Enhanced Reconstitution of Human Erythropoiesis and Thrombopoiesis in an Immunodeficient Mouse Model with Kit<sup>Wv</sup> Mutations

Ayano Yurino,1 Katsuto Takenaka,2 Takui Yamauchi,1 Takuya Nunomura,1 Yasufumi Uehara,1 Fumiaki Jinnouchi,1 Kohta Miyawaki,1 Yoshikane Kikushige,1 Koji Kato,1 Toshihiro Miyamoto,1 Hiromi Iwasaki,2 Yuya Kunisaki,2 and Koichi Akashi1,2,*

1Department of Medicine and Biosystemic Science, Kyushu University Graduate School of Medical Sciences, Fukuoka 812-8582, Japan
2Center for Cellular and Molecular Medicine, Kyushu University Hospital, Fukuoka 812-8582, Japan
*Correspondence: akashi@med.kyushu-u.ac.jp

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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<sup>Wv</sup> mutations into C57BL/6.Rag2<sup>−/−</sup>Il2rg<sup>−/−</sup> mice with NOD-Sirpa (BRGS) strongly promoted human multi-lineage reconstitution. After xenotransplantation of human CD34<sup>+</sup>CD38<sup>−</sup> cord blood cells, these newly generated C57BL/6.Rag1<sup>−/−</sup>Il2rg<sup>−/−</sup>NOD-Sirpa Kit<sup>Wv</sup>/Wv (BRGS<sup>Wv</sup>) 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 erythropoiesis and thrombopoiesis with terminal maturation in the bone marrow. 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 g-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<sup>−/−</sup> (NSG/NOG) (Ito et al., 2002; Shultz et al., 2005), NOD.Rag1<sup>−/−</sup>Il2rg<sup>−/−</sup> (NOD-RG) (Pearson et al., 2008), and BALB/c.Rag1<sup>−/−</sup>Il2rg<sup>−/−</sup> (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 ζ (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<sup>−/−</sup>Il2rg<sup>−/−</sup> 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 cytotoxicity 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.
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 mature 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 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 KitW41 or KitWv 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 KitWv mutation (BRGSK). The homozygous KitWv BRGS mouse (BRGSKWv/+ ) was established by introducing the KitWv allele of the C57BL/6J-KitWv mouse into the BRGS mouse (Yamauchi et al., 2013). Because mice homozygous for KitWv are sterile (Nocka et al., 1990; Sharma et al., 2007), the BRGSKWv/Wv mouse was obtained by mating BRGSKWv/+ pairs.

BRGSKWv/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-KitWv mice (Nocka et al., 1990; Sharma et al., 2007). The number of leukocytes and platelets was not significantly different to that in BRGS mice. BRGSKWv/+ and BRGSKWv/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 BRGSKWv/+ and BRGSKWv/Wv mice do not develop bone marrow failure until at least 30 weeks after birth.

**RESULTS**

**Establishment and Characterization of the BRGSK Mouse Line**

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

BRGSKWv/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-KitWv mice (Nocka et al., 1990; Sharma et al., 2007). The number of leukocytes and platelets was not significantly different to that in BRGS mice. BRGSKWv/+ and BRGSKWv/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 BRGSKWv/+ and BRGSKWv/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, BRGSKWv/+ , and BRGSKWv/Wv mice were sublethally irradiated, and injected with 3.5–5 × 10⁴ 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 BRGSKWv/+ and BRGSKWv/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 BRGSKWv/+ and BRGSKWv/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 BRGSKWv/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; Yahata et al., 2003). Interestingly, even in the non-injected
left femur, the average human CD45+ cell chimerism reached 82.0% and 97.4% in BRGSKWv/+ and BRGSKWv/Wv mice, respectively, compared with 56.7% in BRGS mice (Figure 2B, left panel). These data clearly show that the introduction of the KitWv 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). BRGSKWv/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 BRGSKWv/+ mice (average = 38.9% and 54.6%, respectively) (Figure 2A, right panel). In the non-injected left femur, only BRGSKWv/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 BRGSKWv/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 KitWv mutations into the BRGS strain is critical for the long-term maintenance and self-renewal of human HSCs.

