

Enhanced Reconstitution of Human Erythropoiesis and Thrombopoiesis in an Immunodeficient Mouse Model with *Kit*^{W^v} Mutations

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SUMMARY

In human-to-mouse xenograft models, reconstitution of human hematopoiesis is usually B-lymphoid dominant. Here we show that the introduction of homozygous *Kit*^{W^v} mutations into C57BL/6.*Rag2*^{null}*Il2rg*^{null} mice with NOD-*Sirpa* (BRGS) strongly promoted human multi-lineage reconstitution. After xenotransplantation of human CD34⁺CD38⁻ cord blood cells, these newly generated C57BL/6.*Rag2*^{null}*Il2rg*^{null}NOD-*Sirpa* *Kit*^{W^v/W^v} (BRGSK^{W^v/W^v}) mice showed significantly higher levels of human cell chimerism and long-term multi-lineage reconstitution compared with BRGS mice. Strikingly, this mouse displayed a robust reconstitution of human erythropoiesis and thrombopoiesis with terminal maturation in the bone marrow. Furthermore, depletion of host macrophages by clodronate administration resulted in the presence of human erythrocytes and platelets in the circulation. Thus, attenuation of mouse KIT signaling greatly enhances the multi-lineage differentiation of human hematopoietic stem and progenitor cells (HSPCs) in mouse bone marrow, presumably by outcompeting mouse HSPCs to occupy suitable microenvironments. The BRGSK^{W^v/W^v} mouse model is a useful tool to study human multi-lineage hematopoiesis.

INTRODUCTION

Immunodeficient mice are widely used as hosts for xenogeneic transplantation to study human hematopoiesis in vivo (Doulatov et al., 2012; Goyama et al., 2015; Ishikawa, 2013; Rongvaux et al., 2013; Shultz et al., 2012). Elimination of the mouse lymphoid system, including T, B, and natural killer (NK) cells, is necessary to prevent human graft rejection. Thus, mice with the *scid* mutation (Greiner et al., 1998; McCune et al., 1988; Shultz et al., 1995) or those lacking recombination activating gene 1 or 2 (*Rag1* or *Rag2*) (Goldman et al., 1998; Shultz et al., 2000, 2003) have been used. In addition, these mice also had mutations in the interleukin-2 (IL-2) receptor common γ -chain subunit (*Il2rg*) gene (Ishikawa et al., 2005; Ito et al., 2002; Shultz et al., 2005). Previous studies reported that a non-obese diabetic (NOD) or BALB/c genetic background facilitates human hematopoietic stem cell (HSC) engraftment (Brehm et al., 2010). Therefore, these strains with complete lymphoid depletion, such as NOD-*scid* *Il2rg*^{null} (NSG/NOG) (Ito et al., 2002; Shultz et al., 2005), NOD.*Rag1*^{null}*Il2rg*^{null} (NOD-RG) (Pearson et al., 2008), and BALB/c.*Rag1*^{null}*Il2rg*^{null} (BALB-RG) (Brehm et al., 2010; Traggiai et al., 2004) mice, have been commonly used for recent xenotransplantation experiments.

We found that the strain-specific genetic determinant of human HSC engraftment was a polymorphism in the signal-regulatory protein α (*Sirpa*) gene (Iwamoto et al., 2014; Takenaka et al., 2007; Yamauchi et al., 2013). *SIRPA*

is expressed on the surface of macrophages and binds to its ligand, CD47, which is ubiquitously expressed (Matozaki et al., 2009). This binding activates inhibitory signals for phagocytosis of CD47-expressing cells, termed the “don’t eat me signal” (Jaiswal et al., 2009; Kuriyama et al., 2012; Majeti et al., 2009; Oldenborg et al., 2000). In the setting of human-to-mouse xenotransplantation, this inhibitory signal is necessary to prevent engulfment of human graft cells by host macrophages. Although the binding of *SIRPA* and CD47 is species specific (Subramanian et al., 2006), NOD and BALB/c mouse strains contain unique *SIRPA* immunoglobulin (Ig) domains that cross-react with human CD47 (Iwamoto et al., 2014; Takenaka et al., 2007). With regard to human CD47, NOD-*SIRPA* has the strongest affinity while BALB/c-*SIRPA* has an intermediate affinity (Iwamoto et al., 2014), and it is not recognized by C57BL/6-*SIRPA*. Based on these data, we developed a C57BL/6.*Rag2*^{null}*Il2rg*^{null} mouse line harboring NOD-*Sirpa* (BRGS) (Yamauchi et al., 2013). The efficiency of human cell engraftment in BRGS mice is significantly greater than in NOD-RG mice. Because BRGS mice do not possess a number of other NOD-specific abnormalities, including complement 5 deficiency (Yamauchi et al., 2013), they are also useful for testing the complement-dependent cytotoxic activity of antibodies in vivo (Kikushige and Miyamoto, 2013). Thus, the BRGS mouse line is one of the most efficient and convenient immunodeficient mouse strains for xenotransplantation.

However, in these models, human hematopoietic reconstitution is usually B-lymphoid dominant, and engraftment



of human myeloid, erythroid, and megakaryocyte lineages is limited (Doulatov et al., 2012; Ito et al., 2012; Rongvaux et al., 2013; Yamauchi et al., 2013). This skewed reconstitution could be due to the poor cross-reactivity of mouse cytokines with their corresponding human cytokine receptors (Manz, 2007). Indeed, BALB-RG mice carrying the human thrombopoietin gene displayed enhanced human cell engraftment and myelomonocytic differentiation (Rongvaux et al., 2011). The development of MITRG mice by further humanization of BALB-RG mice, generated by knocking in four human cytokines, namely macrophage colony-stimulating factor, granulocyte/macrophage colony-stimulating factor, IL-3, and thrombopoietin, has facilitated the functional development of human macrophages, monocytes, and NK cells (Rongvaux et al., 2014). The receptor tyrosine kinase KIT, which is expressed on hematopoietic stem and progenitor cells (HSPCs), functions as an essential regulator of the interaction between these cells and their niches by binding to its ligand, stem cell factor (SCF) (Czechowicz et al., 2007; Lyman and Jacobsen, 1998). Transgenic expression of membrane-bound human SCF in NSG mice resulted in improved human cell engraftment and myeloid differentiation (Takagi et al., 2012). However, even in these models, only low levels of human erythroid and megakaryocyte reconstitution were present.

For successful full-lineage engraftment, human HSPCs may need to find suitable niches within the mouse bone marrow. It was shown that immunodeficient mice with loss-of-function *Kit* mutations (Waskow et al., 2009) or those treated with a neutralizing anti-mouse KIT antibody (Czechowicz et al., 2007) accepted donor mouse HSCs without irradiation preconditioning that may be critical for the depletion of host HSCs to open their niches. Recent studies also showed that irradiation preconditioning was not required for human HSPCs to engraft in BALB-RG or NSG mice with *Kit*^{W41} or *Kit*^{Wv} mutations (Cosgun et al., 2014; McIntosh et al., 2015). These studies suggest that impaired KIT signaling renders mouse HSCs uncompetitive in occupying putative niches, therefore allowing human HSCs to access to mouse HSC niches and to expand in the mouse bone marrow.

In the present study, we established a BRGS strain with loss-of-function *Kit*^{Wv} mutation (BRGSK). The homozygous *Kit*^{Wv} BRGS mouse (BRGSK^{Wv/Wv}) is viable. In addition, it supports human hematopoietic reconstitution more efficiently after transplantation of human cord blood (CB) cells when compared with BRGS mice. In BRGSK^{Wv/Wv} mice, human myeloid reconstitution was dramatically improved and all types of myeloid cells were observed. More strikingly, abundant human erythroid and megakaryocyte cell lineages developed in BRGSK^{Wv/Wv} mouse bone marrow. This BRGSK mouse line is a useful tool for in vivo studies of human multi-lineage hematopoiesis.

