Erythroid-Specific Expression of β-globin from *Sleeping Beauty*-Transduced Human Hematopoietic Progenitor Cells

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Abstract

Gene therapy for sickle cell disease will require efficient delivery of a tightly regulated and stably expressed gene product to provide an effective therapy. In this study we utilized the non-viral *Sleeping Beauty* (SB) transposon system using the SB100X hyperactive transposase to transduce human cord blood CD34⁺ cells with DsRed and a hybrid IHK–β-globin transgene. IHK transduced cells were successfully differentiated into multiple lineages which all showed transgene integration. The mature erythroid cells had an increased β-globin to γ-globin ratio from 0.66±0.08 to 1.05±0.12 (p=0.05), indicating expression of β-globin from the integrated SB transgene. IHK–β-globin mRNA was found in non-erythroid cell types, similar to native β-globin mRNA that was also expressed at low levels. Additional studies in the hematopoietic K562 cell line confirmed the ability of chS4 insulator elements to protect DsRed and IHK–β-globin transgenes from silencing in long-term culture conditions. Insulated transgenes had statistically significant improvement in the maintenance of long term expression, while preserving transgene regulation. These results support the use of *Sleeping Beauty* vectors in carrying an insulated IHK–β-globin transgene for gene therapy of sickle cell disease.

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Introduction

Hematopoietic stem cells (HSCs) are an attractive target for genetic modification to treat diseases such as sickle cell anemia, β-thalassemia, and severe combined immunodeficiency (SCID), among others [1,2]. The ability of HSCs to be harvested with relative ease, withstand ex vivo manipulation and their long-established clinical use in transplants allows for numerous potential gene therapy strategies. This is supported by a recent report of successful treatment of a β-thalassemia patient using a recombinant hemoglobin-expressing lentivirus to transduce autologous CD34⁺ cells [3]. However, as in other viral vector clinical trials, significant and potentially problematic clonal expansion was observed.

HSCs give rise to a diverse number of cell types and maintain their ability to self-renew [4,5]. The current methods for gene transfer in clinical trials rely principally on the use of modified retroviruses such as Moloney murine leukemia virus (MMLV) and lentivirus, which have been known to insert into actively transcribed genes and potentially promote oncogenesis [6–8]. The development of leukemia in 4 out of 20 patients enrolled in a trial of MLV-based therapy for X-SCID continues to be a cautionary example to the field of gene therapy [9,10].

Non-viral vectors offer a more easily implemented and potentially safer method to genetically modify cells [11–13]. Non-viral vectors, however, are challenged by relatively low gene transfer efficiency and the difficulty of maintaining long-term stable expression [13–15]. Transposons can be utilized to provide an integration mechanism for stable copy number and long-term expression important for non-viral gene therapies [16–19]. The *Sleeping Beauty* (SB) transposon system has become increasingly useful in fields ranging from cancer biology and stem cell research to gene therapy over the last decade since its resurrection from the salmonid genome [17,20,21]. The recent development of the SB100X transposase and its utility in modifying primary CD34⁺ cells are evidence of the system’s continued improvements and potential clinical significance [22,23].

In this study, we used the SB transposon to develop an optimal expression system for erythroid-specific gene expression in specific lineages [24]. Typical viral vectors drive erythroid-specific β-globin expression using 2.7 kilobases (kb) from the β-globin locus control region and promoter [25]. This large size makes the approach impractical for the SB system, which loses efficiency in a linear fashion from 2-kb cargos up to a practical limit of 6–10 kb [16,22]. The 1-kb erythroid promoter IHK can provide high-level...
expression of β-globin in hematopoietic cells [26,27]. The IHK promoter is composed of the ALAS2 intron 3 strong erythroid enhancer, the HS40 core element upstream from the γ-globin gene, and the Ankyrin-1 promoter [27].

Even with improved integration efficiency and transgene design, the SB transposon system is still subject to epigenetic changes in the host genome such as DNA methylation, and histone modification [28–30]. In a recent report, the heterologous chicken HS4 β-globin insulator elements (cHS4) flanking a fluorescent transgene were employed in SB vectors and provided protection against progressive silencing of transposon integration sites [21]. This chicken HS4 insulator element was able to block enhancer activity, eliminating or diminishing the influence of enhancer elements on genes in close proximity [31,32]. In addition, it has been shown to protect transgenes from silencing by epigenetic modifications such as CpG methylation when the transgene is flanked on both sides by cHS4 insulator elements [33,34]. Furthermore, this enhancer blocking activity of the insulator may mitigate the potential transactivation of neighboring host genes by SB-mediated transgene insertion, which has been observed in a few insertion loci in previous studies [28,35].

