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Generation of transgenic mouse line expressing Kusabira Orange throughout body, including erythrocytes, by random segregation of provirus method

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ABSTRACT

Fluorescent-protein transgenic mice are useful for obtaining marked somatic cells to study kinetics of development or differentiation. Fluorescence-marked hematopoietic stem cells in particular are commonly used for studying hematopoiesis. However, as far as we know, no transgenic mouse line is described in which a fluorescent protein is stably and constitutively expressed in all hematopoietic cells, including erythrocytes and platelets. Using the random segregation of provirus (RSP) method, we generated from retrovirally transduced mouse embryonic stem cells a transgenic mouse line expressing a red/orange fluorescent protein, Kusabira Orange (KuO). KuO transgenic mouse line cells carry only one proviral integration site and stably express KuO in all hematopoietic-lineage elements, including erythrocytes and platelets. Moreover, bone-marrow transplantation in KuO transgenic mice demonstrated normal hematopoieisis. KuO transgenic mice likely will prove useful for study of hematopoiesis that includes erythropoiesis and megakaryopoiesis.

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1. Introduction

Red/Orange fluorescent proteins such as Discosoma red (DsRed) (excitation/emission wave lengths 558/583 nm), tdTomato (554/571 nm), mCherry (587/610), and Kusabira Orange (KuO) (548/561 nm) [1–3] are widely used to visualize cells, to track cells and their progeny, or to quantify molecules in living cells. Whilst high level expression of oligomeric red/orange fluorescent proteins may induce growth defects and instability of fluorescent-protein expression, monomeric red fluorescent protein (mRFP; 584/607) induces almost no toxicity [4,5]. Although previously immunogenicity of the enhanced green fluorescent protein (EGFP) [6,7] was reported, we found that mouse hematopoietic stem cells (mHSCs) transduced with humanized Kusabira Orange (KuO) behaved like wild-type mHSCs in recipient mice after transplantation [8]. Furthermore, no toxicity was apparent in KuO-expressing cloned pigs generated by nuclear transfer of KuO-transduced fibroblasts [9]. These results indicate that KuO is an appropriate fluorescent protein for biological research, including research into hematopoiesis.

Moloney murine leukemia virus (MoMLV)-based retroviral vectors have been most commonly used for gene transduction. MoMLV vectors, however, show low level transcriptional activity in embryonic or hematopoietic stem cells, due to promoter methylation or to promoter binding of transcriptional inhibitory factors [10–12].

We have improved the retroviral vector: GCDsap by long terminal repeat (LTR) modification to reduce susceptibility to promoter methylation. Using GCDsap carrying enhanced green fluorescent protein (EGFP), we successfully generated mouse embryonic stem cells (mESCs) expressing high levels of EGFP and, from those cells, EGFP transgenic mice (retro-GFP mice). EGFP was stably expressed even in F1 progeny (after germline transmission) [13]. In these retro-GFP mice, EGFP was expressed throughout the body, including

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leukocytes and platelets, but was not expressed in erythrocytes. This may owe to the location of the retroviral integration site, on the closed chromatin region in erythroid precursors. This lack of expression of EGFP in erythroid-lineage elements is a major restriction upon use of retro-GFP mice. As far as we know, no fluorescent-protein transgenic mouse is described in which fluorescent protein is expressed in all hematopoietic lineages, including erythroid precursors and erythrocytes.

We generated KuO-expressing transgenic mice derived from mESCs transduced by GCDsap carrying KuO (GCDsapKuO). In these mice, KuO was highly expressed in both solid tissues and hematopoietic cells, including HSCs and the five blood lineages (ending in neutrophils/macrophages, T-cells, B-cells, platelets, and erythrocytes). Finding the open chromatin region in Ter119 expressing-erythroid precursors was achieved through random segregation of provirus (RSP) after germline transmission from GCDsapKuO-infected mESCs that carry multiple proviruses. KuO transgenic mice showed no obvious abnormality in development, exhibited no tendency to hemorrhage, and did not develop tumors.