RESULTS

Establishment and Characterization of the BRGSK Mouse Line

The heterozygous *Kit*^{Wv} BRGS mouse line (BRGSK^{Wv/+}) was established by introducing the *Kit*^{Wv} allele of the C57BL/6J-*Kit*^{Wv} mouse into the BRGS mouse (Yamauchi et al., 2013). Because mice homozygous for *Kit*^{Wv} are sterile (Nocka et al., 1990; Sharma et al., 2007), the BRGSK^{Wv/Wv} mouse was obtained by mating BRGSK^{Wv/+} pairs.

BRGSK^{Wv/Wv} mice had a white coat color (Figure 1A) and developed severe macrocytic anemia with a significant decrease in erythrocyte count (Figure 1B), as seen in homozygous C57BL/6J-*Kit*^{Wv} mice (Nocka et al., 1990; Sharma et al., 2007). The number of leukocytes and platelets was not significantly different to that in BRGS mice. BRGSK^{Wv/+} and BRGSK^{Wv/Wv} mice had a short median life span of 58 weeks and 35 weeks, respectively (Figure 1C). However, serial analysis of BRGSK mice revealed that aging did not exacerbate macrocytic anemia (data not shown). Their bone marrow cellularity and the number of HSCs and megakaryocyte-erythrocyte progenitors (MEPs) were similar to those in BRGS mice; in addition, they did not decline with age (Figure 1D). These results suggest that BRGSK^{Wv/+} and BRGSK^{Wv/Wv} mice do not develop bone marrow failure until at least 30 weeks after birth.

BRGSK Mice Have Improved Human Cell Engraftment Efficiency Compared with BRGS Mice

Six- to 8-week-old BRGS, BRGSK^{Wv/+}, and BRGSK^{Wv/Wv} mice were sublethally irradiated, and injected with $3.5\text{--}5 \times 10^3$ human lineage-depleted (Lin⁻) CD34⁺CD38⁻ CB cells. Injections were administered into the right femur. We used intrafemoral injections because they provided higher engraftment levels than intravenous injections in the xenotransplantation model (McKenzie et al., 2005; Yamauchi et al., 2013). At 8–12 weeks after transplantation, both BRGSK^{Wv/+} and BRGSK^{Wv/Wv} mice displayed higher levels of human hematopoietic reconstitution in the bone marrow compared with BRGS control mice. In the injected right femur, the average frequency of human CD45⁺ cells in BRGSK^{Wv/+} and BRGSK^{Wv/Wv} mice was 92.2% and 97.8%, respectively, and was 80.1% in BRGS mice (Figure 2A, left panel). We previously showed that human cell chimerism in male NOD-RG and BRGS recipients was lower than that in female recipients (Yamauchi et al., 2013). However, under the same transplantation protocol, both male and female BRGSK^{Wv/Wv} mice showed a similar excellent reconstitution (Figure S2). It was also reported that human cell chimerism in the non-injected side was very low (McDermott et al., 2010; McKenzie et al., 2005; Yamauchi et al., 2003). Interestingly, even in the non-injected

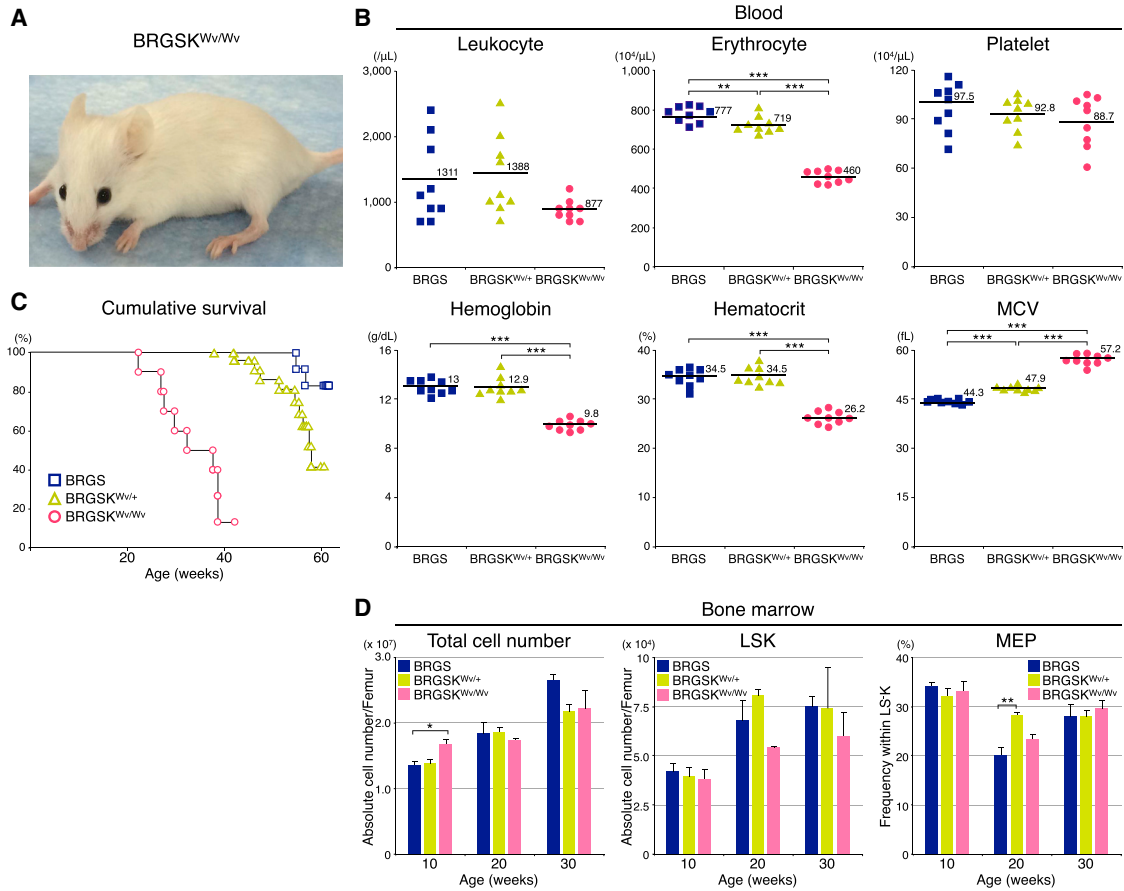


Figure 1. BRGSK^{Wv/Wv} Mice Develop Severe Macrocytic Anemia

(A) The appearance of the BRGSK^{Wv/Wv} mouse at 8 weeks of age.

(B) Frequencies of blood leukocytes, erythrocytes, and platelets, and the levels of hemoglobin, hematocrit, and mean corpuscular volume (MCV) in 8-week-old BRGS, BRGSK^{Wv/+}, and BRGSK^{Wv/Wv} mice (n = 9 mice per strain). All BRGSK^{Wv/Wv} mice had macrocytic anemia. The numbers and horizontal bars indicate mean values in each strain. **p < 0.01, ***p < 0.001.

(C) Kaplan-Meier survival curves for BRGS (n = 12), BRGSK^{Wv/+} (n = 30), and BRGSK^{Wv/Wv} (n = 10) mice. Log-rank test: p < 0.0001.

(D) Changes in the absolute number of femoral bone marrow cells and Lin⁻SCA-1⁺c-KIT⁺ (LSK) cells, and in the frequency of MEPs in BRGS, BRGSK^{Wv/+}, and BRGSK^{Wv/Wv} mice (n = 4 mice per group). Error bars indicate SEM. *p < 0.05, **p < 0.01. LS⁻K, Lin⁻SCA-1⁻c-KIT⁺.

left femur, the average human CD45⁺ cell chimerism reached 82.0% and 97.4% in BRGSK^{Wv/+} and BRGSK^{Wv/Wv} mice, respectively, compared with 56.7% in BRGS mice (Figure 2B, left panel). These data clearly show that the introduction of the *Kit*^{Wv} allele into the BRGS strain has a significant impact on the level of human cell reconstitution, and that it also accelerates the homing of human HSCs to bone marrow niches via the circulation.

