells were analyzed at 12 weeks after the 1st 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.

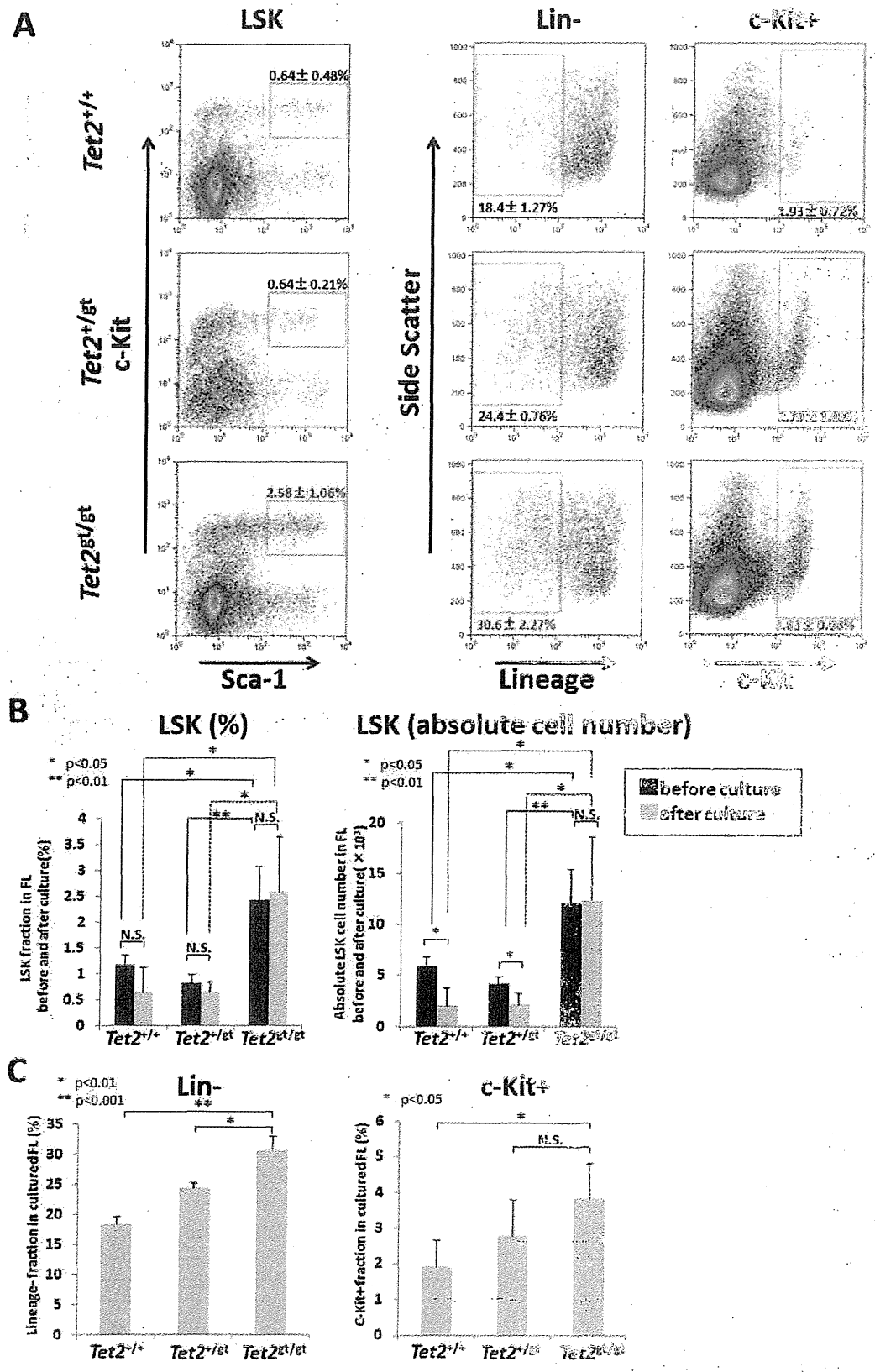

Figure 5 | *Tet2*-mutant FL cells are resistant to differentiative stress in liquid culture. WT or *Tet2*-mutant FL cells were cultured for 7-days with various cytokines (rmSCF, rmIL-6, rhFLT3L, rhTPO, and rmIL-3), and LSK, Lin$^-$ and c-Kit$^+$ cells were analyzed by flow cytometry as described in the 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.