2. Materials and methods

2.1. Mice

C57BL/6 mice congenic for the Ly5 locus (B6-Ly5.2) mice were purchased from Japan SLC (Shizuoka, Japan) and Nihon Clea (Tokyo, Japan). B6-Ly5.1 mice and recombination activating 2 knockout (Rag2 KO) mice were bred and maintained at Sankyo Labo Service (Tokyo, Japan). All procedures were approved by the Animal Care and Use Committee, University of Tsukuba, and by the Institute of Medical Science, University of Tokyo.

2.2. Retroviral vectors

The retroviral vector [GCD/huKO(KuO)] was constructed by insertion of KuO cDNA which was isolated from phKO1-MC1 (AM-V0045; MBL, Tokyo, Japan) by digesting with NcoI and NotI. The fragment containing KuO cDNA was subcloned into the NcoI-NotI digested [GCDsap] vector [13].

The vector was converted to a corresponding retrovirus packaged in a VSV-G envelope by transduction into 293 gp [14]. The virus titer of GCD/KuO was approximately 2.0×10^7 infectious units (I.U.)/ml as assessed on Jurkat cells.

2.3. Transduction into ES cells

Mouse ES cells (E14) were cultured according to supplier's recommendations [15]. Single cell suspensions were transduced once or twice by adding 100–400 μl of concentrated virus supernatant with or without centrifugation (2000 rpm) for 30 min at 32 °C. KuO-expressing cells were counted and mean florescent protein intensity (M.I.) was quantified by flow cytometry (FACS Calibur; Becton–Dickinson, Franklin Lakes, NJ).

2.4. Blastocyst injection and generation of chimeric mice

Chimeric mice were generated conventionally; un-clonal KuO-transduced ESCs were microinjected into day 3.5 blastocysts of B6-Ly5.2 mice followed by transfer into host uteri as described [16]. The resultant chimeric mice were mated with B6-Ly5.1 mice. Sample tissues of chimeras and their progeny were observed under fluorescence microscopy using Axioplan 2 and AxioVision 3.1 software (Carl Zeiss, Oberkochen, Germany).

2.5. Southern blot analysis and inverse PCR

High-molecular-weight DNA was obtained from mouse tails. Genomic DNA was digested with BamHI, electrophoresed (1% agarose gel), transferred to a nylon membrane (Hybond-XL: Amersham Biosciences GE Healthcare, UK), and hybridized to [α-^{32}P]dCTP (deoxycytidine 5′-triphosphate)-labeled KuO cDNA. As the restriction enzyme BamHI cut the vector at only one site, the number of fragments hybridized with the probe was considered to be the number of provirus copies integrated into the host genome. Hybridization signals were detected with an auto image analyzer (FLA 5100, Fuji Film, Tokyo, Japan) after exposure to an imaging plate.

To analyze the retroviral integration sites of transgenic mice, inverse PCR was performed as described [17]. After 50 ng of genomic DNA was digested with Taq1 the fragments containing LTRs were self-ligated and were subjected to nested PCR using the LTR-specific primers F1:TCCGAATCGTGGACTCGCTG, F2:GGGTCTCCTCAGATTGATTG, R1:AGACAAGGATTGGAACTAGACTTG, and R2:GAACGT TTATTCGAATGAATTCG. The amplified fragments were sequenced and the genomic coordinates of the integration sites were determined by interrogation of the mouse genome database of the National Center for Biotechnology Information (NCBI).