In this study, the IHK-β-globin gene was used in combination with the SB100X transposase system to provide erythroid-specific expression of β-globin when integrated into primary CD34+ cells, and differentiated into multiple lineages. In addition, our results confirmed previous reports that the incorporation of insulator elements into the SB construct(s) protects transgenes against long-term inactivation, possibly from epigenetic changes, which reduce expression of integrated transgenes over time [21,35]. Together, the results support the potential use of a SB IHK-β-globin expression system for the gene therapy of sickle cell disease in human clinical trials.

Results

SB100X transduces CD34+ cells in cis or trans

To test the utility of a cis SB100X construct, 5 × 10^5 CD34+ cells from freshly isolated cord blood were mock-nucleofected without DNA, nucleofected with either 10 µg of pKT2/CAGGS-DSRed, 10 µg of pKT2/CAGGS-DSRed plus 5 µg of UbC-SB100X, or 10 µg of pKT2/meIF-SB100X-CAGGS-DSRed and recovered for 2 hours (Figure 1A). Cells were evaluated in standard CFU assays. In the non-transposase pKT2/CAGGS-DSRed condition, we observed no DSRed positive colonies at 14–16 days and comparable viability to the cis plasmid post-nucleofection (data not shown). The ability of SB100X to transduce hematopoietic cells and the effect of nucleofected DNA on colony yields was assessed. The cis SB100X vector averaged 5% DSRed+ colonies compared with 8% for the trans SB100X vector (Figure 1B). Nucleofection resulted in DNA-induced toxicity with 51% viability with the cis vector and 34% in trans when normalized to mock-nucleofected controls (Figure 1C). DSRed+ cells were observed in BFU-E, CFU-GM, and CFU-GEMM cell types in both trans and cis, but not in the non-transposase controls (Figure 1D, cis shown; Figures S1 and S2). In addition, some single colonies displayed heterogeneous expression patterns (Figure 1E, cis shown; Figure S3), suggesting variable epigenetic changes.

Nucleofected CD34+ cells differentiate in vitro

To assess the erythroid specificity of the IHK promoter, we differentiated nucleofected CD34+ cells from cord blood into multiple lineages from both myeloid and lymphoid classes. CD34+ cells (7.5 × 10^5 to 1 × 10^6, >90% CD34+) obtained from three separate umbilical cords were nucleofected with 10 µg of pKT2/meIF-SB100X-IHK-β-Globin (Figure 2A). Cells were incubated for two hours in recovery media with cytokines and seeded into differentiation cultures for granulocytic, erythroidic, B-cell, T-cell, and myelocyte/monocyte development. Differentiated cultures were assessed for granulocyte, erythrocyte, B-cell, T-cell, and myelocyte/monocyte development by identification of surface markers using flow cytometry (Figure 2D). Granulocytes showed robust differentiation with over 90% of CD15+ cells after ten days. In contrast, B-cell cultures demonstrated ~10% CD19 expression at 3 weeks, consistent with previous reports on differentiation without specific enrichment [50]. T-cell cultures were associated with >90% CD7+ cells and 30% CD1a/CD7+ cells at 4 weeks. Myelocyte/monocyte cultures showed just over 40% of cells expressing CD14, CD33, or both. Erythroid cultures demonstrated that >95% of cells expressed CD235a and ~20% had enucleated by 18 days as determined by DRAQ5 staining. The erythroid cultures were followed from a low density seeding (day 0) through expansion and seeding onto a confluent MS-5 feeder layer (days 8 and 11, respectively) to maturation and hemoglobinization (days 15 and 18; Figure 2B). By day 18, nucleated erythroid cells, enucleated biconcave cells, and recently enucleated cup-shaped cells were apparent throughout the culture (black arrows left-to-right; Figure 2C).