We then analyzed the long-term repopulating activity of human HSCs at 20–24 weeks after transplantation, because it was reported that constant reconstitution (greater than 20 weeks) in xenogeneic hosts reflects the successful self-renewal of human HSCs in mouse bone marrow (Notta et al., 2011). BRGSK^{Wv/Wv} mice maintained a high level of human cell reconstitution (average = 86.4%) in the right

femur, whereas human cell chimerism significantly declined in both BRGS and BRGSK^{Wv/+} mice (average = 38.9% and 54.6%, respectively) (Figure 2A, right panel). In the non-injected left femur, only BRGSK^{Wv/Wv} mice maintained considerable levels of human cell chimerism (average = 53.8%) (Figure 2B, right panel). Furthermore, when we purified 10⁶ human CD45⁺ cells from cryopreserved bone marrow cells of primary BRGSK^{Wv/Wv} recipients and transplanted them into irradiated adult BRGS mice, multi-lineage engraftment of human hematopoietic cells was observed in 7 of 15 secondary recipients at 8 weeks after the secondary transplantation (Figure 2C). Collectively, the introduction of homozygous *Kit*^{Wv} mutations into the BRGS strain is critical for the long-term maintenance and self-renewal of human HSCs.

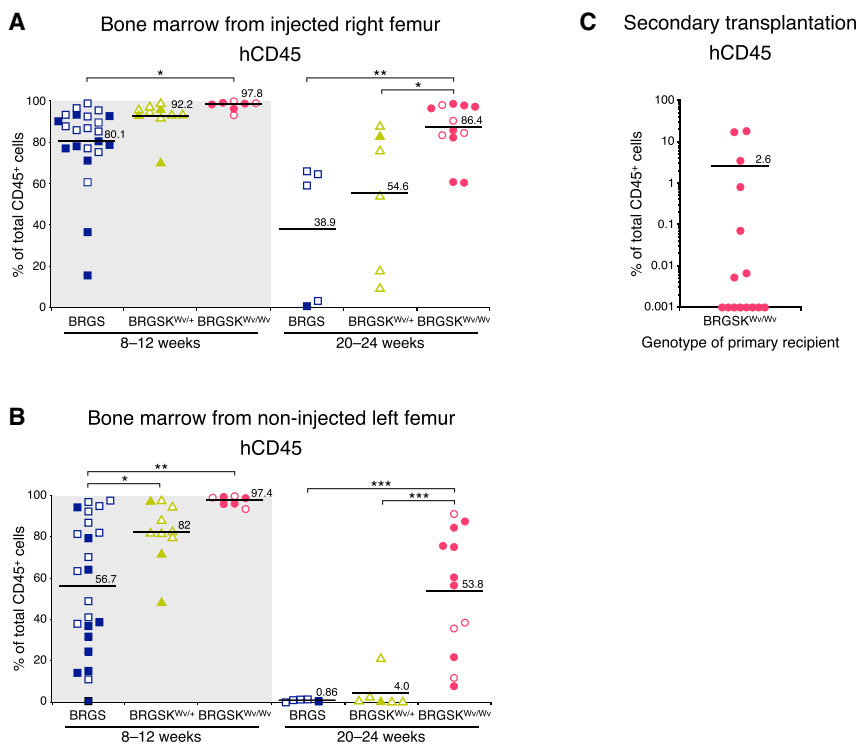


Figure 2. Efficient Long-Term Human Hematopoietic Reconstitution and Self-Renewal of Human HSCs in BRGSK^{Wv/Wv} Mice

(A and B) Human cell chimerism in the bone marrow of the right femur injected with $3.5\text{--}5 \times 10^3$ human CD34⁺CD38⁻ CB cells (A), and of the non-injected left femur (B) at 8–12 weeks and 20–24 weeks after transplantation. Open and closed symbols represent females and males, respectively (n = 5–23 mice per strain). The numbers and horizontal bars indicate mean values from four independent experiments in each strain. *p < 0.05, **p < 0.01, ***p < 0.001.

(C) Human cell chimerism in secondary BRGSK recipients injected with human CD45⁺ cells purified from cryopreserved bone marrow cells of primary BRGSK^{Wv/Wv} recipients. At 8 weeks after the secondary transplantation, 7 of 15 secondary recipients reconstituted human hematopoiesis in the bone marrow. The number and horizontal bar indicate mean value from four independent experiments. See also Figures S1 and S2.

BRGSK^{Wv/Wv} Mice Efficiently Reconstitute All Human Myeloid Lineages

Figure 3 shows the lineage analysis of human CD45⁺ cells reconstituted in the injected right femur. In BRGS and BRGSK^{Wv/+} mice, greater than 90% of engrafted human CD45⁺ cells were CD19⁺ B cells, and only up to 6% of CD33⁺ myeloid cells were observed at 8–12 weeks after transplantation (Figure 3B, left panel). In contrast, BRGSK^{Wv/Wv} mice had an average of 65% CD19⁺ B cells, resulting from a high level of myeloid reconstitution. CD33⁺ myeloid cells reached an average of 32.5% within the total human CD45⁺ population, and reached 57.3% at 20–24 weeks after transplantation (Figure 3B, right panel). NK cell reconstitution was also significantly improved in BRGSK^{Wv/Wv} mice. The enhanced multilineage reconstitution in BRGSK^{Wv/Wv} recipients was also seen in the non-injected left femur (Figure S3). Reconstituted B cells were polyclonal and expressed IgM with either the Igκ or Igλ light chain, and human T cell maturation was achieved in the BRGSK^{Wv/Wv} thymus (Figure S4). The gradual decline in human B cells and the increase in myeloid and NK cell frequencies in BRGSK recipient bone marrow after engraftment were compatible with our previous data in BRGS mice (Yamauchi et al., 2013).

We analyzed subsets of human CD33⁺ myeloid cells reconstituted in the injected right femur at 20–24 weeks after

transplantation. As shown in Figure 4, all myeloid lineage cells were present in BRGS and BRGSK mice. Neutrophils, eosinophils, basophils, and mast cells were separated by fluorescence-activated cell sorting (FACS) as side scatter (SSC)^{high}CD125⁻, SSC^{high}CD125⁺, SSC^{low}CD14⁻CD117⁻CD125^{low}FcεR1α⁺, and SSC^{low}CD14⁻CD117⁺ populations, respectively (Figure 4A). SSC^{low}CD11b⁺CD14⁺ monocytes, SSC^{low}CD14⁻CD11c⁺CD123⁻HLA-DR⁺ conventional dendritic cells (cDCs), and SSC^{low}CD14⁻CD11c⁻CD123^{high}HLA-DR⁺ plasmacytoid dendritic cells (pDCs) were also evident. Figure 4B shows the morphology of each myeloid subset purified from BRGSK^{Wv/Wv} recipients. We then tested the expression profiles of genes related to myeloid cell function or development. We used the Molecular Signatures Database v5.1 (<http://www.broadinstitute.org/gsea/msigdb/index.jsp>) to identify genes closely related to each myeloid subset. *TPSB2* and *CPA3* were chosen as representative genes for mast cells, *RNASE2* and *CLC* for eosinophils, *MPO* and *CXCR2* for neutrophils, *LYZ*, *MAFB*, and *SLC7A7* for monocytes, *CD1C* and *HLA-DR* for cDCs, *IRF7* and *TNFRSF21* for pDCs, and *FLT3* for both cDCs and pDCs. As shown in Figure 4C, each myeloid subpopulation expressed genes representative of their cellular properties, suggesting that they developed normally in the BRGSK^{Wv/Wv} mouse model. There were no significant differences in the composition of myeloid cells among BRGS and BRGSK strains (Figure 4D).