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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

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bjh short report

Outcome of children with refractory anaemia with excess of blast (RAEB) and RAEB in Transformation (RAEB-T) in the Japanese MDS99 study

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Summary

We report the outcome of 16 children with refractory anaemia with excess of blasts (RAEB; $n = 4$) and RAEB in transformation (RAEB-T; $n = 12$) following induction therapy with etoposide, cytarabine and mitoxantrone (ECM) prior to haematopoietic stem cell transplantation (HSCT). The median observation period was 77 months (range 5–123). Complete remission rate was 81% following induction; no toxic deaths occurred. Eight-year event-free survival and overall survival was 50% and 56%, respectively. None of the three patients with a complex karyotype survived, suggesting karyotype is a crucial prognostic factor for survival. This study indicates the safety and high remission rate of ECM and high survival rates after HSCT for paediatric RAEB and RAEB-T.

Keywords: refractory anaemia with excess of blast, refractory anaemia with excess of blast in transformation, childhood, haematopoietic stem cell transplantation.

Myelodysplastic syndrome (MDS) is a rare haematological disorder in childhood, accounting for <5% of all paediatric haematopoietic neoplasias (Passmore *et al*, 2003; Niemeyer & Kratz, 2008) . In the French–American–British (FAB) Cooperative Group classification, refractory anaemia with excess blasts (RAEB) is defined by dysplastic morphology in two or more cell lineages with more than 5% blasts and RAEB in transformation (RAEB-T) is defined as RAEB with 20–29% blasts.

There is no standardized therapy for paediatric RAEB and RAEB-T. Conventional acute myeloid leukaemia (AML)-type chemotherapy without haematopoietic stem cell transplantation (HSCT) resulted in survival rates below 30% (Sasaki *et al*, 2001; Woods *et al*, 2002). Although the only curative treatment strategy is considered to be allogeneic HSCT, the therapeutic utility of AML-type remission induction therapy before HSCT remains unclear.

To evaluate safety and efficacy of AML-type remission induction therapy before allogeneic HSCT, we conducted the MDS99 study for paediatric RAEB and RAEB-T and report patient outcomes.

Patients and methods

Nineteen patients with paediatric RAEB and RAEB-T who were enrolled in the observational study MDS99 between October 1999 and June 2004 were treated according to the study protocol. Diagnosis was confirmed by central review of morphology performed by the MDS committee of the Japanese Society of Paediatric Haematology (JSPH) according to the FAB classification (Hasegawa *et al*, 2009). None of the patients had undergone previous chemotherapy or radiotherapy, or had a history of inherited bone marrow failure syndrome or aplastic anaemia. Remission induction therapy

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consisted of etoposide 150 mg/m^2 2-h infusion (days 1–5), cytarabine 200 mg/m^2 12-h infusion (days 6–12), mitoxantrone 5 mg/m^2 intravenous injection (days 6–10) and intrathecal injection of methotrexate, cytarabine and hydrocortisone (day 6). This ECM regimen was part of the previously reported Japanese AML treatment regimen (Tomizawa *et al*, 2007; Tsukimoto *et al*, 2009). Complete remission (CR) was defined morphologically as <5% blasts and recovery of normal haematopoiesis. After remission induction therapy, HSCT was performed without consolidation therapy. Conditioning regimens and stem cell sources were determined by each physician. All treatments were performed with informed consent from the patients' parents or guardians according to the Declaration of Helsinki. This study was approved by the Institutional Review Board of each participating institution. Survival curves were calculated by the Kaplan–Meier method.