**Figure 1. BRGSKWv/Wv Mice Develop Severe Macrocytic Anemia**
(A) The appearance of the BRGSKWv/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, BRGSKWv/+ and BRGSKWv/Wv mice (n = 9 mice per strain). All BRGSKWv/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), BRGSKWv/+ (n = 30), and BRGSKWv/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, BRGSKWv/+ and BRGSKWv/Wv mice (n = 4 mice per group). Error bars indicate SEM. *p < 0.05, **p < 0.01. LS K, Lin−/SCA-1−/c-KIT+.
**BRGSK<sup>Wv/Wv</sup> 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<sup>Wv/+</sup> 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<sup>Wv/Wv</sup> 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<sup>Wv/Wv</sup> mice. The enhanced multilineage reconstitution in BRGSK<sup>Wv/Wv</sup> recipients was also seen in the non-injected left femur (Figure S3).

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<sup>Wv/Wv</sup> mice. Neutrophils, eosinophils, basophils, and mast cells were separated by fluorescence-activated cell sorting (FACS) as side scatter (SSC)<sup>high</sup>CD125<sup>+</sup>, SSC<sup>high</sup>CD125<sup>+</sup>, SSC<sup>low</sup>CD14<sup>−</sup>CD117<sup>−</sup>FcR<sup>+</sup>, and SSC<sup>low</sup>CD14<sup>++</sup>CD117<sup>−</sup>CD123<sup>++</sup>HLA-DR<sup>+</sup> populations, respectively (Figure 4A). SSC<sup>low</sup>CD11b<sup>−</sup>CD14<sup>+</sup>CD123<sup>−</sup>HLA-DR<sup>+</sup> conventional dendritic cells (cDCs), and SSC<sup>low</sup>CD14<sup>−</sup>CD11c<sup>−</sup>CD123<sup>−</sup>HLA-DR<sup>−</sup> plasmacytoid dendritic cells (pDCs) were also evident. Figure 4B shows the morphology of each myeloid subset purified from BRGSK<sup>Wv/Wv</sup> 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, RNAS2 and CCL 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<sup>Wv/Wv</sup> mouse model. There were no significant differences in the composition of myeloid cells among BRGS and BRGSK strains (Figure 4D).
BRGS and BRGSKWv/+ mice. Strikingly, BRGSKWv/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 BRGSKWv/+ and BRGSKWv/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 BRGSKWv/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). BRGSKWv/Wv mice displayed every stage of human erythropoiesis defined by the expression pattern of CD71 and CD235a.
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 BRGSKWv/Wv recipient bone marrow. High numbers of CD235a+ erythroid cells were present in BRGSKWv/Wv 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). BRGSKWv/Wv 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 BRGSKWv/Wv mice.
To visualize the broad distribution of human erythroid cells in BRGSK<sup>Wv/Wv</sup> 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<sup>+</sup> or mouse TER119<sup>+</sup> 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<sup>+</sup> cells formed islands, mouse TER119<sup>+</sup> 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<sup>Wv/Wv</sup> mice.

Systemic Depletion of Macrophages Releases Human Erythrocytes and Platelets into the Circulation of BRGSK<sup>Wv/Wv</sup> 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.,...
2013; Takagi et al., 2012). They were also undetectable in BRGSKWv/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 BRGSKWv/Wv mice with human hematopoietic reconstitution (data not shown). Previous studies reported that after xenotransplantation of a high dose (1–5 × 10⁵ cells) of human CD34⁺ fetal liver cells, which have strong erythroid expansion potential (Holyoake 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 BRGSKWv/Wv mice transplanted with human CB cells. Injection of clodronate liposomes (100–200 µL 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 BRGSKWv/Wv mouse bone marrow; whereas mouse macrophages in BRGSKWv/Wv mice might still prevent these cells from circulating, presumably by engulfing them in the bone marrow and/or spleen.