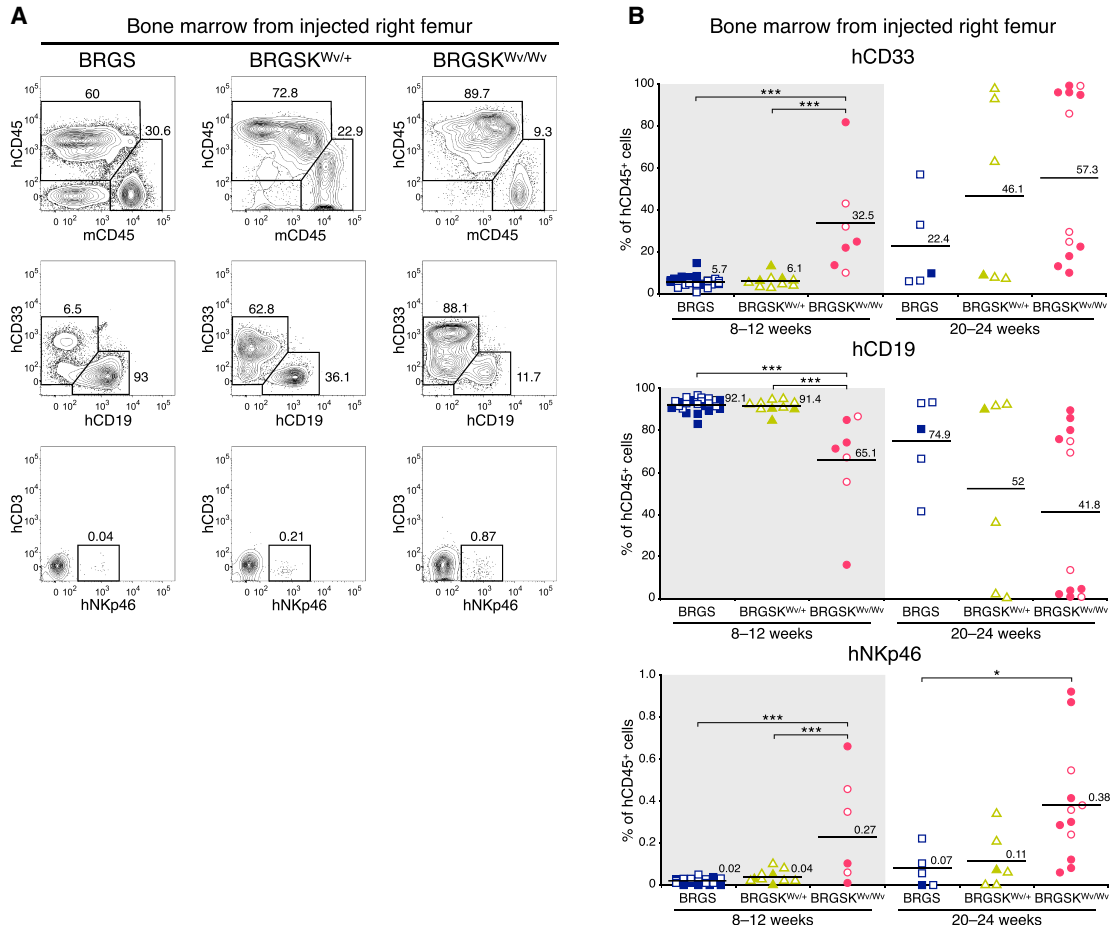


Figure 3. Human Myeloid Reconstitution Is Enhanced in BRGSK^{Wv/Wv} Mice

Human myeloid and lymphoid reconstitution was evaluated in the bone marrow from the right femur of BRGS, BRGSK^{Wv/+}, and BRGSK^{Wv/Wv} mice. (A) Representative FACS plots at 20 weeks after transplantation. Human lineage cells were analyzed within the human CD45⁺ population. Numbers in FACS plots indicate the percentages of gated cells.

(B) Frequencies of human CD33⁺ myeloid cells, CD19⁺ B cells, and NKp46⁺ NK cells at 8–12 weeks and 20–24 weeks after transplantation. Open and closed symbols represent females and males, respectively (n = 5–23 mice per strain). The numbers and horizontal bars indicate mean values from four independent experiments in each strain. *p < 0.05, ***p < 0.001.

See also Figures S3 and S4.

BRGSK^{Wv/Wv} Mice Reconstitute and Enhance Human Erythropoiesis and Thrombopoiesis in the Bone Marrow

The reconstitution of human erythroid and megakaryocyte lineages has been very limited by the xenogeneic transplantation of human HSCs (Ishikawa et al., 2005; Takagi et al., 2012). As shown in Figures 5A and 5B, CD235a⁺ erythroid cells were almost absent in the bone marrow of BRGS and BRGSK^{Wv/+} mice. Strikingly, BRGSK^{Wv/Wv} mice had a high level of human erythroid reconstitution, reaching an average of 42.2% and 13.7% of the total erythroid population at 8–12 weeks and 20–24 weeks after transplantation, respectively. In terms of thrombopoiesis, human CD41⁺ platelets began to reconstitute in all strains at

8–12 weeks after transplantation (Figure 5B, left panel), and BRGSK^{Wv/+} and BRGSK^{Wv/Wv} mice developed 32.7% and 49.1% of human platelets, respectively, at 20–24 weeks after transplantation (Figure 5B, right panel). Human erythroid and platelet reconstitution was also present in the non-injected left femur of BRGSK^{Wv/Wv} mice, although the level of chimerism was low (Figure S3).

Representative FACS plots of the analysis of human erythroid development are shown in Figure 5A. CD71 (transferrin receptor) expression on human erythroid lineage cells gradually decreases as cells become mature (Giarratana et al., 2005; Miharada et al., 2006). BRGSK^{Wv/Wv} mice displayed every stage of human erythropoiesis defined by the expression pattern of CD71 and CD235a.

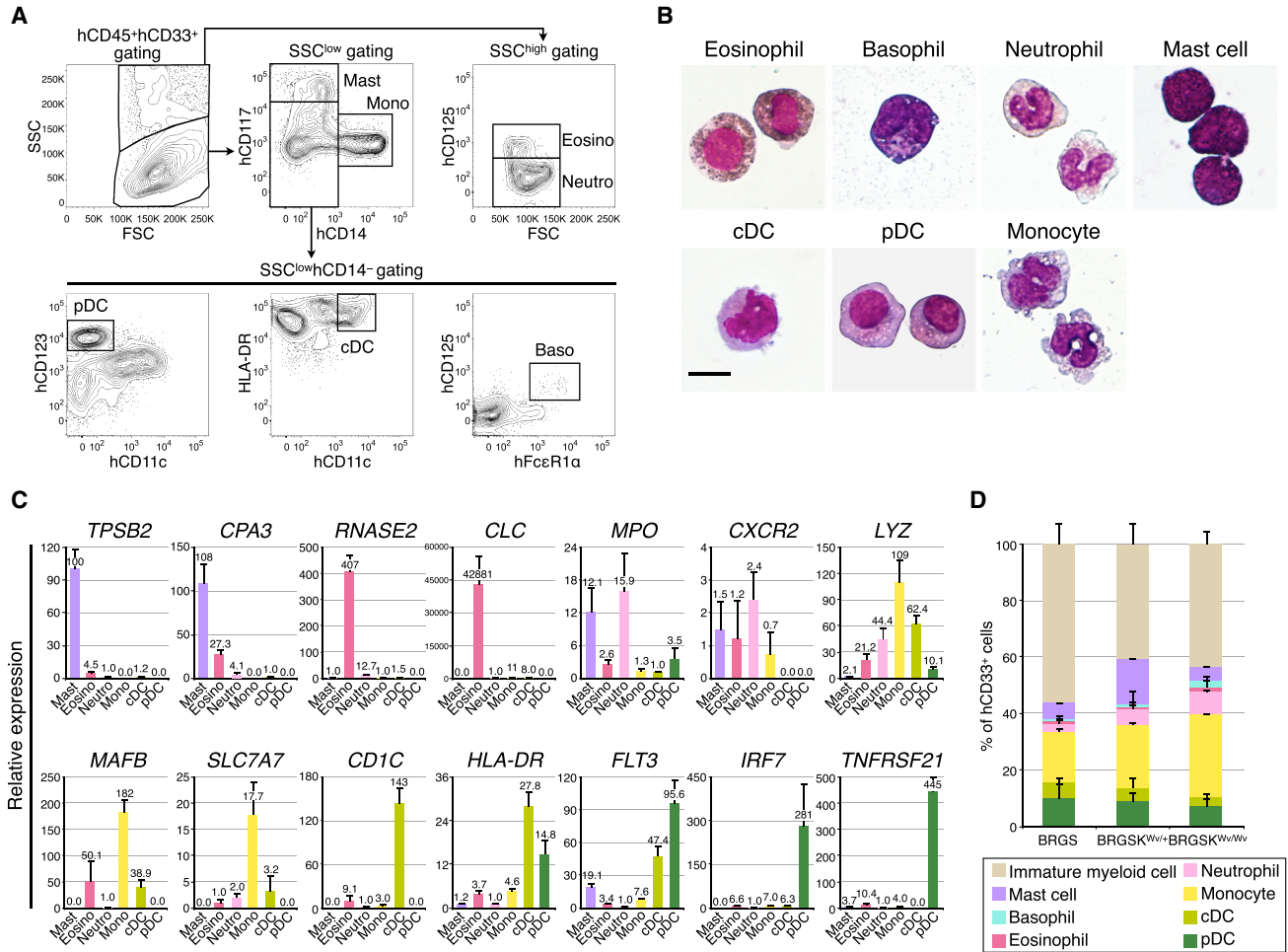


Figure 4. Terminal Differentiation of Human Myeloid Cells in BRGSK^{W^v/W^v} Mice