IHK-β-globin transgene mediates erythroid-specific hemoglobin expression

To test IHK-β-globin transgene expression, purified genomic DNA, RNA, and protein was isolated from differentiated erythrocytes, granulocytes, myelocytes, monocytes, B-cells, and T-cells and examined for IHK-β-globin. The IHK-β-globin gene was detected in genomic DNA from all trials, while SB100X coding sequence was found in 10 of 13 trials using a highly sensitive PCR assay (Figure 3, trial 1). Optimization of multiplex genomic PCR demonstrated an ability to detect SB100X DNA down to 3 copies (Figure S4). In two of the three erythroid trials the SB100X-coding sequence was not detectable, likely attributable to a lower seeding density and a higher level of expansion compared to other lineages. Expression of β-globin in the differentiated populations was determined by western blotting with conditions optimized to detect as little as 3 ng of adult hemoglobin spiked into 50 µg of white blood cell lysate (Figure S5). Both IHK-β-globin transduced and control erythroid cultures showed robust β-globin expression, while expression was not detectable in the non-erythrocyte lineages (Figure 4; Figures S6 and S7). HPLC analysis of hemoglobin from control and IHK transduced-erythroid cells showed a significant increase (p<0.05) in the ratio of β-globin to γ-globin from 0.66±0.08 to 1.05±0.12 in the three IHK transduced erythroid cultures relative to the threec control cultures (Figure 5).

β-globin transcript is increased in non-erythroid progeny of IHK-transduced CD34+ cells while SB100X transcript is undetectable

RNA from differentiated cells derived from IHK-transduced CD34+ cells was subjected to RT-PCR using primers specific for β-actin and β-globin (Figure 6; Figures S8 and S9). All sets of samples showed similar levels of β-actin. Erythroid cells had increased β-globin transcript expression with the IHK-β-globin compared to control erythroid cells. Interestingly, the β-globin transcript was found in both the WBC control and at a higher level in the non-erythroid cells derived from IHK-transduced CD34+ cells. Because copies of SB100X-coding sequence were found to be carried in some transduced cells, RT-PCR for SB100X RNA was...
also performed which showed no detectable SB100X transcript (data not shown), thereby excluding the possibility of SB transposon mobility at this stage.

Protection of transgene from progressive silencing by cHS4 insulator elements

cHS4 insulator elements were introduced into the SB transposon vector system to test whether cHS4 insulator elements can provide protection against progressive silencing of the transgene [21,28,30]. The CAGGS-DsRed transgene cassette was cloned into a helper-independent cis SB vector containing the hyperactive SB transposase, SB100X, as pKT2/melF-SB100X-Ins-CAGGS-DsRed-Ins (Figure 7A). In this construct, each boundary of the CAGGS-DsRed transgene cassette was flanked by a complete 1.2-kb cHS4 insulator element placed in parallel to limit potential homologous recombination between the two elements. K562 erythroid cells were transfected with pKT2/melF-SB100X-Ins-CAGGS-DsRed-Ins or non-insulated control, pKT2/melF-SB100X-CAGGS-DsRed vectors. Two days after transfection, cells positive for DsRed were sorted into 96-well plates at a density less than 1 cell per well for both groups of transfected cells. Single-cell clones were isolated by either manual selection or FACS/Aria cell sorting. These clones were examined for the expression of DsRed on the 96-well plates, and DsRed-positive clones were further expanded to 24-well plates. Initially, 67 independent DsRed-positive clones from cells transfected with the insulated pKT2/melF-SB100X-Ins-CAGGS-DsRed-Ins vector and 56 individual DsRed-positive clones from K562 cells transfected with non-insulated control pKT2/melF-SB100X-CAGGS-DsRed vector were isolated by FACS/Aria or manual selection.

Between 2 and 4 weeks after transfection, each single-cell-derived clonal population was collected and analyzed for DsRed expression by flow cytometry. The expression of DsRed in clones was assessed once again at 13 to 15 weeks post-transfection, by using parameters normalized to the initial analysis at 2 to 4 weeks after transfection. Clones derived from cHS4-insulated SB constructs maintained the mean intensity of DsRed expression as well as the proportion of DsRed-positive cells at 13–15 weeks post-transfection when compared with the initial data obtained at 2–4 weeks. This was in contrast to clones from the non-insulated control SB vectors demonstrating long-term silencing as noted by significant decrease in the mean intensity of DsRed expression and percent DsRed+ cells (Figures 7B and C). While the majority of clones in both trials maintained relatively stable expression, comparison of clones showing the greatest losses and gains in percent DsRed+ cells for each vector demonstrated the potential for non-insulated transgenes to be highly down-regulated and insulated transgens to gain expression over time. DsRed expression profiles of the 5 highest ranked clones for negative and positive shifts from each SB vector during long-term culture
are compared (Figures 7D and E, respectively) while many clones showed little change between the time points.