2.6. Cell surface analysis

Hematopoietic cells were stained with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse Gr-1 (RB6-8C5; eBioscience, San Diego, CA), CD11b (M1/70; BD Pharmingen, Franklin Lakes, NJ), and CD41 (MWReg30; BD Pharmingen) antibodies; allophycocyanin (APC)-conjugated rat anti-mouse CD4 (RM4-5; eBioscience), CD8a (53–6.7; eBioscience), Ter119 (Ter119; BD Pharmingen), and CD117 (c-KIT: 2B8; eBioscience) antibodies; Phycoerythrin-Cyanian 7 (PE-Cy7)-conjugated rat anti-mouse B220 (RA3-6B2; eBioscience), Ly-6A (Sca-1:D7; eBioscience) antibodies; biotin-conjugated rat anti-mouse CD4 (RM4-5; eBioscience), CD8a (53–6.7; eBioscience), Ter119 (Ter119; BD Pharmingen), Gr-1 (RB6-8C5; eBioscience), CD11b (M1/70; BD Pharmingen), B220 (RA3-6B2; eBioscience), and CD127 (IL-7b receptor alpha [A7R34]; eBioscience); and APC/Cy7-conjugated streptavidin (BioLegend,San Diego, CA). After being washed with phosphate-buffered saline containing 2% fetal calf serum (FCS), stained cells were analyzed by flow cytometry using FACS Calibur or CantII equipment (Becton–Dickinson, Franklin Lakes, NJ).

2.7. HSC purification and transplantation

HSC purification and transplantation were carried out according to published protocols [18]. In brief, bone-marrow (BM) cells of Ly5.1 mice or KuO mice were suspended in phosphate-buffered saline (PBS). Low-density cells (<1.077 g/mL) were isolated using Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, England). Cells were suspended in 3% FCS in PBS and were stained with a lineage antibody mix consisting of biotinylated anti-Gr-1, -Mac1, -B220, -CD4, -CD8, -Ter119, and -interleukin-7 receptor antibodies. Lineage marker-expressing cells were depleted using magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were stained with fluorescein isothiocyanate-conjugated anti-CD34, APC-conjugated anti-c-Kit, PE-Cy7-conjugated anti-Sca-1, and the lineage antibody-mix antibodies. The biotinylated antibodies were developed with streptavidin-APC-Cy7. CD34$^-$ c-Kit$^+$Sca-1$^+$Linage$^-$ cells (34$^-$KSL cells) were sorted by flow cytometry (MoFlo, Beckman Coulter, Fullerton, CA).

Cells were mixed with competitor cells (total BM cells) at a desired ratio (e.g., 100 CD34$^-$KSL cells: 1×10^6 competitor

cells). Cells in 200 μl of medium were injected into each of a group of lethally irradiated Ly5.2 mice or 8 Gy-irradiated Rag2 knockout mice. To perform secondary transplantation, BM cells collected from each femur of all recipient mice were pooled. Each secondary-transplant recipient mouse received BM cells equivalent to one-quarter of the total femoral BM of a primary-transplant recipient mouse. Peripheral blood cells of the recipient mice were analyzed 4, 12, 17, 22, and 28 weeks after transplantation.

3. Results

3.1. KuO-expressing chimeric mice and progeny from transduced ES cells

To use the RSP method (Fig. 1A) of finding open chromatin region in all hematopoietic cells, including erythroid precursors, we initially generated mESCs carrying multiple provirus copies [14]. We infected mESCs with GCDsapKuO (Fig. 1B) by following three