(A) Representative FACS plots of human myeloid subsets within the hCD45⁺hCD33⁺ population at 20 weeks after transplantation in the bone marrow of a BRGSK^{W^v/W^v} recipient. SSC, side scatter; FSC, forward scatter; Mast, mast cell; Mono, monocyte; Eosino, eosinophil; Neutro, neutrophil; Baso, basophil; cDC, conventional dendritic cells; pDC, plasmacytoid dendritic cells.

(B) Morphology of each myeloid subset purified from BRGSK^{W^v/W^v} recipient bone marrow by FACS. Representative dot plots and images of four independent experiments are shown. Scale bar, 10 μm.

(C) Relative mRNA expression levels of representative functional genes in human myeloid subsets purified from BRGSK^{W^v/W^v} recipient bone marrow. The numbers and error bars indicate mean values and SEM, respectively (n = 4 independent experiments).

(D) Percentage of each myeloid subset within the CD33⁺ population at 20–24 weeks after transplantation. The data are the summary of five BRGS, six BRGSK^{W^v/+}, and 12 BRGSK^{W^v/W^v} mice. Error bars indicate SEM from four independent experiments.

CD71⁻CD235a⁺ mature erythrocytes were clearly detectable, although the number of this population was low. The morphology of each purified fraction is shown in Figure 5C: CD71⁺CD235a⁻ cells were proerythroblasts and basophilic erythroblasts, CD71⁺CD235a⁺ cells were polychromatic to orthochromatic erythroblasts, and CD71⁻CD235a⁺ cells were enucleated mature erythrocytes.

Figure 6A shows the immunohistochemical analysis of the reconstituted human erythroid cells and megakaryocytes in BRGSK^{W^v/W^v} recipient bone marrow. High numbers of CD235a⁺ erythroid cells were present in

BRGSK^{W^v/W^v} mice but not in BRGS mice. These erythroid cells were functionally mature as they produced human hemoglobin subunit α protein (Figure 6A), and expressed human hemoglobin subunit β transcripts (Figure 6B). BRGSK^{W^v/W^v} mice also contained a number of megakaryocytes that were strongly stained with human CD41 and scattered throughout the bone marrow, which were not found in BRGS mice. These data indicate the enhanced functional maturation of human erythroid cells and megakaryocytes in the bone marrow of BRGSK^{W^v/W^v} mice.

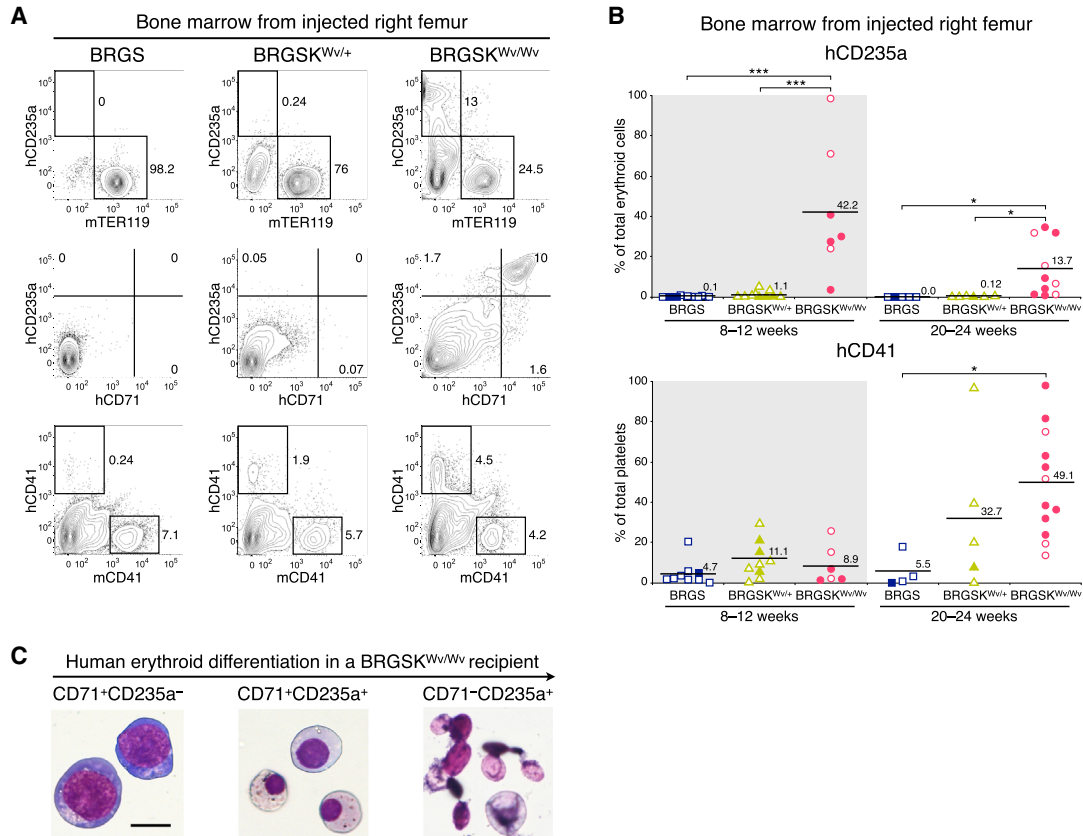


Figure 5. Terminal Differentiation of Human Erythropoiesis and Thrombopoiesis Is Enhanced in BRGSK^{Wv/Wv} Mice

Analysis of human erythroid and megakaryocyte reconstitution in the bone marrow from the right femur of BRGSK, BRGSK^{Wv/+}, and BRGSK^{Wv/Wv} mice.

(A) Representative FACS plots at 20 weeks after transplantation. Chimerism of erythroid cells was analyzed within the human and mouse CD45⁻ population. Platelets were analyzed within the Lin⁻ population, and scatter gates specific for the platelet fraction were set. Numbers in FACS plots indicate the percentages of gated cells.

(B) Frequencies of human CD235⁺ erythroid cells and CD41⁺ platelets. Open and closed symbols represent females and males, respectively (n = 4–23 mice per strain). The numbers and horizontal bars indicate mean values from four independent experiments in each strain. *p < 0.05, ***p < 0.001.

(C) Morphology of CD71⁺CD235a⁻ proerythroblasts/basophilic erythroblasts, CD71⁺CD235a⁺ polychromatic to orthochromatic erythroblasts, and CD71⁻CD235a⁺ enucleated mature erythrocytes in a BRGSK^{Wv/Wv} recipient. Representative images of three independent experiments are shown. Scale bar, 10 μm.

See also Figure S3.

To visualize the broad distribution of human erythroid cells in BRGSK^{Wv/Wv} recipient bone marrow, we performed three-dimensional (3D) confocal immunofluorescence imaging experiments by using whole-mount tissues of the mouse sternum (Kunisaki et al., 2013) stained with human CD71 and mouse TER119. Human hematopoiesis was efficiently reconstituted, even in the sternal bone marrow (Figure 6C). Interestingly, human and mouse erythropoiesis appeared to form “erythroblastic islands” (Figure 6D). Either human CD71⁺ or mouse TER119⁺ erythroid cells formed colony-like structures around the vascular network throughout the sternum, as visualized by VE-cadherin/PECAM-1 staining; however, where

human CD71⁺ cells formed islands, mouse TER119⁺ islands did not co-exist, and vice versa (Figure 6E). These results suggest that human erythroid reconstitution is achieved by outcompeting host erythropoiesis for putative “erythroid” niches in the bone marrow of BRGSK^{Wv/Wv} mice.