To test the effect of the insulators on long-term \( \beta \)-globin transgene expression, we transfected K562 cells with the cHS4-insulated \( \beta \)-globin-expressing vector pKT2/meIF-SB100X-II\( \beta \)g and the non-insulated control pKT2/meIF-SB100X-I\( \beta \)g. Two days after transfection, single cells were sorted manually and cultured in 96-well plates for two weeks, transferred to 24-well plates for 2-weeks and analyzed for genomic IHK by PCR. For IHK\( ^{\text{+}} \) clones, hemin and non-hemin treated samples were collected at 4 and 14 weeks. Quantification of \( \beta \)-globin protein expression in hemin-induced samples at both time points showed a
IHK is its use of erythroid enhancer and promoter elements, to provide therapeutic expression [22]. An interesting feature of transposition and to lower the number of insertion sites necessary SB drive hemoglobin expression [22,27]. It is important to reduce the vectors makes it well suited for the small size of IHK relative to promoter than the previous studies have shown that IHK is potentially a more active progeny, while maintaining tight, erythroid-specific expression. IHK-globin, can express hemoglobin in mature erythroid progeny. Hypotonic lysates from fetal RBCs from the donor cords, erythrocytes from non-transduced CD34 cells, and erythrocytes from IHK-transduced CD34 cells were analyzed by RP-HPLC to determine relative quantities of individual globin chains. Data from three trials are presented as mean ± SEM, * indicates p<0.05, paired t-test.

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significant (p<0.001) loss of expression in the non-insulated vectors relative to the insulated vectors (Figure 8A). The change represents a mean loss of 13.1±81.6% of β-globin expression in the insulated group and of 66.1±50.3% of β-globin expression in the non-insulated group. In summary, the results were similar to those generated with the insulated and non-insulated SB vectors for DsRed. To investigate the effect of the insulators on IHK-β-globin regulation we tested the response of clones in each group to induction with hemin, which stimulates hemoglobin production and upregulation of erythroid-associated genes [37]. Induced clones from the insulated and non-insulated groups showed no significant differences (1.88±1.29 fold and 1.77±1.75 fold increases, respectively) in β-globin expression upon hemin induction (Figure 8B).

Discussion

In this study we have shown that primary human CD34+ cells can be transduced with the SB construct, pKT2/melF-100X-IHK-β-globin, can express hemoglobin in mature erythroid progeny, while maintaining tight, erythroid-specific expression. Previous studies have shown that IHK is potentially a more active promoter than the β-globin LCR, albeit in a viral vector. The small size of IHK relative to β-globin LCR/promoter-based vectors makes it well suited for the SB system as a mechanism to drive hemoglobin expression [22,27]. It is important to reduce the overall size of the SB transgene cargo to promote efficient transposition and to lower the number of insertion sites necessary to provide therapeutic expression [22]. An interesting feature of IHK is its use of erythroid enhancer and promoter elements, which are not directly involved in normal β-globin production. While the IHK acts as a strong promoter in erythroid cells, we have previously shown that the native introns and untranslated regions of the β-globin gene are crucial for high-level expression of protein [26].

Analysis of the transgene content and expression of pKT2/melF-100X-IHK-β-globin transduced cells showed that each of the nucleofected trials retained the IHK transgene in each lineage after 10 to 28 days of differentiation. In this study, as previously shown, we did find a low-level of transcription in non-erythroid cells, which was translationally suppressed and failed to produce detectable β-globin [26]. In that the inserted transposon still contains IR/DRs necessary for transposition, the potential remobilization of the transposon is a significant concern. We found that SB transposase-coding DNA can be detected at low levels within a transduced population but does not express detectable mRNA as determined by RT-PCR, consistent with a recent study of SB transduced cells [38].

In order to achieve maximum efficiency of transposon insertion into the host genome, we employed an enhanced transposase, SB100X, which was shown to be ~100 times as active as the original version of the Sleeping Beauty transposase [39]. Stable gene transfer efficiencies of almost 50% were achieved with SB100X in the human cells differentiated from transfected hematopoietic CD34+ cells in remarkable contrast to stable gene transfer efficiencies of the original SB transposase (0.2 to 1%) or of second-generation hyperactive SB11 [16]. These previous results support the potential use of SB100X as a gene transfer agent for human CD34+ hematopoietic cells, and further development of the SB system in general.