Results

The characteristics of 19 study patients are shown in Tables I and II. Survival status was as of April 2011. In further analysis, we focused on 16 patients who underwent ECM regimen as remission induction therapy. Of these patients, 13 patients achieved complete remission and CR rate was estimated to be 81%. Median time between initiation of chemotherapy and remission evaluation (remission induction duration) was 41 d (range 29–155). Grade 3 or 4 non-haematological toxicities according to the National Cancer Institute-Common Toxicity Criteria were reported in nine patients (infection 4, allergic reaction 4 and mucositis 1), but no toxic death was observed. One patient [unique patient number (UPN) 19] who underwent another induction regimen did not achieve CR. Among 16 patients, one patient (UPN 8) who achieved CR after ECM regimen did not receive HSCT because of parental refusal and she died of primary disease (DOD). The other 15 patients underwent allogeneic HSCT (12 in CR and 3 in non-CR). Outcomes of all patients are shown in Figure S1. All preconditioning regimens were myeloablative [total body irradiation (TBI) regimen, $n = 15$; non-TBI, $n = 3$]. Cyclosporine or tacrolimus plus methotrexate were used for graft-*versus*-host- disease (GVHD) prophylaxis.

Table I. Details of patients with RAEB/RAEB-T in the MDS99 study.

Number of patients	19
Gender: male/female	10/9
Age at diagnosis (years): median (range)	6 (1–14)
Disease type: RAEB/RAEB-T	7/12
Remission induction: ECM/other/none	16/1/2
HSCT type	
MSD-BMT	2
MSD-PBSCT	1
MFD-BMT	2
UD-BMT	5
UD-CBT	8
Interval between diagnosis and HSCT (months): median (range)	5 (2–8)
Observation period in months: median (range)	77 (5–123)
Outcome: alive/dead	11/8

RAEB, refractory anaemia with excess blasts; RAEB-T, RAEB in transformation; HSCT, haematopoietic stem cell transplantation; ECM, etoposide, cytarabine, mitoxantrone; MSD, human leucocyte antigen (HLA)-matched sibling donor; BMT, bone marrow transplantation; PBSCT, peripheral blood stem cell transplantation; MFD, HLA-matched family donor; UD, unrelated donor; CBT, cord blood transplantation.

As for the 12 patients who underwent HSCT in CR after ECM regimen, eight patients achieved continuous CR (CCR), three suffered transplantation-related mortality (TRM) [invasive aspergillosis (IA), sepsis and interstitial pneumonitis (IP)] and one suffered DOD. One of the three patients who failed to attain CR after ECM regimen failed to engraft but achieved CCR after the second HSCT, whereas the other two succumbed to disease after HSCT.

Cytogenetic abnormalities were observed in 12 of 19 patients. The 19 patients were classified into four groups as follows; structurally complex karyotype [defined as more than or equal to three chromosomal aberrations in the presence of at least one structural aberration (Gohring *et al*, 2010); $n = 3$], monosomy 7 ($n = 2$), other abnormal karyotype ($n = 7$) and normal karyotype ($n = 7$). Two of the three patients with structurally complex karyotype achieved CR after ECM regimen but the other did not. All these patients died after HSCT (2 DOD, 1 TRM). One of the two patients with monosomy 7 DOD without HSCT due to parental refusal and the other remains in CCR after HSCT without remission induction therapy. Of seven patients with other abnormal karyotypes, six achieved CR with ECM regimen and are alive without disease after HSCT, whereas the other patient failed to achieve CR with ECM regimen and suffered DOD after HSCT. Of seven patients who showed normal karyotype and underwent HSCT, four remain CCR and three died (2 DOD, 1 TRM).

Acute GVHD II–IV was seen in 13 patients and chronic GVHD in 8. No influence of GVHD was observed on survival rate.