Systemic Depletion of Macrophages Releases Human Erythrocytes and Platelets into the Circulation of BRGSK^{Wv/Wv} Mice

In previous human-to-mouse xenogeneic transplantation models, circulating human erythrocytes or platelets were not usually observed (Chen et al., 2009; Rongvaux et al.,

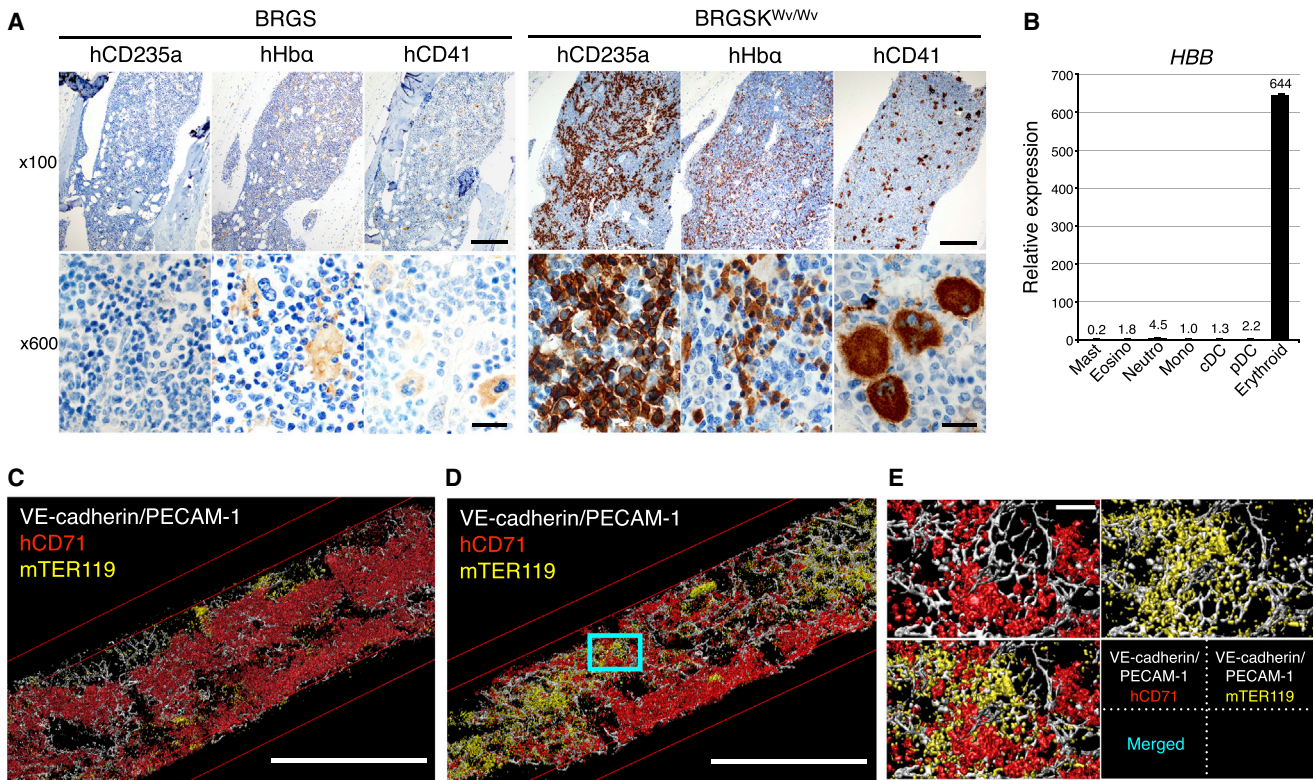


Figure 6. Immunohistochemistry and 3D Confocal Immunofluorescence Imaging of Human Megakaryo-Erythroid Lineage Cells in BRGSK^{Wv/Wv} Mice

(A) Immunohistochemical analysis of human CD235a, hemoglobin subunit α (Hb α), and CD41 expression in the femoral bone marrow of BRGS and BRGSK^{Wv/Wv} mice (magnification: top row, $\times 100$; bottom row, $\times 600$). Representative images of four independent experiments are shown. Scale bars: top row, 200 μ m; bottom row, 20 μ m.

(B) Relative mRNA expression levels of human hemoglobin subunit β (*HBB*) in human myeloid and CD235a⁺ erythroid cells purified from BRGSK^{Wv/Wv} recipient bone marrow. The numbers and error bars indicate mean values and SEM, respectively (n = 4 independent experiments). Mast, mast cell; Eosino, eosinophil; Neutro, neutrophil; Mono, monocyte.

(C–E) 3D reconstructed images of whole-mount sternal bone marrow from BRGSK^{Wv/Wv} recipients stained with human CD71 (red), mouse TER119 (yellow), VE-cadherin (white), and PECAM-1 (white) antibodies at 20 weeks after transplantation. Representative images from BRGSK^{Wv/Wv} recipients with high erythroid chimerism (C) and with intermediate erythroid chimerism (D) are shown. Scale bars, 800 μ m. (E) Extended images of the square area in (D). Data shown are representative of six independent experiments. Scale bar, 50 μ m.

2013; Takagi et al., 2012). They were also undetectable in BRGSK^{Wv/Wv} mice (Figure 7A). We considered that mature human blood cells might still be engulfed by mouse macrophages, even after the introduction of NOD-*Sirpa*, because hemosiderin was deposited in the spleen of BRGSK^{Wv/Wv} mice with human hematopoietic reconstitution (data not shown). Previous studies reported that after xenotransplantation of a high dose ($1\text{--}5 \times 10^5$ cells) of human CD34⁺ fetal liver cells, which have strong erythroid expansion potential (Holoake et al., 1999), the elimination of host macrophages by clodronate administration accelerated the peripheral distribution of human mature cells (Hu and Yang, 2012; Hu et al., 2011). We therefore tested whether clodronate

treatment could induce human erythrocytes and platelets into the circulation of BRGSK^{Wv/Wv} mice transplanted with human CB cells. Injection of clodronate liposomes (100–200 μ L per injection) was started at 13 weeks after transplantation, and continued for 3 weeks. Strikingly, a statistically significant number of circulating human CD71⁺CD235a⁺ mature erythrocytes and human CD41⁺ platelets appeared after clodronate treatment (Figure 7B). Our findings show that human hematopoiesis can progress up to the mature erythrocyte and platelet stages in BRGSK^{Wv/Wv} mouse bone marrow, whereas mouse macrophages in BRGSK^{Wv/Wv} mice might still prevent these cells from circulating, presumably by engulfing them in the bone marrow and/or spleen.