We have also shown that cHS4 insulator elements can mitigate the tendency for SB-inserted transgenes to be inactivated over the long-term [30]. Long-term stable expression of a therapeutic transgene is critical in the successful gene delivery for genetic diseases such as β-thalassemia or sickle cell anemia. There are numerous reports of transgene silencing associated with delivery by either retroviral vectors or non-viral SB vectors [30,40]. Most of this long-term inactivation of transgenes can be attributed to epigenetic changes such as DNA methylation or chromatin modifications in the inserted transgenes [29,30,41]. The chicken HS4 insulator element employed here has previously been shown to block enhancer activity, eliminating or diminishing the influence of enhancer elements on genes in close proximity whether the enhancer is included within the transgene or placed close to the insertion site in the host genome [31,32]. In addition, the cHS4 insulator is known to protect transgene from silencing by
epigenetic modification such as CpG methylation when the transgene is flanked by cHS4 insulator elements [33,34].

In this study, cHS4 insulator elements successfully protected both CAGGS-DsRed and IHK–β-globin transgenes from progressive silencing in the context of SB transposon system.

Importantly, we showed that a cHS4-flanked transgene prevented sharp inactivation of the transgene and in some cases increased transgene expression over the 14-week culture period. The results are consistent with a previous report in which the insulators prevented long-term silencing of a yellow fluorescent protein.
transgene integrated into host genome by SB [21]. This is especially important in potential gene therapies where increasing vector copy numbers to compensate for inactivation increases the risk of harmful mutagenesis [14]. Insulators also play a role in preventing local changes in gene expression due to insertion sites. The appropriate regulation of an insulated IHK-β-globin transgene is also important for potential therapies and in this study we found no significant differences in transgene expression upon differentiation between K562 cells transduced with insulated or non-insulated vectors. Insulators combined with the erythroid-specific IHK promoter could potentially confer long-term therapeutic levels of hemoglobin for the treatment of sickle cell disease, β-thalassemia, and other hemoglobinopathies utilizing CD34+ cells.

This combination of a highly efficient non-viral vector, a cargo protected from epigenetic changes, and a tightly regulated transgene is potentially of great utility in a clinical setting. Further in vitro and in vivo studies are needed to evaluate the pKT2/mefIF-SB100X-ILlglg and variant vectors in primary hematopoietic cells over long term experiments and the impact of such vectors on disease models. Expanding on the results of this study will lead to the development of a vector that will be suitable for clinical trials.

Methods

Sleeping Beauty transposon constructs

The cis pKT2-mefIF-SB100X and trans pKT2-RV SB transposon vectors were produced using standard molecular cloning techniques (Figure 1). The hyperactive SB100X transposase gene was driven by the constitutive murine eukaryotic initiation factor 4A1 promoter (Invitrogen, Carlsbad, CA) [22]. In order to protect the transgenes from long-term silencing, we constructed SB transposon vectors containing two insulator elements flanking multiple restriction endonuclease recognition sites derived from pIRES2-EGFP (Invitrogen) [31,42]. Chicken HS4 insulator elements (1.2 kb) were transferred to the BamHI and SacI sites of pIRES2-EGFP from the plasmid DNA construct, pC13-1 (kindly provided by Dr. Gary Felsenfeld, NIDDK, NIH), by digestion of pC13-1 with XbaI or SacI, respectively [31]. Ends of released insulator elements and digested pIRES2-EGFP vectors were modified to be compatible with the ligation. After successful transfer of two cHS4 insulator elements into pRES2-EGFP, a DNA fragment encompassing two cHS4 insulator elements and a partial multicloning site (MCS) between the insulators was cloned into pKT2-mefIF-SB100X or pKT2-RV SB vectors by modification of DNA ends with Klenow, T4 DNA polymerase and Antarcitic Phosphatase (New England Biolabs, Ipswich, MA). This resulted in the insulated SB vectors, pKT2/mefIF-SB100X-Ins-MCS-Ins or pKT2/RV-Ins-MCS-Ins. The two insulators encompassing the partial multicloning site were inserted in the same, parallel direction to minimize loss of transgenes by recombination. As a final step, the 3.2 kb IHK-β-globin transgene fragment (Figure S10) was derived from pT2/IHK-β-globin/ cIF-SB10 by digestion with PstI, and introduced into the partial MCS of pKT2/mefIF-SB100X-Ins-MCS-Ins and pKT2/RV-Ins-MCS-Ins. These constructs are hereafter referred to as pKT2/mefIF-SB100X-IHgIβg and pKT2/RV-IHgIβg, respectively. Control SB-IHK-β-globin constructs with no insulator elements were made by cloning the 3.2- kb IHK-β-globin transgene fragment into pKT2-mefIF-SB100X and pKT2-RV vectors by EcoRV digestion and blunt-end ligations (pKT2/mefIF-SB100X-IHgIβg and pKT2/RV-IHgIβg, respectively).

