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Fucci-guided purification of hematopoietic stem cells with high repopulating activity

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ABSTRACT

Fluorescent ubiquitination-based cell cycle indicator (Fucci) technology utilizing the cell cycle-dependent proteolysis of ubiquitin oscillators enables visualization of cell cycle progression in living cells. The Fucci probe consists of two chimeric fluorescent proteins, FucciS/G_2/M and FucciG_1, which label the nuclei of cells in S/G_2/M phase green and those in G_1 phase red, respectively. In this study, we generated Fucci transgenic mice and analyzed transgene expression in hematopoietic cells using flow cytometry. The FucciS/G_2/M-#474 and FucciG_1-#639 mouse lines exhibited high-level transgene expression in most hematopoietic cell populations. The FucciG_1-#610 line expressed the transgene at high levels predominantly in the hematopoietic stem cell (HSC) population. Analysis of the HSC (CD34$^-$KSL: CD34$^{-/\text{low}}$c-Kit$^+$Sca-1$^+$lineage marker$^-$) population in the transgenic mice expressing both FucciS/G_2/M and FucciG_1 (#474/#610) confirmed that more than 95% of the cells were in G_0/G_1 phase, although the FucciG_1(red) intensity was heterogeneous. An in vivo competitive repopulation assay revealed that repopulating activity resided largely in the FucciG_1(red)$^{\text{high}}$ fraction of CD34$^-$KSL cells. Thus, the CD34$^-$KSL HSC population can be further purified on the basis of the Fucci intensity.

1. Introduction

Hematopoiesis is a hierarchical differentiation process by which all blood cell types are generated from hematopoietic stem cells (HSCs). HSCs are capable of self-renewal and multilineage differentiation. In the adult bone marrow (BM), HSCs are predominantly quiescent and reside in a specific microenvironment, referred to as the niche, where HSC quiescence, self-renewal, proliferation, and differentiation are thought to be strictly regulated in order to maintain the HSC pool and sustain lifelong production of blood cells [1]. HSCs give rise to highly proliferative progenitors with limited or no self-renewal capacity and lineage-restricted differentiation potential, producing terminally differentiated hematopoietic cells. Cell cycle regulation plays a critical role in hematopoiesis [2]. Although many intrinsic and extrinsic factors are involved in hematopoiesis, the regulatory mechanisms underlying hematopoietic cell proliferation and differentiation are still unclear. Visualizing the progress of the cell cycle in hematopoietic cells including HSCs will provide valuable information for better understanding how cell cycle progression and hematopoiesis are coordinated.

Fluorescent ubiquitination-based cell cycle indicator (Fucci) technology makes it possible to visualize cell cycle progression in living cells [3]. This technology utilizes the cell cycle-dependent proteolysis of two ubiquitin oscillators, human Cdt1 and geminin. The original Fucci probe was generated by fusing monomeric Kusabira Orange 2 (mKO2) and monomeric Azami Green (mAG) to the ubiquitination domains of Cdt1 (hCdt1(30/120)) and geminin (hGem(1/110)), respectively. The resulting mKO2-hCdt1(30/120) and mAG-hGem(1/110) fusion proteins label the nuclei of cells in G_1 phase red and those in S/G_2/M phase green, respectively. Using Fucci probes, the cell cycle behavior of individual cells was visualized in vitro and in vivo [3–7].

In this study, we generated transgenic mice expressing Fucci probes and analyzed transgene expression in hematopoietic cells including HSCs. We also analyzed the relationship between Fucci signal intensity and the repopulating activity of HSCs.

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2. Materials and methods

2.1. Fucci transgenic mice

Generation of Fucci transgenic mice expressing mAG-hGem(1/110) and mKO2-hCdt1(30/120) under the control of the CAG promoter has been reported previously [3]. Transgenic mouse lines (FucciS/G_2/M-#474, -#492, -#504 and FucciG_1-#596, -#610, -#639) were backcrossed to C57BL/6N (B6-Ly5.2) mice for more than ten generations and can be obtained from RIKEN BioResource Center (Tsukuba, Japan). All animal experiments were approved by the Animal Experiment Committee at the RIKEN Tsukuba Institute.