The 8-year event-free survival (EFS) and overall survival (OS) of 16 patients who underwent ECM regimen was 50 ± 13% and 56 ± 12%, respectively (Fig 1). No serious late effect was observed among long-term survivors other than secondary hypogonadism due to HSCT.

Discussion

This study aimed to evaluate the safety and efficacy of the ECM regimen, which is an AML-type remission induction therapy, before allogeneic HSCT for paediatric RAEB and RAEB-T. This regimen has been used for AML remission induction and its safety and efficacy have already been

Table II. Clinical characteristics of 19 patients in the MDS 99 study for RAEB/RAEB- T.

UPN	Age (years)	Diagnosis	Cytogenetics	Response to ECM	Interval between diagnosis and HSCT (months)	Preconditioning	HSCT type	aGVHD (Grade)	cGVHD	Outcome	Survival (months)
1	13	RAEB-T	Other	CR	6	TBI	UD-CBT	II	No	CCR	106+
2	4	RAEB-T	Complex	Non-CR	4	Non-TBI	UD-CBT	II	No	DOD	11
3	10	RAEB-T	Other	CR	5	TBI	UD-CBT	III	No	CCR	119+
4	10	RAEB	Other	CR	3	TBI	UD-CBT	III	Yes	CCR	123+
5	10	RAEB	Normal	Non-CR	5	TBI	UD-BMT	II	No	DOD	10
6	6	RAEB-T	Normal	CR	5	TBI	UD-CBT	II	No	TRM (sepsis)	8
7	4	RAEB-T	Normal	CR	3	TBI	MFD-BMT	II	Yes	CCR	77+
8	11	RAEB-T	Monosomy 7	CR	NA	NA	NA	NA	NA	DOD	5
9	11	RAEB-T	Other	CR	4	TBI	MFD-BMT	III	Yes	CCR	106+
10	1	RAEB-T	Complex	CR	8	TBI	UD-CBT	II	No	TRM (IP)	11
11	3	RAEB-T	Normal	Non-CR	2	TBI	UD-CBT	I	Yes	CCR	107+
12	3	RAEB	Normal	CR	5	Non-TBI	MSD-BMT	I	Yes	CCR	107+
13	4	RAEB	Normal	CR	6	TBI	UD-BMT	IV	Yes	TRM (IA)	16
14	14	RAEB-T	Other	CR	7	TBI	UD-BMT	II	No	CCR	104+
15	13	RAEB-T	Other	CR	5	TBI	MSD-BMT	I	No	CCR	102+
16	6	RAEB-T	Complex	CR	5	TBI	UD-BMT	I	Yes	DOD	21
17	5	RAEB*	Monosomy 7	NA	2	Non-TBI	MSD-PBSCT	III	Yes	CCR	72+
18	6	RAEB*	Normal	NA	5	TBI	UD-BMT	I	No	CCR	92+
19	3	RAEB†	Other	NA	4	TBI	UD-CBT	II	No	DOD	7

UPN, unique patient number; ECM, etoposide, cytarabine, mitoxantrone; HSCT, haematopoietic stem cell transplantation; aGVHD, acute GVHD; cGVHD, chronic GVHD; RAEB, refractory anaemia with excess blasts; RAEB-T, RAEB in transformation; CR, complete remission; NA, not applicable; TBI, total body irradiation; MSD, human leucocyte antigen (HLA)-matched sibling donor; MFD, HLA-matched family donor; UD, unrelated donor; CBT, cord blood transplantation; BMT, bone marrow transplantation; PBSCT, peripheral blood stem cell transplantation; +, patient is alive.

*Patients who did not receive any pre-transplant chemotherapy.

†Patient who underwent pre-transplant chemotherapy other than ECM regimen.