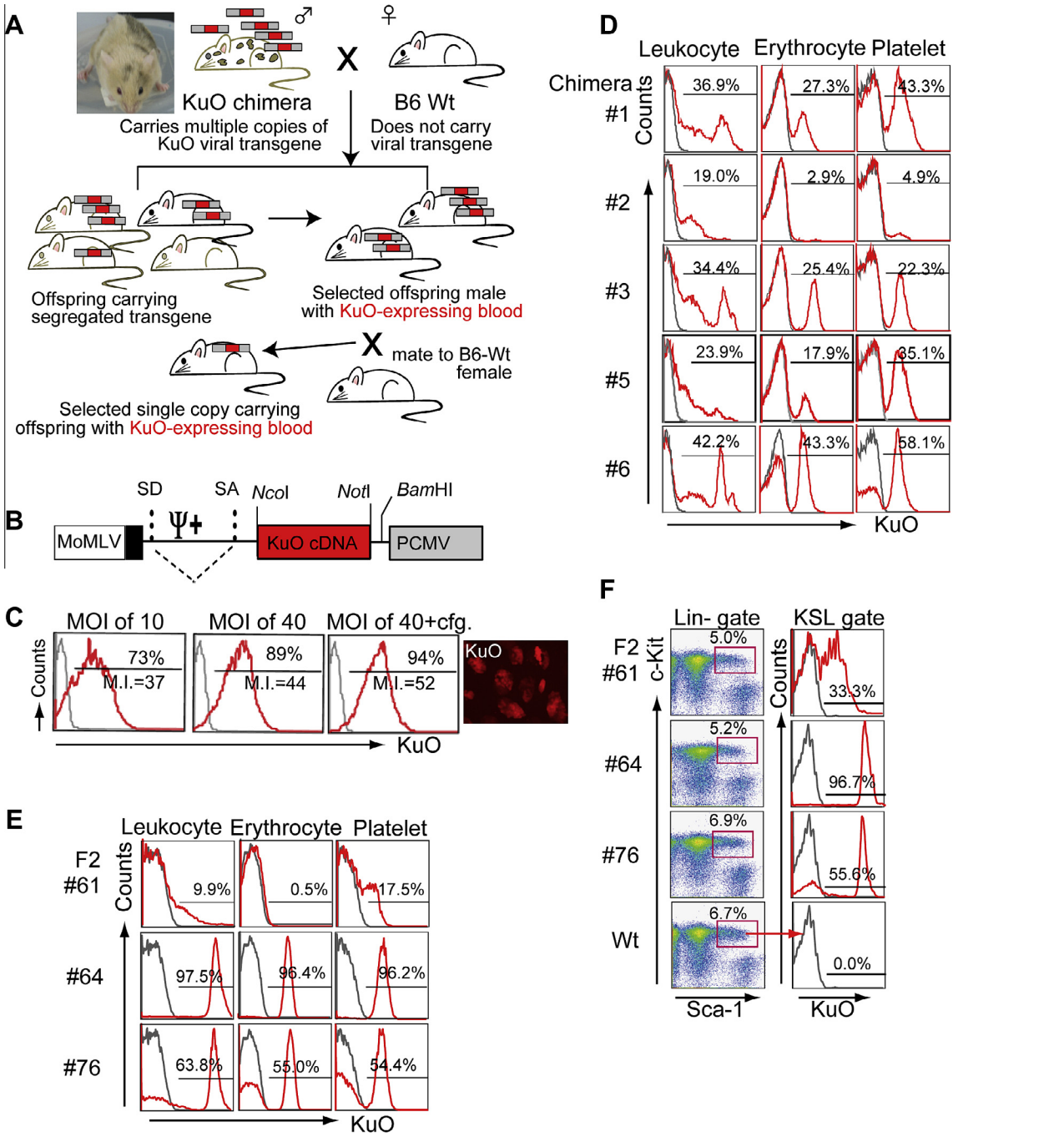

Fig. 1. Structure of retroviral vector GCDsapKuO and generation of KuO-expressing mice derived from KuO-transduced embryonic stem cells (ESCs). (A) Experimental outline of random segregation of provirus (RSP) method for generation of KuO transgenic mice carrying single copy of integrated provirus. (B) Vector construction of GCDsapKuO. (C) KuO expression pattern in ESCs infected by three different protocols (Single infection at multiplicity of infection (MOI) of 10, duple infection at MOI of 40, and duple infection at MOI of 40 with low-speed centrifugation) and KuO expressed ESCs (MOI of 40 with low speed centrifuged infection) were observed by fluorescence microscopy. (D) FACS analysis of KuO expression in peripheral blood (leukocytes, platelets, and erythrocytes of chimeric mice). (E and F) FACS analysis of KuO expression in perpheral blood and BM of F2 mice. Grey line, wild-type mouse; red line, KuO mouse (C, D, E and F).

protocols: Single infection at multiplicity of infection (MOI) of 10, duple infection at MOI of 40, and duple infection at MOI of 40 with low-speed centrifugation. Efficiencies of infection were 73%, 89%, and 94% respectively (Fig. 1C). We chose the protocol with highest efficiency of infection to generate ESCs carry the highest numbers of provirus copies.