2.2. Analysis of transgene expression

Hematopoietic cells were isolated from BM, peripheral blood (PB), spleen, and thymus of Fucci transgenic mice (3–6 months of age). The cells were stained with cell surface marker antibodies. The following antibodies were used: APC-, APC-Cy7-, PE-Cy7-, or PerCP-Cy5.5-conjugated anti-B220, anti-CD3, anti-CD4, anti-CD8, anti-NK1.1, anti-Gr-1, anti-Mac-1, anti-CD41, anti-Ter119, anti-c-Kit, anti-Sca-1, anti-FcγR, anti-IL-7Rα, and anti-CD34 (all antibodies purchased from eBioscience, San Diego, CA). Lineage marker (Lin) antibodies consist of biotinylated anti-Gr-1, anti-Mac-1, anti-B220, anti-IgM, anti-CD4, anti-CD8, and anti-Ter119. The biotinylated antibodies were developed with APC-Cy7-conjugated streptavidin (eBioscience). Fluorescence-activated cell sorting (FACS) analysis was performed with a FACSCalibur or a FACSAriaIII equipped with four lasers (405, 488, 561, and 633 nm) (BD Biosciences, San Jose, CA).

2.3. Competitive repopulation assay

B6-Ly5.2 mice were purchased from Charles River Laboratories Japan. B6-Ly5.1 mice were obtained from RIKEN BioResource Center. B6-Ly5.1/Ly5.2 F1 mice were obtained by mating pairs of B6-Ly5.1 and B6-Ly5.2 mice. BM cells isolated from Fucci transgenic mice (B6-Ly5.2) were stained with biotinylated Lin antibodies. The cells were then stained with eFluor660-conjugated anti-CD34, PE-Cy5.5-conjugated anti-Sca-1, and PE-Cy7-conjugated anti-c-Kit antibodies (eBioscience). The biotinylated antibodies were developed with APC-eFluor780-conjugated streptavidin (eBioscience). Twenty FACS-sorted FucciG_1(red fluorescence)^{high} or FucciG_1(red fluorescence)^{low} CD34$^{-/low}$c-Kit$^+$Sca-1$^+$Lin$^-$ (CD34$^-$KSL) cells were mixed with 2×10^5 total BM competitor cells from B6-Ly5.1/5.2 F1 mice and transplanted into lethally (9.5 Gy) irradiated B6-Ly5.1 mice. At various time points after transplantation, PB cells of the recipient mice were collected and stained with biotinylated anti-Ly5.2 (BD Biosciences), APC-conjugated anti-Ly5.1, PE-Cy7-conjugated anti-Mac-1, PE-Cy7-conjugated anti-Gr-1, PE-Cy7-conjugated anti-B220, eFluor450-conjugated anti-CD4, and eFluor450-conjugated anti-CD8 antibodies (eBioscience). The biotinylated antibody was developed with APC-eFluor780-conjugated streptavidin. FACS analysis was performed with a FACSAriaIII. Donor chimerism was determined as the percentage of Ly5.2$^+$ cells.

3. Results

3.1. Analysis of transgene expression in hematopoietic cells of Fucci transgenic mice

We generated eight transgenic mice expressing mAG-hGem(1/110) (FucciS/G_2/M) and 16 transgenic mice expressing mKO2-hCdt1(30/120) (FucciG_1) [3]. Analysis of whole body sections from newborn mice revealed high-level transgene expression in FucciS/G_2/M transgenic mouse lines (#474, #492, #504, and #514) and FucciG_1 lines (#596, #610, #639, and #659). Then, these eight mouse lines were further analyzed by FACS for the transgene expression in various hematopoietic cell populations of BM cells (Fig. 1). The Fucci transgenes were expected to be highly expressed apparently in all tissues of the FucciS/G_2/M-#504 and FucciG_1-#596 lines [3]. However, the FACS data indicated that these two lines and FucciG_1-#659 line expressed transgenes at very low levels in all hematopoietic cell populations we analyzed.

FucciS/G_2/M-#474 had the highest transgene expression in most hematopoietic cell populations. Note that high-level transgene expression was observed preferentially in B lymphoid cells from FucciS/G_2/M-#492 mice. FucciS/G_2/M-#492 was successfully used to visualize the localization of activated proliferating memory B cells in the spleen [8]. FucciG_1-#610 and FucciG_1-#639 expressed the transgene at high levels, especially in HSCs (CD48$^-$KSL or CD34$^-$KSL) and mature hematopoietic cell populations, respectively.