SB vectors of cHS4-insulated pKT2/meIF-SB100X-Ins-MCS-Ins and pKT2/RV-Ins-MCS-Ins in a similar manner. CAGGS-DSRed transgene was derived from digestion of pCAGGS-DSRed with SpeI and HindIII, where DSRed transgene was obtained with PCR from pDsRed-Express (Clontech, Mountain View, CA) and cloned into pCAGGS vector [43]. The CAGGS-DSRed transgene fragment was then blunted by Klenow (New England Biolabs) and cloned into SacI recognition site of pKT2/mefIF-SB100X-Ins-MCS-Ins and pKT2/RV-Ins-MCS-Ins, making pKT2/mefIF-SB100X-InsCAGGS-DSRed-Ins and pKT2/RV-Ins-CAGGS-DSRed-Ins, respectively. Non-insulated control vectors, pKT2/mefIF-SB100X-CAGGS-DSRed and pKT2/RV-CAGGS-DSRed, were generated by directly cloning SpeI-HindIII CAGGS-DSRed transgene into original pKT2-mefIF-SB100X and pKT2-RV, via blunt-end ligation.

K562 cell culture, transfection, and isolation of single-cell clones

K562 (ATCC catalog number CCL-243) cells were maintained in RPMI 1640 (Invitrogen) supplemented with 10% FBS (Omega Scientific, Tarzana, CA). Eighteen hours prior to transfection, 2×10^6 cells were freshly plated in 100-mm Petri dishes in RPMI 1640 medium without antibiotics. Four μg of plasmid DNA (pKT2/mefIF-SB100X-Ins-CAGGS-DSRed-Ins or non-insulated control vectors, pKT2/mefIF-SB100X-CAGGS-DSRed) was transfectected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells were passaged to fresh medium after 18 hours and DSRed single-cell clones were sorted into 96-well plates via FACSAria (BD Biosciences, San Jose, CA) or manual isolation under epifluorescence microscopy. Single-cell clones were then transferred to 24-well plates and maintained for long-term (≥12 weeks) studies.

K562 cells were also transfectected with pKT2/mefIF-SB100X-IHgIβg and pKT2/mefIF-SB100X-IHgIβg as above and PCR screening of clones for the IHK sequence and confirmation of β-globin expression at 4 weeks after transfection was used to select clones for long-term culture. β-globin expression was induced with 20 μM hemin added to the culture media for 72 hours prior to collection for western blot analysis.
Western blot analysis

For analysis of K562 derived clones, western blot analysis was conducted as previously described [26]. Nitrocellulose blots were stained simultaneously with mouse antibodies against human β-globin (sc-21757; Santa Cruz Biotechnology, Santa Cruz, CA) and β-actin (AC-15; Sigma-Aldrich, St. Louis, MO) at 1:1000 dilutions. Blots were visualized using HRP-conjugated goat anti-mouse secondary antibody (32430; Thermo Scientific, Rockford, IL), Super Signal Dura substrate (34076, Thermo Scientific), and X-ray film (BioMax; Eastman Kodak, Rochester, NY). For analysis of CD34+ derived cells, western blot analyses were conducted as described previously [26,44]. Pre-cast SDS-PAGE gels (Mini-PROTEAN TGX 4–20%; Bio-Rad, Hercules, CA) were loaded with differentiated cell lysates, resolved at 100 volts, and cut into upper and lower sections with the aid of a pre-stained ladder at 25–30 kDa (GE Healthcare, Little Chalfont, United Kingdom). The upper portion was transferred and analyzed for β-globin as previously described [26]. The lower portion was transferred and analyzed for β-globin using a modified technique as reported [44]. The modified transfer for β-globin was performed for 20 min at 30 volts and the membranes were fixed into pre-warmed and equilibrated recovery medium (10 ng/ml Flt-3L (all Invitrogen) and cultured in 6-well plates for 10 days) consisting of X-VIVO 10 without phenol red or gentamycin removed into pre-warmed and equilibrated recovery medium + remove any residual red blood cells. Cells were washed three times and background correction.