To assess expression ratio among hematopoietic cells or toxicity of KuO throughout ontogenesis, GCDsapKuO-transduced ESCs (KuO ESCs) carrying various proviral copy numbers and harboring a variety of integration sites were microinjected into 3.5-day blastocysts of C57BL/6 (B6) mice. The birth rate of chimeric mice, determined by coat color, was 77.8% (seven out of nine mice; Supplementary Table 1). Five out of seven mice showed 70–100% chimerism judged by coat color. In order to investigate KuO expression in hematopoietic cells in chimeric mice, we performed flow-cytometry analysis of leukocytes, erythrocytes, and platelets in peripheral blood. KuO was expressed in leukocytes of all seven chimeric mice. Moreover, in five out of seven mice (71%; #1, #2, #3, #5, and #6) KuO was expressed not only in leukocytes but also in erythrocytes and platelets. Respective expression ratios in individual mice were, for leukocytes, 36.9%, 19.0%, 34.4%, 23.9% and 42.2%; for erythrocytes, 27.3%, 2.9%, 25.4%, 17.9% and 43.3%; and for platelets, 43.3%, 4.9%, 22.3%, 35.1% and 58.1% (Fig. 1D).

The chimeric mice #1, #3, and #6 in which high levels of KuO expression were observed in erythrocytes were next crossed to B6 mice. Germline transmission was observed from all three chimeras. F1 mice were then back-crossed to B6 mice, yielding F2 mice. Unlike retro-GFP mice [13], expression patterns of KuO were similar among leukocytes, erythrocytes and platelets in F2 mice (Fig. 1E, Supplementary Fig. 1), In one mouse in which KuO expression was low in peripheral leukocytes (9.9%), KuO expression also was low in erythrocytes (only 0.5%) and in platelets (17.5%). By contrast, in one mouse in which KuO expression was high in peripheral leukocytes (97.5%), KuO expression also was high in erythrocytes (96.4%) and in platelets (96.2%). Furthermore, KuO expression patterns in the hematopoietic progenitor cell fraction