Next, we generated transgenic mice expressing both FucciS/G_2/M and FucciG_1 by cross-breeding FucciS/G_2/M-#474 with FucciG_1-#610, and analyzed transgene expression in cells from various hematopoietic organs. As shown in Fig. 2, transgene expression was detected in mature hematopoietic cell populations from the PB, BM, spleen, and thymus. As expected, the number of FucciG_1 (red)-positive cells increased with differentiation from immature to mature cells (e.g., immature CD4$^+$CD8$^+$ T cells vs. mature CD4^{+-}CD8$^-$ or CD4$^-$CD8$^+$ T cells in the thymus). On the other hand, the number of FucciS/G_2/M(green)-positive cells increased with the differentiation of HSCs into multipotent progenitors (MPPs) and lineage-restricted progenitors, common lymphoid progenitors (CLPs), common myeloid progenitors (CMPs), granulocyte/macrophage progenitors (GMPs), and megakaryocyte/erythrocyte progenitors (MEPs).

3.2. Analysis of Fucci fluorescence intensity and repopulating activity of HSCs

Next, we analyzed the HSC population of #474/#610 mice in detail. More than 95% of CD34$^-$KSL cells were FucciG_1(red)-positive (Fig. 3A), confirming that HSCs are predominantly in G_0/G_1 phase. Interestingly, heterogeneous fluorescence intensities were found in the FucciG_1(red)-positive population. The proportion of FucciG_1(red)^{high} cells was significantly higher in the HSC (CD34$^-$KSL) population than in the MPP (CD34$^+$KSL) population. This finding suggests that FucciG_1(red)^{high} cells are more quiescent or stay longer in G_0/G_1 phase than FucciG_1(red)^{low} cells. The FucciG_1(red) intensity was also heterogeneous in other HSC populations using CD150 and CD48 markers [9] (Supplementary Fig. 2), though these HSC populations substantially overlap with each other [10–12].

To assess whether FucciG_1 expression status correlates with repopulating activity, the FucciG_1(red)^{high} or FucciG_1(red)^{low} fraction of CD34$^-$KSL cells was sorted by FACS (Fig. 3B) and subjected to an in vivo competitive repopulation assay. As shown in Fig. 3C, repopulating activity was found to reside mainly in the FucciG_1 (red)^{high} cell population. We also cultured FucciG_1(red)^{high} and FucciG_1(red)^{low} CD34$^-$KSL cells in vitro with a combination of cytokines (stem cell factor, thrombopoietin, fibroblast growth factor-1, and insulin-like growth factor-2) [13]. No significant differences were found in the timing of the first cell division and the duration of the cell cycle between these two cell populations (data not shown). The cell cycle state of CD34$^-$KSL cells appears to not influence the induction of cell proliferation under the in vitro culture conditions.