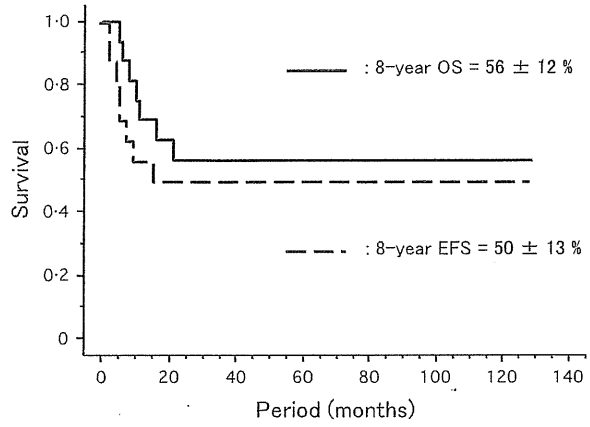

Fig 1. Survival curves of children with RAEB and RAEB-T enrolled in the MDS99 study.

reported in previous Japanese AML studies (Tomizawa *et al*, 2007; Tsukimoto *et al*, 2009). In the present study, remission induction rate with ECM regimen was as high as 81%, which was comparable with that of previous AML studies (Tomizawa *et al*, 2007; Tsukimoto *et al*, 2009). The duration of remission induction (median 41 d) was also acceptable although more than 60 d were required in two patients. Grade 3 or 4 non-haematological toxicities were reported in nine out of 16 patients, however, no toxic death was observed. Although there are several reports regarding the safety and efficacy of AML-type remission induction therapy for paediatric RAEB and RAEB-T, the results are not conclusive (Hasle *et al*, 1996; Chan *et al*, 1997; Creutzig *et al*, 1998; Webb *et al*, 2002; Woods *et al*, 2002). Considering the results of this study, we conclude that the ECM regimen is safe, with a high remission induction rate, for paediatric RAEB and RAEB-T. One might speculate that the advantage of AML-type remission induction therapy was that children with RAEB and RAEB-T became transfusion-independent and less susceptible to infection after remission was obtained and consequently they would be in a much more stable state than non-CR patients.

In spite of the high remission induction rate of the ECM regimen, EFS was not satisfactory. In our study, post-transplantation events were relapse in four, TRM in three and rejection in one. Recently, the European Working Group on Childhood MDS reported results of HSCT for advanced MDS in children using a uniform transplantation regimen (Strahm *et al*, 2011). In their report, EFS was reported as 59% and the cumulative incidence of TRM and relapse incidence were 21% each (Strahm *et al*, 2011). Although Strahm *et al* (2011) did not recommend specific pre-transplant therapy, they concluded the efficacy of AML-type remission induction therapy was restricted to more advanced MDS (i.e. MDS related AML). The major differences between the study reported by Strahm *et al* (2011) and ours included preparative regimens and stem cell sources. The earlier study used a uniform regimen consisting of busulfan, cyclophosphamide and melphalan and their stem cell sources were almost exclusively bone marrow [MSD in 40% and unrelated donor (UD) 60%] (Strahm *et al*, 2011). We mainly used TBI regimen and UD-cord blood was predominantly used as stem cell source. Recently, Madureira *et al* (2011) described the outcome of childhood MDS patients after cord blood transplantation (Madureira *et al*, 2011) with a reported OS of 42%, which is comparable to our study.

A structurally complex karyotype has been reported to be the strongest prognostic factor in advanced childhood MDS (Gohring *et al*, 2010). In the present study, all three patients with a structurally complex karyotype did not achieve CCR, regardless of haematological status at the time of HSCT. Children with a structurally complex karyotype will need novel approaches in addition AML-type remission induction therapy and myeloablative HSCT, whereas a current treatment scheme may be adequate for those with normal karyotype and other abnormal karyotypes. Considering the rarity of the disease, an international collaboration is warranted to conduct a prospective trial in order to explore an appropriate therapeutic strategy.

Conflicts of interest

The authors declare no conflict of interest.

Author contributions

In this study, A.K., D.H. and A.M. wrote the paper. A.K., D. H. and Y.O. analysed the data. D.H., Y.O., K.H. and S.K. performed the research. J.O., T.N. and A.M. designed the research study.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Schema of therapeutic procedure in the MDS 99 study.

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

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