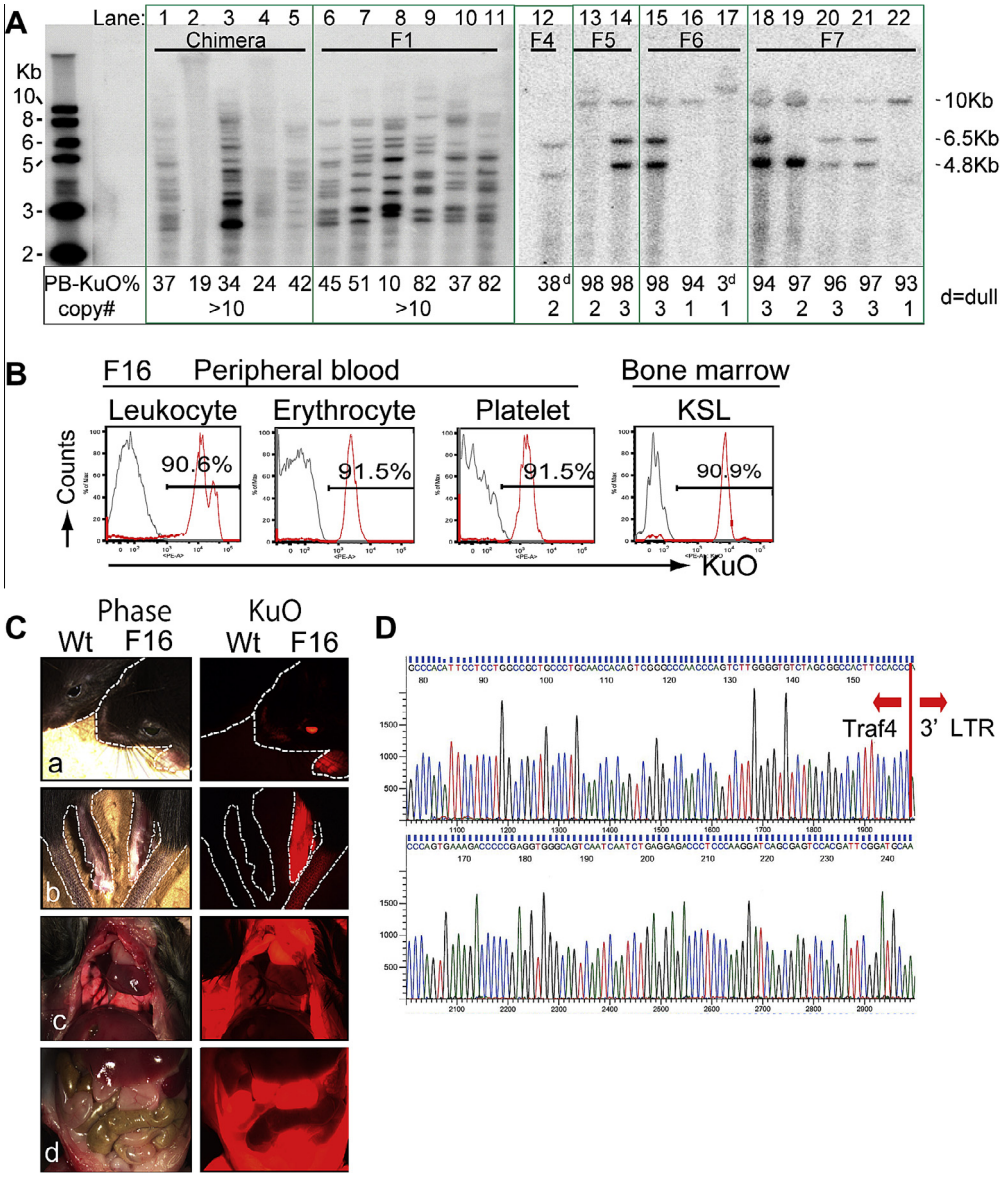

Fig. 2. Generation of KuO-expressing transgenic mouse carrying single transgene. (A) Number of provirus copies in chimeric mice and offspring mice were analyzed by southern blot. Percentages of KuO-expressing cells in the peripheral blood are indicated. (B) KuO expression levels in peripheral blood, BM, and erythrocytes of F16 mice carrying a single copy of provirus. Grey line, wild-type mouse; red line, KuO mouse. (C) KuO expression was observed in eyes and paw (a), paw and tail (b), thorax (c), and abdomen (d). (D) Provirus integration site was analyzed by inverse PCR.

(Lineage$^-$/c-Kit$^+$/Sca1$^+$ [KSL] cells) in BM were the same as those in peripheral blood (Fig. 1F, Supplementary Fig. 1).

Next, we investigated by fluorescence microscopy whether KuO was expressed in systemic organs other than hematopoietic cells. Strong KuO fluorescence was observed in brain, heart, liver, lung, muscle, skin, spleen, thymus, uterus, tail, and elsewhere, as well as in E13.5 whole embryos (Supplementary Fig. 2).

These results indicate that provirus integration into open chromatin in erythroid precursors was enabled by high MOI infection with GCDsap and that high levels of expression of KuO did not interfere with mouse development.