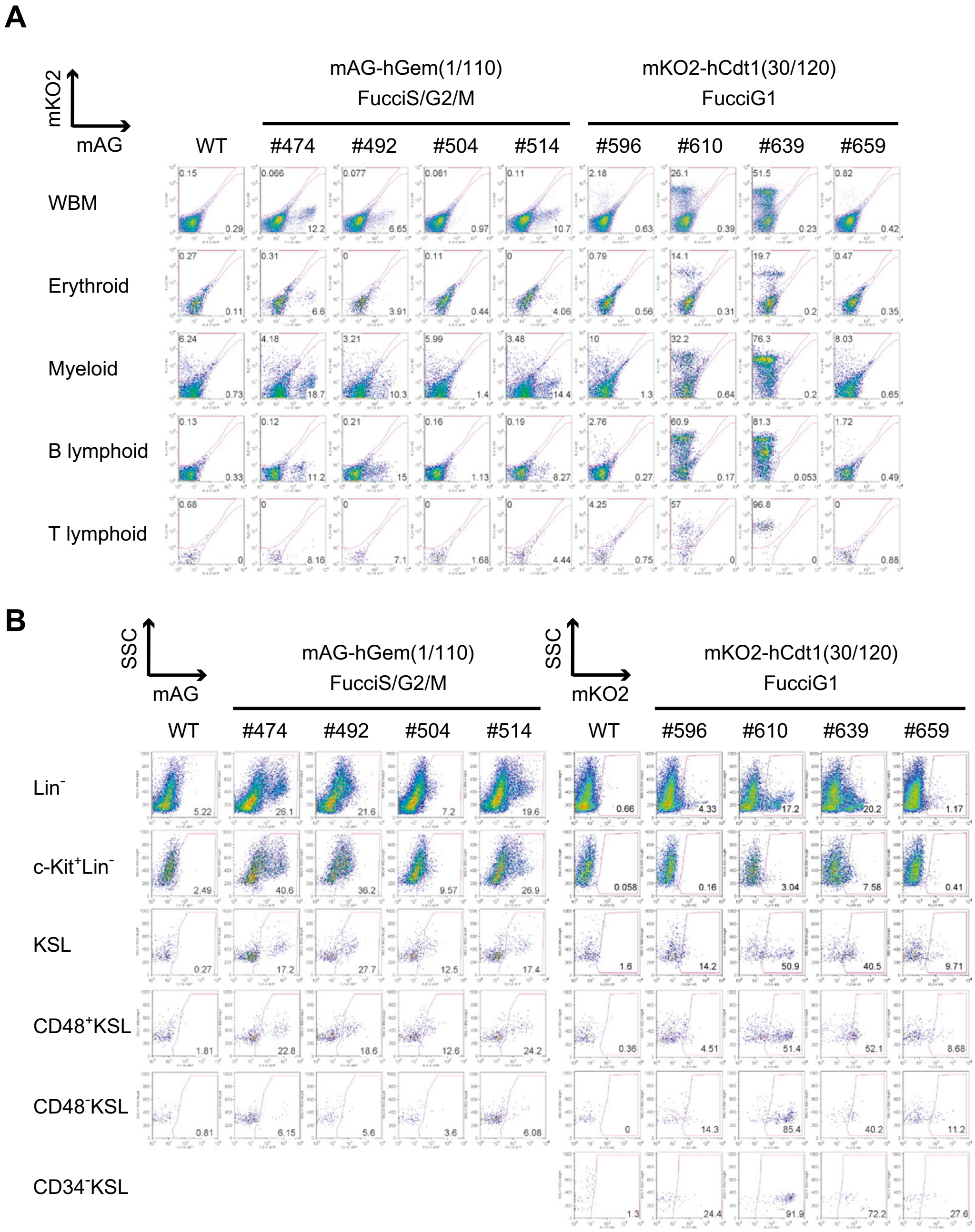

Fig. 1. FACS analysis of transgene expression in hematopoietic cells of FucciS/G_2/M and FucciG_1 transgenic mice. BM cells were isolated from FucciS/G_2/M transgenic mouse lines (#474, #492, #504, and #514) and FucciG_1 lines (#596, #610, #639, and #659). The cells were stained with cell surface marker antibodies, and FucciS/G_2/M(mAG) or FucciG_1(mKO2) transgene expression was analyzed by flow cytometry. Shown are representative FACS profiles of (A) whole BM, erythroid (Ter119$^+$), myeloid (Gr-1$^+$ or Mac-1$^+$), B-lymphoid (B220$^+$), T-lymphoid (CD4$^+$ or CD8$^+$), (B) Lin$^-$, c-Kit$^+$Lin$^-$, KSL, CD48$^+$KSL, CD48$^-$KSL, and CD34$^-$KSL cells.

4. Discussion

We generated Fucci transgenic mouse line #474/#610 expressing both FucciS/G_2/M and FucciG_1 in various hematopoietic cells, especially HSCs. Thus, #474/#610 mice are useful for studying the cell cycle dynamics of HSC differentiation into mature hematopoietic cells. Using #474/#610 mice, we were able to visualize endomitosis in megakaryoblasts differentiated from HSCs, MPPs, CMPs, and MEPs [7]. It would be interesting to analyze hematopoietic cell cycle dynamics in mice generated by crossing #474/#610 mice with mutant mice associated with hematopoietic abnormalities in future studies.