Figure 6. Immunohistochemistry and 3D Confocal Immunofluorescence Imaging of Human Megakaryo-Erythroid Lineage Cells in BRGSKWv/Wv Mice
(A) Immunohistochemical analysis of human CD235a, hemoglobin subunit α (Hba), and CD41 expression in the femoral bone marrow of BRGS and BRGSKWv/Wv mice (magnification: top row, ×100; bottom row, ×600). Representative images of four independent experiments are shown. Scale bars: top row, 200 µm; bottom row, 20 µm.
(B) Relative mRNA expression levels of human hemoglobin subunit β (HBB) in human myeloid and CD235a⁺ erythroid cells purified from BRGSKWv/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 BRGSKWv/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 BRGSKWv/Wv recipients with high erythroid chimerism (C) and with intermediate erythroid chimerism (D) are shown. Scale bars, 800 µm. (E) Extended images of the square area in (D). Data shown are representative of six independent experiments. Scale bar, 50 µm.
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 KitWv (BRGSKitWv/Wv) repopulated robust human erythropoiesis and thrombopoiesis as well as myelopoiesis.

Among the Kit mutations, KitW, the null mutant, is the most severe type, and KitWv and KitW41, which lack the intracellular signaling portion of KIT, are less severe (Nocka et al., 1990). Mice homozygous for KitW are lethal, those homozygous for KitWv are viable but sterile, and those homozygous for KitW41 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 KitWv 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 KitW41 or KitWv 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 × 10⁴ to 3 × 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 BRGSKitWv/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 (3.5–5 × 10^3 cells) of human CD34^+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 BRGSKWv/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 BRGSKWv/Wv mice. In addition, maturation of human erythroid cells was imperfect, as the number of CD71^−CD235a^+ mature erythrocytes was limited. Human erythroid reconstitution was not improved further by injecting recombinant human erythropoietin into BRGSKWv/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 BRGSKWv/Wv mice.

We did not observe circulating erythrocytes or platelets, even in BRGSKWv/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 BRGSKWv/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. Clodronate 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 (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 BRGSKWv/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 10^7 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 BRGSKWv/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 BRGSKWv/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**

C57BL/6-J-Kit^{Wv} mice were purchased from the Jackson Laboratory. BRGS mice were developed in our laboratory (Yamauchi et al., 2013). The BRGSK^{Wv/+} mouse was generated by breeding BRGS and heterozygous C57BL/6-J-Kit^{Wv} mice, and the BRGSK^{Wv/Wv} mouse line was maintained by crossing BRGSK^{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 KitWv allele on radiation sensitivities. Groups of 6- to 10-week-old heterozygous or homozygous C57BL/6j-KitWv mice were irradiated by a 77.7-TBq (2.1 kCi) 137Cs γ-iradiator with a dose rate of 70 Gy/min and monitored for 8 weeks. As shown in Figure S1A, most C57BL/6j-KitWv heterozygotes receiving greater than 550 cGy irradiation died (Figure S1A), and all C57BL/6j-KitWv 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-KitWv mice. BRGSKWv/+ and BRGSKWv/Wv 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 3.5–5 × 10⁵ sorted Lin−CD34+CD38−/−CB cells were injected intravenously into 6- to 8-week-old BRGSK and control BRGS mice. BRGS, BRGSKWv/+, and BRGSKWv/Wv 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. 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 μL per mouse for the first injection and then 100 μL 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 BRGSKWv/Wv 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. Kunisaki performed the whole-mount imaging experiments. Y. Ki-kushige, K.K., T.M., H.I., and K.A. designed the experiments, reviewed the data, and edited the manuscript.

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