3.2. Generation of KuO transgenic mouse carrying only one copy of provirus

To investigate the relationship between expression of KuO and provirus copy numbers, peripheral blood cells were analyzed by flow cytometry and provirus copy numbers were estimated by southern blot. More than 15 copies with different provirus integration patterns were observed in chimeric mice (Fig. 2A, lane 1–5). The F1-mice derived from chimeric mice #1 or #3 carried around 10 provirus copies, with various proportion of KuO-expressing leukocytes, erythrocytes and platelets from 10% to 82%. Furthermore, after backcrossing, proviruses were segregated into 3 independent integration sites revealed as 10 Kb, 6.5 Kb, and 4.8 Kb bands in Southern hybridization. Proportion of hematopoietic-lineage elements expressing KuO in mice carrying 3 copies of provirus (10 Kb, 6.5 Kb, and 4.8 Kb) was 98% (Fig. 2A, lanes 14, 15, 18, 20, and 21). In mice carrying 2 copies of provirus (6.5 Kb and 4.8 Kb) it was about 38% (Fig. 2A, lane 12). In mice carrying a single copy (10 Kb) it was approximately 93% (Fig. 2A, lanes 16 and 22). Thus, the integration site represented by 10 Kb band appeared to give the highest expression in hematopoietic-lineage elements.

We next used inverse PCR to analyze the proviral integration site in mice carrying a single copy (10 kb by southern blot analysis). We identified the integration site as on Chromosome 11 in the region of the tumor necrosis factor receptor associated factor 4 (Traf4) gene enhancer.

We generated a KuO transgenic mouse line that carries a single proviral copy (Fig. 2B and C, Supplementary Fig. 3). We could not detect any tumors or other abnormalities in the mice during a year of observation.

These results indicate that the RSP method is useful for investigating open chromatin regions in specific cells and that the Traf4 enhancer region is one of the open chromatin region in erythroid precursors (Fig. 2D).

3.3. Reconstitution of BM hematopoiesis by transplantation of KuO-expressing HSCs

Since KuO was expressed not only in lymphocytes but also in erythrocytes and platelets in KuO mice, we next performed bone marrow transplantation to identify that KuO is a red fluorescent protein suitable for studying hematopoiesis by comparison of reconstituting abilities of hematopoietic cells.

CD34$^{-/low}$ c-Kit$^+$Sca-1$^+$Lin$^-$ (CD34$^-$KSL) cells from B6-Ly5.1 or KuO mice were transplanted, with competitor cells, into wild-type mice irradiated with 9.8 Gy or Rag2 KO mice irradiated with 8.0 Gy. Expression of KuO in hematopoietic-lineage elements was then analyzed 4, 12, 17, 22, and 28 weeks after transplantation. Platelets, erythrocytes, and leukocytes gradually appeared beginning 4 weeks after transplantation in all transplantation groups. We did not observe significant differences in KuO chimerism between recipients of wild-type HSCs and recipients of KuO HSCs (Fig. 3). These results indicate that KuO expression did not interfere with normal differentiation of HSCs. The results also indicate that KuO, an exogenic protein to mice, was not immunogenic at least