It is generally accepted that HSCs in the adult BM are almost exclusively in quiescent G_0 phase. Our results with Fucci probe also suggest that most HSCs remain in G_0/G_1 phase. FucciG_1(red)^{high} cells in HSC populations appear to be in G_0 phase for a long period of time, but the Fucci probe used in this study cannot distinguish between G_0 and G_1 phase. Recently, a fusion protein of the fluorescent protein mVenus and a p27K$^-$ mutant lacking CDK inhibitory activity (mVenus-p27K$^-$) was shown to be capable of visualizing cells in G_0 phase [14]. In combination with Fucci probe, mVenus-p27K$^-$ probe is also capable of distinguishing between cells in G_0 phase and cells in G_1 phase during the G_0–G_1 transition. Therefore, the relationship between FucciG_1(red) intensity and duration of G_0 phase in HSC populations could be analyzed using mVenus-p27K$^-$ probe. Unfortunately, the expression of mVenus-p27K$^-$ was reported to be low or undetectable in hematopoietic cells from mVenus-p27K$^-$ transgenic mice. However, time-lapse analysis of

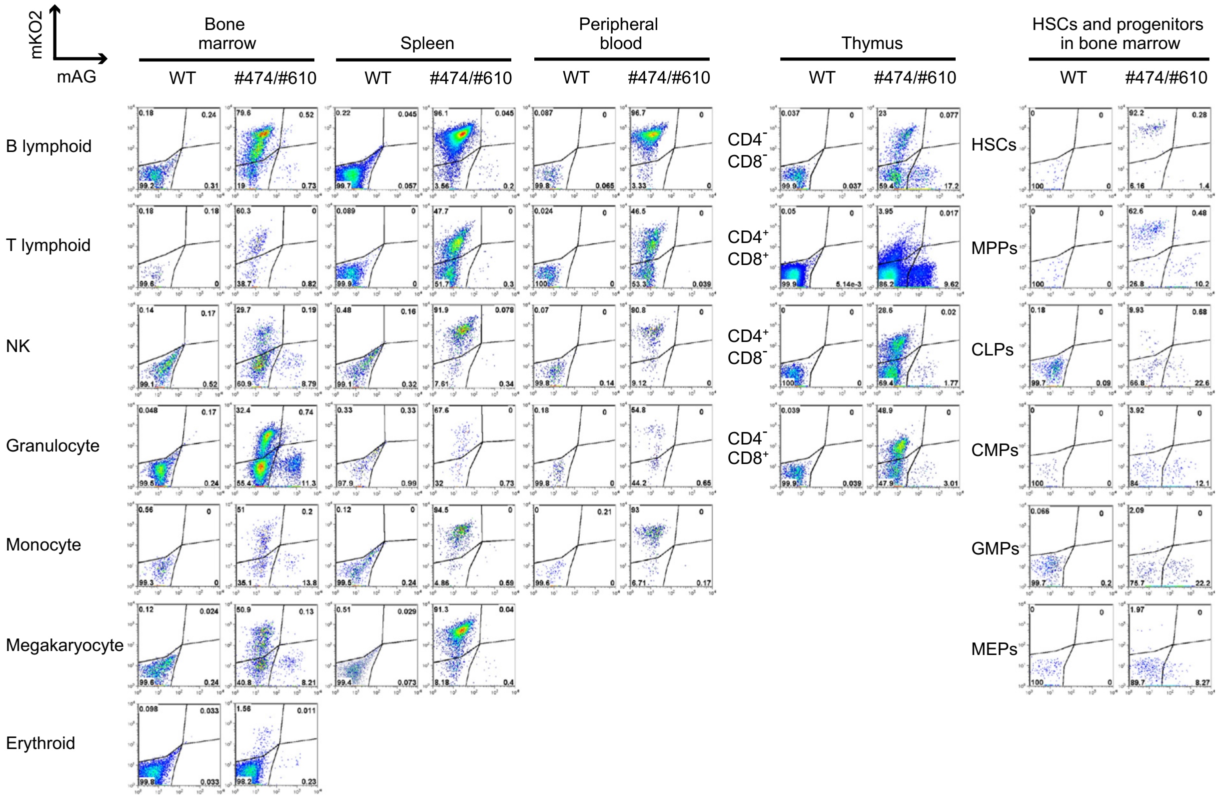

Fig. 2. FACS analysis of transgene expression in hematopoietic cells of Fucci transgenic mouse line #474/#610. Hematopoietic cells were isolated from BM, spleen, PB, and thymus of Fucci transgenic mouse line #474/#610 and stained with cell surface marker antibodies. FucciS/G_2/M(mAG) and FucciG_1(mKO2) transgene expression was analyzed by flow cytometry. Representative FACS profiles of hematopoietic subpopulations are shown. Gating for hematopoietic subpopulations are shown in Supplementary Fig. 1.