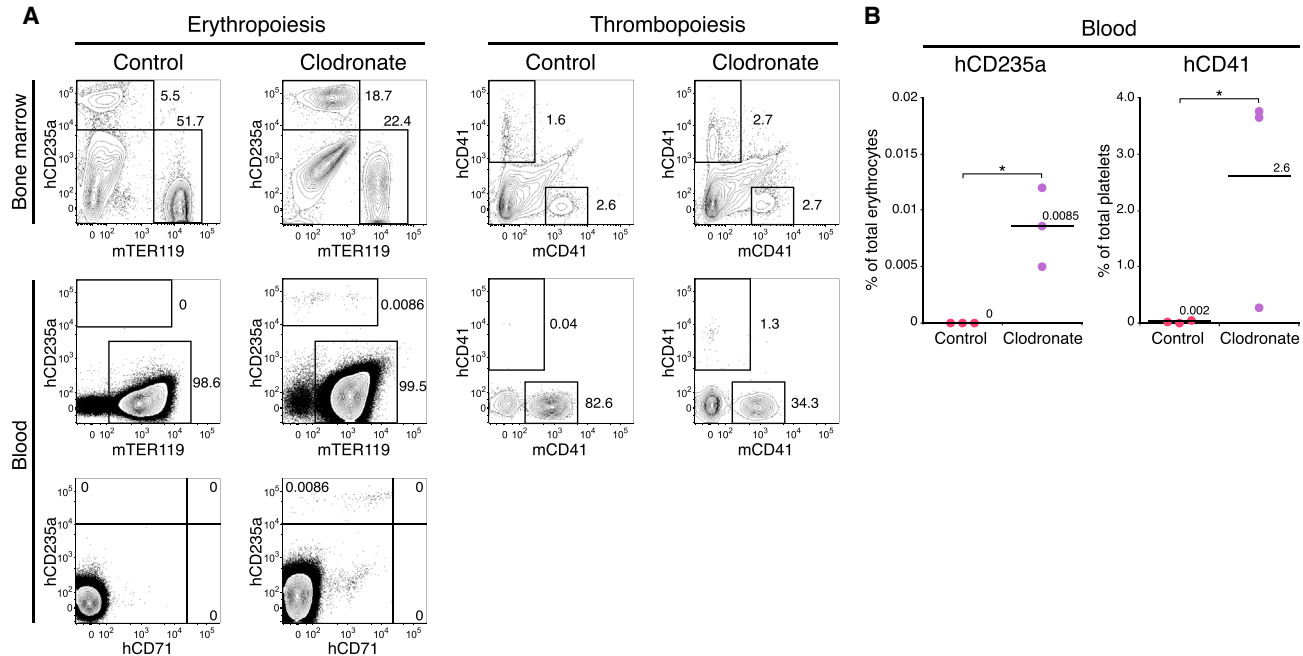


Figure 7. Effect of Systemic Macrophage Depletion on the Peripheral Distribution of Human Erythrocytes and Platelets in BRGSK^{Wv/Wv} Mice

BRGSK^{Wv/Wv} recipients at 13 weeks after transplantation were injected intraperitoneally with clodronate (n = 3) or control (n = 3) liposomes for 3 weeks (200 μ l for the first injection and 100 μ l every 4 days thereafter). Human erythroid and megakaryocyte reconstitution were analyzed at 16 weeks after transplantation.

(A) Representative FACS plots are shown. Chimerism of erythroid cells was analyzed within the human and mouse CD45⁻ population. Platelets were analyzed within the Lin⁻ population, and scatter gates specific for the platelet fraction were set. Note that all circulating CD235a⁺ cells were CD71⁻ mature erythrocytes. Numbers in FACS plots indicate the percentages of gated cells.

(B) Frequencies of human CD235⁺ erythrocytes and CD41⁺ platelets in the peripheral blood. The numbers and horizontal bars indicate mean values from three independent experiments. *p < 0.05.

DISCUSSION

Although many immunodeficient mouse strains have been developed, it has become clear that most models display B-lymphoid dominant reconstitution after the xenogeneic transplantation of human HSPCs (Doulatov et al., 2012; Ito et al., 2012; Rongvaux et al., 2013; Yamauchi et al., 2013). Genetically delivered human cytokines have greatly improved myelomonocytic differentiation (Rathinam et al., 2011; Rongvaux et al., 2011, 2014; Takagi et al., 2012; Willinger et al., 2011); however, human erythroid and megakaryocyte reconstitution is still limited (Chen et al., 2009; Rongvaux et al., 2011; Takagi et al., 2012). In the present study, we showed that BRGS mice homozygous for *Kit*^{Wv} (BRGSK^{Wv/Wv}) repopulated robust human erythropoiesis and thrombopoiesis as well as myelopoiesis.

Among the *Kit* mutations, *Kit*^W, the null mutant, is the most severe type, and *Kit*^{Wv} and *Kit*^{W41}, which lack the intracellular signaling portion of KIT, are less severe (Nocka et al., 1990). Mice homozygous for *Kit*^W are lethal, those homozygous for *Kit*^{Wv} are viable but sterile, and those

homozygous for *Kit*^{W41} are fertile (Nocka et al., 1990). In addition, these phenotypes correspond to the degree of impairment in hematopoiesis (Sharma et al., 2007). Based on these data, we chose to introduce the *Kit*^{Wv} mutation into the BRGS strain, as it should provide the strongest inhibition of KIT signaling while still preserving the viable phenotype.

Previous studies reported that the introduction of *Kit*^{W41} or *Kit*^{Wv} mutations into BALB-RG or NSG mice could render mouse HSCs uncompetitive to donor human HSCs (Cosgun et al., 2014; McIntosh et al., 2015). They also showed that human HSPCs injected at a high dose (5 \times 10⁴ to 3 \times 10⁵ CD34⁺ CB cells), without irradiation preconditioning, could outcompete mouse HSCs for engraftment and differentiate into myeloerythroid cells. This is probably because the introduction of loss-of-function *Kit* mutations might render host HSCs uncompetitive in occupying HSC niches. The BRGSK^{Wv/Wv} mouse also reconstituted human hematopoiesis, without irradiation, after a high dose of human HSPC transplantation (data not shown). However, when these mice were irradiated,



only a small number ($3.5\text{--}5 \times 10^3$ cells) of human $\text{CD34}^+\text{CD38}^-$ CB cells was sufficient to achieve a high level of human cell chimerism of erythroid and megakaryocyte lineage cells. Thus, the introduction of homozygous Kit^{Wv} mutations into mice with severe combined immunodeficiency, in combination with irradiation preconditioning, allows the robust reconstitution of human erythropoiesis and thrombopoiesis with terminal maturation.

Mice with *Kit* mutations display macrocytic anemia (Nocka et al., 1990) and impairment of megakaryopoiesis and myelopoiesis (Chervenick and Boggs, 1969; Ebbe et al., 1973) as the major hematopoietic phenotypes. These changes might be explained by the decreased affinity between HSPCs and their niches by attenuated KIT signaling. In this context, *Kit* mutations might help open the putative “myeloid,” “erythroid,” or “megakaryocyte” niches, resulting in the ample reconstitution of human myeloid, erythroid, and megakaryocyte cell lineages in the bone marrow. Indeed, robust human erythropoiesis occurred in the sternal bone marrow of $\text{BRGSK}^{\text{Wv}/\text{Wv}}$ mice, where human erythroid cells colonized, forming “islands” independently of mouse erythroid cells. This suggested that these cells migrated and competed for mouse “erythroid” niches in the bone marrow. However, the level of human CD235a^+ or CD41^+ cell chimerism was lower than that of human CD45^+ cell chimerism, even in $\text{BRGSK}^{\text{Wv}/\text{Wv}}$ mice. In addition, maturation of human erythroid cells was imperfect, as the number of $\text{CD71}^- \text{CD235a}^+$ mature erythrocytes was limited. Human erythroid reconstitution was not improved further by injecting recombinant human erythropoietin into $\text{BRGSK}^{\text{Wv}/\text{Wv}}$ mice (data not shown). Therefore, some other unknown determinants, critical for the faithful regeneration of human hematopoiesis, may still be lacking in the bone marrow microenvironment of $\text{BRGSK}^{\text{Wv}/\text{Wv}}$ mice.

We did not observe circulating erythrocytes or platelets, even in $\text{BRGSK}^{\text{Wv}/\text{Wv}}$ mice with a very high chimerism of human hematopoiesis. In the bone marrow, we detected cells at every stage of human erythroid differentiation, including mature enucleated erythrocytes that synthesized hemoglobin, and mature human megakaryocytes with multiple nuclei. Of note, nearly 50% of platelets in the bone marrow were of human origin. Furthermore, elimination of phagocytic cells by clodronate successfully released a fraction of mature erythrocytes and platelets into the circulation. Thus, the final developmental step, migration from the bone marrow to the circulation and sustainment in the circulation, might not yet be “humanized” even in $\text{BRGSK}^{\text{Wv}/\text{Wv}}$ mice harboring *NOD-Sirpa*. It is possible that the xenogeneic activation of mouse macrophages to engulf human cells does not depend solely on the SIRPA-CD47 interaction, and that other unknown mechanisms of macrophage activation against human cells exist. Clodr-

onate administration, however, was unable to deplete all host macrophages, because a considerable number of residual macrophages were present, at least in the spleen (data not shown). Macrophages reside throughout the body, including around blood vessels in the bone marrow niches (Ehninger and Trumpp, 2011). New strategies to completely inhibit mouse macrophage activity against human cells should be developed to establish a more faithful humanized model with circulating mature human blood cells.