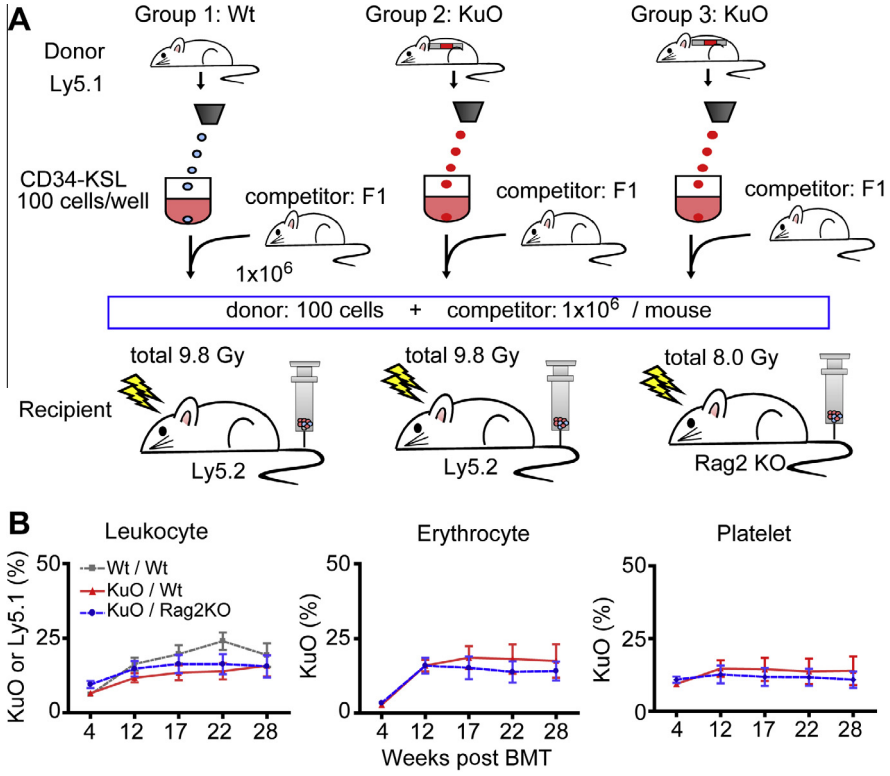

Fig. 3. KuO-expressing HSCs were reconstituted in wild-type or Rag2 KO mice. (A) Schematic diagram of BM transplantation. (B) Time course of engraftment (percentage of transplanted-cell progeny in peripheral blood). Donor (D)/recipient (R), grey; wild-type (D)/wild-type (R), red; KuO (D)/wild-type (R), blue; KuO (D)/Rag2 KO (R).

in this experimental HSC transplantation setting. They permit the inference that KuO is a fluorescent protein without detectable immunogenicity, suitable for analysis of hematopoiesis.

4. Discussion

In this study we used the RSP method to generate KuO transgenic mice in which KuO was expressed in all hematopoietic cells, including erythrocytes. RSP technique requires generation of ESCs carrying randomly integrated provirus in high copy number. To achieve this, we performed retroviral infection at high MOI with low speed centrifugation, thereby generating ESCs carrying more than 15 integrated copies of provirus. We then backcrossed KuO transgenic mice and chromosome segregation was utilized to find a provirus integration site where chromatin was open in all hematopoietic cells, including erythrocyte precursors.

Using FACS, we observed two peaks in the expression pattern of KuO in hematopoietic cells from early-generation of offspring. Some provirus copies may have been transcribed in reverse orientation from an endogenous promoter, with the resultant transcript acting as an antisense nucleotide against KuO transcribed from retrovirus LTR.

Retroviral vector integration activates the transcription of silent genes and induces side effects such as oncogenesis. However, KuO mice harboring a single proviral integration site in the Traf4 enhancer region did not show tumor formation during one year of observation. Furthermore, expression levels of KuO in different cell populations in BM of KuO mice were the same as those in wild-type mouse BM. These results indicate that the Traf4 enhancer region is relatively insusceptible to epigenetic modification in hematopoietic cells, whilst other integration sites may be influenced by epigenetic modification results in transcriptional silencing. Moreover, we found that KuO was stably expressed in embryo, adult tissue, HSCs, and terminally differentiated hematopoietic cells, indicating that the Traf4 enhancer region persists as open chromatin throughout mouse development and hematopoietic cell maturation.

Unlike previously reported fluorescent-protein transgenic mice, our KuO transgenic mouse makes it possible to trace not only hematopoietic stem, progenitor cells, or lymphoid cells, but also platelets and erythrocytes. Therefore, this transgenic mouse line likely will provide a useful tool for the study in particular of erythropoiesis and megakaryopoiesis in vivo.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.017.

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