Analyzed using established guidelines [45]. Briefly, multiple CD34+ cells were separated using Miltenyi MACS CD34 magnetic beads (Miltenyi Biotechnology, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. CD34+ purity was assessed using flow cytometry and was >90% in all cases [Figure S11]. Cells were counted and resuspended at 0.75 to 1×10⁶ cells per 100 μl of CD34+ complete Nucleofection solution (Lonza, Basel, Switzerland). Cells were mixed with plasmid DNA and nucleofected using the Amaxa Nucleofector II with program U-008. Nucleofected cells were immediately removed into pre-warmed and equilibrated recovery medium consisting of X-VIVO 10 without phenol red or gentamycin (Lonza), 100 ng/ml Stem Cell Factor (SCF), 10 ng/ml IL-3, 10 ng/ml Flt-3L (all Invitrogen) and cultured in 6-well plates for 2 hours prior to differentiation.

Differentiation of CD34+ cells

Erythroid differentiation was carried out on MS-5 stromal cells as previously described [46,47]. Briefly, 2×10⁵ cells were plated in 2 mL serum-free expansion medium containing 100 ng/ml SCF, 5 ng/ml IL-3, 3 U/ml erythropoietin (EPO), and 1 mM hydrocortisone (Sigma-Aldrich) and cultured in 6-wells plates at 37°C and 5% CO₂ for 4 days. The cells were then diluted into 6 ml of the same medium in a 25 cm² flask and cultured for an additional 4 days. Expanded cells were washed with basal medium and resuspended in 25 ml of basal medium with 3 U/ml EPO and plated onto a newly confluent 75 cm² flask of MS-5 feeder cells and cultured for 3 days. For final maturation, erythroid cells were co-cultured on feeder cells for 10 days using basal medium without cytokines with one medium change after 5 days at which time 2×10⁶ cells were harvested for nucleic acid purification. T-cell differentiation was carried on OP9 stromal cells as previously described [48]. Briefly, 5×10⁶ nucleofected cells were resuspended in αMEM (Invitrogen) with 20% FBS (Atlanta Biologicals, Lawrenceville, GA), 5 ng/ml IL-7, and 5 ng/ml Flt3L (both Peprotech, Rocky Hill, NJ) onto sub-confluent OP9-DL1 cells. Fresh media with cytokines was replaced every 3 to 4 days. B-cell differentiation was carried out on MS-5 stromal cells as reported [36]. Briefly, 1.5×10⁶ nucleofected CD34+ cells were resuspended in 15 ml of αMEM with 10% FBS, 10 ng/ml SCF, and 10 ng/ml G-CSF and seeded onto a confluent MS-5 layer in a 75 cm² flask. Ten milliliters of fresh medium was added after 7 days and cells were maintained with twice weekly half-media changes. Myeloid and granulocyte differentiations were performed on MS-5 stromal cells as described [25,49]. Briefly, 10⁵ nucleofected CD34+ cells were seeded in 1 ml IMDM with 10% FBS, 2 mM L-salanyl-glutamine, 50 ng/ml SCF, and 50 ng/ml IL-3 in six-well plates. After 3 days in culture, cells were washed and seeded into 25 cm² flasks in basal medium plus 50 ng/ml SCF, 50 ng/ml IL-3, and 10 ng/ml G-CSF. Cells were maintained at 2×10⁶ to 8×10⁶ cells per ml in complete medium with cytokines for 10 days until harvest.

Colonization assays

Colonization assays were performed using MethoCult 4435 as recommended by the manufacturer (StemCell Technologies, Vancouver, Canada). Cells were seeded in quadruplicate and colonies were scored after 14–16 days using an Olympus IX70 epifluorescence light microscope (Olympus, Tokyo, Japan). Digital images were batch processed using ImageMagick 6.0 (imagemagick.org).