In $\text{BRGSK}^{\text{Wv}/\text{Wv}}$ mice, the level of myeloid reconstitution was up to 30% and 60% at 8–12 weeks and 20–24 weeks after transplantation, respectively. These levels might be comparable with those in MITRG mice in which four critical myeloid cytokines were humanized (Rongvaux et al., 2014), although a direct comparison is difficult owing to differences in dose, source, and injection methods of human HSPCs. Specifically, MITRG mice were reconstituted with 10^5 human CD34^+ fetal liver cells by intrahepatic injection. The humanization of key cytokines should be important for the functional maturation of myeloid cells (Rathinam et al., 2011; Rongvaux et al., 2011, 2014; Willinger et al., 2011). However, the introduction of homozygous Kit^{Wv} mutations was sufficient for reconstituted myeloid cells to mature morphologically and to express genes related to their functions. It will be of interest to test whether the $\text{BRGSK}^{\text{Wv}/\text{Wv}}$ mouse model can further improve human multi-lineage reconstitution potential by humanization of key cytokines.

In summary, we introduced the loss-of-function Kit^{Wv} mutation into the BRGS mouse strain and achieved a highly efficient, long-term, and multi-lineage engraftment of human HSCs. This line can reconstitute robust human erythropoiesis and thrombopoiesis without exogenous human cytokine support. The $\text{BRGSK}^{\text{Wv}/\text{Wv}}$ mouse line is a useful tool to help increase our understanding of the biology of human multi-lineage hematopoiesis and a variety of malignant hematopoietic disorders.

EXPERIMENTAL PROCEDURES

Mice

$\text{C57BL}/6\text{J-Kit}^{\text{Wv}}$ mice were purchased from the Jackson Laboratory. BRGS mice were developed in our laboratory (Yamauchi et al., 2013). The $\text{BRGSK}^{\text{Wv}/+}$ mouse was generated by breeding BRGS and heterozygous $\text{C57BL}/6\text{J-Kit}^{\text{Wv}}$ mice, and the $\text{BRGSK}^{\text{Wv}/\text{Wv}}$ mouse line was maintained by crossing $\text{BRGSK}^{\text{Wv}/+}$ pairs. All mice were bred and maintained under specific pathogen-free conditions at the Kyushu University Animal Facility. *Rag2*, *Il2rg*, *Sirpa*, and *Kit* genes were genotyped by direct sequencing after PCR amplification to identify the genotype of BRGSK mice. Primer sequences are described in our previous report (Yamauchi et al., 2013) and the JAX website (<http://jaxmice.jax.org/strain/000049.html>).



Determination of the Irradiation Dose

To determine the irradiation dose for BRGSK mice, we first evaluated the effect of the *Kit*^{W^v} allele on radiation sensitivities. Groups of 6- to 10-week-old heterozygous or homozygous C57BL/6J-*Kit*^{W^v} mice were irradiated by a 77.7-TBq (2.1 kCi) ¹³⁷Cs γ -irradiator with a dose rate of 70 cGy/min and monitored for 8 weeks. As shown in Figure S1, most C57BL/6J-*Kit*^{W^v} heterozygotes receiving greater than 550 cGy irradiation died (Figure S1A), and all C57BL/6J-*Kit*^{W^v} homozygotes irradiated with greater than 200 cGy died within 3 weeks (Figure S1B). In xenotransplantation experiments, BRGSK mice were irradiated with ~75% of the sublethal dose for C57BL/6J-*Kit*^{W^v} mice. BRGSK^{W^v/+} and BRGSK^{W^v/W^v} mice received 450 cGy and 150 cGy of irradiation preconditioning, respectively.

Xenogeneic Transplantation of Human HSCs into Immunodeficient Mice

CB cells were collected during normal full-time deliveries after obtaining informed consent in accordance with the Declaration of Helsinki (provided by the Japanese Red Cross Kyushu Cord Blood Bank). Mononuclear cells were concentrated by standard gradient centrifugation, and CD34-enriched CB cells were obtained using a Lineage Cell Depletion Kit (Miltenyi Biotec). A total of $3.5\text{--}5 \times 10^3$ sorted Lin⁻CD34⁺CD38⁻ CB cells were injected intrafemorally into 6- to 8-week-old BRGSK and control BRGS mice. BRGS, BRGSK^{W^v/+}, and BRGSK^{W^v/W^v} mice were irradiated at a sublethal dose (550, 450, and 150 cGy, respectively). After transplantation, mice were given sterile water containing prophylactic enrofloxacin (Baytril; Bayer HealthCare). Animal experiments were conducted according to the guidelines of the Institutional Animal Committee of Kyushu University.

Flow Cytometric Analysis of Human Hematopoietic Chimerism

At 8, 12, 16, 20, or 24 weeks after transplantation, samples of bone marrow, spleen, thymus, and peripheral blood were harvested from recipient mice, and single-cell suspensions were analyzed and sorted using either FACSARIA2 or FACSARIA3 (BD Biosciences). After lysis of erythrocytes with Pharm Lyse (BD Biosciences), cells were stained with monoclonal antibodies. For the analysis of erythroid cells and platelets, untreated samples were stained and analyzed by setting scatter gates specific for each cell fraction. FACS-purified human cells were confirmed morphologically by May-Giemsa staining. Details about the antibodies used are provided in Supplemental Experimental Procedures.

Immunohistochemistry and Whole-Mount Confocal Immunofluorescence Imaging

Sections prepared from paraformaldehyde-fixed paraffin-embedded mouse femoral bone marrow tissues were immunostained with antibodies. Sternal bones were harvested for immunofluorescence imaging and transected with a surgical blade into two fragments as described previously (Kunisaki et al., 2013). The fragments were sagittally bisected to expose the bone marrow cavity and then fixed in 4% paraformaldehyde. Whole-mount tissues of the sterna were

blocked and permeabilized in PBS containing 20% normal goat serum and 0.5% Triton X-100, then stained with antibodies for 2–3 days. Images were acquired using an Axio Examiner D1 microscope (Zeiss) with a confocal scanner unit, CSUW1CU (Yokogawa), and reconstructed in 3D using Image-Pro software (Media Cybernetics). Details about the antibodies used are provided in Supplemental Experimental Procedures.

Clodronate Treatment

Macrophages were depleted in vivo by intraperitoneal injection of clodronate-encapsulated liposomes (Clophosome-A; FormuMax Scientific). Clodronate liposomes were given at 200 μ L per mouse for the first injection and then 100 μ L per mouse every 4 days for 3 weeks. Control mice were treated at the same time with empty liposomes (Control anionic liposomes; FormuMax Scientific).

Gene-Expression Analysis

Reconstituted human CD235a⁺ erythroid cells and each myeloid subpopulation within the CD33⁺ population were purified from BRGSK^{W^v/W^v} mouse bone marrow, and examined for gene expression. Each target population was sorted directly to TRIzol (Life Technologies), and the total RNA extracted was reverse transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen). Pre-amplification was performed using TaqMan PreAmp Master Mix (Applied Biosystems). cDNA samples were subjected to real-time qPCR using the Biomark system (Fluidigm). Expression of the *GAPDH* gene was used for normalization. Primers and probes used in this assay were obtained from Applied Biosystems.

Statistical Analysis

Student's t test was used for single comparisons, and one-way ANOVA followed by Tukey's HSD (honestly significant difference) test for multiple comparisons. All statistical analyses were performed using JMP software (version 11.0; SAS Institute, www.sas.com) and p values of less than 0.05 were considered statistically significant. The Mantel-Cox log-rank test was used to obtain survival curves.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at <http://dx.doi.org/10.1016/j.stemcr.2016.07.002>.

AUTHOR CONTRIBUTIONS

A.Y. and K.T. coordinated the project, designed and performed the experiments, analyzed the data, and wrote the manuscript. A.Y., Y.U., and Y. Kunisaki performed the whole-mount imaging experiments. T.Y., T.N., F.J., and K.M. performed the research. Y. Kikushige, K.K., T.M., H.I., and K.A. designed the experiments, reviewed the data, and edited the manuscript.

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