Flow cytometry

Cell surface markers were characterized using fluorochrome-conjugated antibodies specific for human antigens: CD34-Alexa Fluor647 (4H11; eBioscience, San Diego, CA), CD1a-FTTC (H149; BD Pharmingen), CD3-V450 (HIT3a; BD Horizon), CD7-Alexa Fluor700 (M-T701; BD Pharmingen), CD14-V450 (MphiP9; BD Horizon), CD15-PE-Cy7 (HI98; BD Pharmingen), CD19-Alexa Fluor700, CD33-AlexaFluor700, CD45-V500 (HI30; BD Horizon), CD45-PerCP-Cy5.5 (ID2; eBioscience), CD71-PE (OKT9; eBioscience), and CD233a-FTTC (H1R2; eBioscience). Cells were labeled in staining buffer (1X Hanks Balanced Salt Solution without Ca²⁺ or Mg²⁺+2% FBS +1 mM EDTA) in antibody dilutions of 1:100 at a concentration of 10⁶ cells per ml. Viability was assessed using 7-AAD (Southern Biotech, Birmingham, AL) or eFluor780 Fixable Viability Dye (eBioscience) and dead cells excluded from analysis. DNA content was assessed using DRAQ5 which was recommended by the manufacturer’s instructions (eBioscience). Data acquisition was performed using a BD LSRII flow cytometer (BD Biosciences). Cell sorting was performed using a BD FACSAria (BD Biosciences). Flow cytometric data was analyzed using FlowJo 7.6.4 software (Tree Star Inc).

Sample collection

Cells were harvested and frozen in liquid nitrogen until processed. DNA, RNA, and protein were purified using the Qagen AllPrep Mini kit (Qiagen, Valencia, CA). Briefly, progressive collection from sample
lysat began with DNA collection using DNeasy columns, followed by RNA collection with RNeasy columns, and protein precipitation and washing using Buffer APP as recommended by the manufacturer. For HPLC analysis of hemoglobin, 1.5 × 10^7 non-adherent erythroid cells were pelleted and lysed using 50 μl of HPLC grade water (Invitrogen); crude lysate was centrifuged at 13,000 G, and cleared lysate was frozen in liquid nitrogen and stored at −80°C prior to analysis.

**PCR analysis**

SB and IHK DNA detection was done in a multiplex format using Qiagen HotStarTaq hot-start PCR system using primers IG2, BAG3, and SS9, for HS40/Ankyrin-1 junction in the IHK promoter, an Intron/Exon junction in β-Actin, and the SB100X coding sequence, respectively (Table S1). Sixty ng of genomic DNA was used from each test condition and the reaction was assembled according to the manufacturer’s instructions and amplified using a two-phase cycle to test condition and the reaction was assembled according to the manufacturer’s instructions and amplified using a two-phase cycle to promote amplification of low-weights of SB100X DNA (Figure S4, Table S1). RT-PCR for β-globin and β-actin was conducted as previously described [26]. Briefly, 20 ng of total RNA was amplified with primers specific for β-actin and β-globin using the Titan One Tube RT-PCR kit (Roche Applied Sciences, Indianapolis, IN) according to the manufacturer’s instructions (Primers BAM1 and BGM1; Table S1). RT-PCR for SB100X was performed using primer set SS9 which is specific for the SB coding sequence and 100 ng of total RNA.

**HPLC analysis**

Globin chains were identified using a modification of the method described earlier [50]. An Agilent 1200 HPLC system (Santa Clara, CA) was used as a C4 column, a water/acetonitrile/trifluoroacetic acid gradient run at 0.7 ml/min at 25°C, and spectroscopic detection at 220 nm.

**Statistical analysis**

Statistical significance of data was performed using SigmaStat 3.5 software (Systat Software, Chicago, IL).

**Supporting Information**

Figure S1 DsRed-β CFUs imaged with a bright field filter (top row), pan-fluorescence filter (middle row), and rhodamine fluorescence filter (bottom row). (TIF)

Figure S2 Homogeneous DsRed-β CFUs imaged with a bright field filter (top row), pan-fluorescence filter (middle row), and rhodamine fluorescence filter (bottom row). (TIF)

Figure S3 Heterogeneous DsRed-β CFUs imaged with a bright field filter (top row), pan-fluorescence filter (middle row), and rhodamine fluorescence filter (bottom row). (TIF)

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