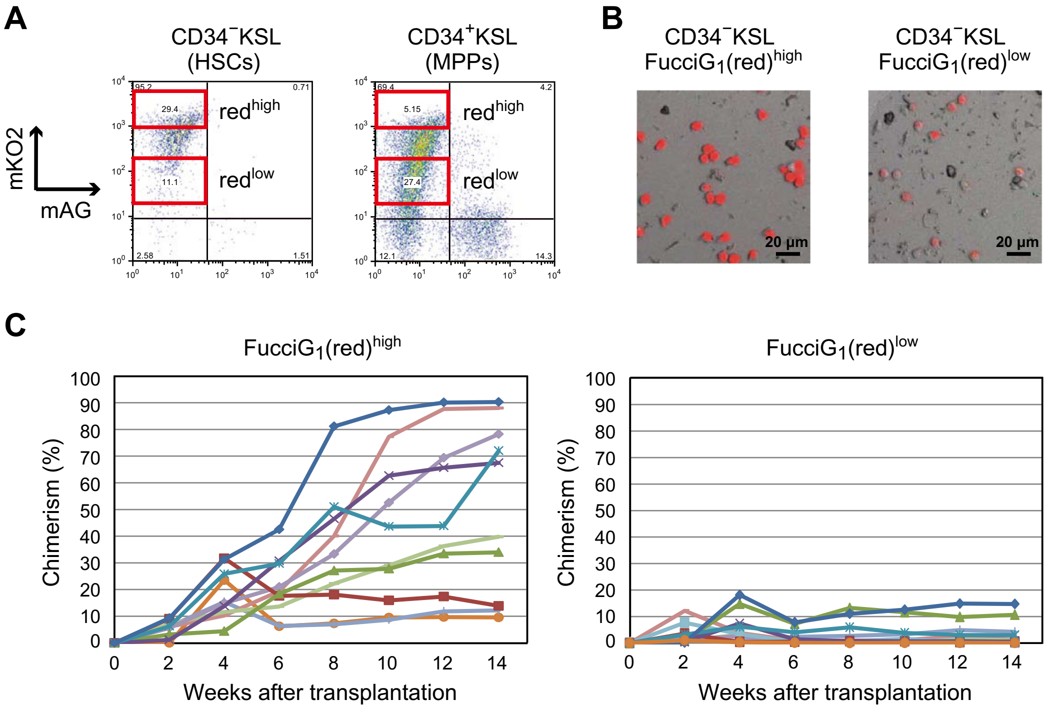

Fig. 3. FucciG_1 fluorescence intensity and repopulating activity of HSCs from Fucci transgenic mouse line #474/#610. (A) FACS profiles of HSC (CD34⁻KSL) and MPP (CD34⁺KSL) populations of BM cells from Fucci transgenic mice (#474/#610). FucciG_1(red)^{high} and FucciG_1(red)^{low} populations are indicated. (B) Representative fluorescence and DIC images of FACS-sorted FucciG_1(red)^{high} and FucciG_1(red)^{low} CD34⁻KSL cells. (C) Twenty FACS-sorted FucciG_1(red)^{high} or FucciG_1(red)^{low} CD34⁻KSL cells were subjected to a competitive repopulation assay. At the indicated time points after transplantation, PB cells of the recipient mice were analyzed by flow cytometry, and donor chimerism was determined. Data from two independent transplantation experiments are shown ($n = 5$ or 6 mice per experiment).

HSC division in the BM niche of mice transplanted with HSCs expressing Fucci and mVenus-p27K$^-$ probes, if possible [15–17], will provide valuable information about HSC biology.

Purification of HSCs is important when studying their self-renewal and differentiation, especially at a clonal level. Substantial progress has been made in isolating murine HSCs using a combination of cell surface markers and flow cytometry. CD34$^-$KSL cells are highly purified HSCs. However, single-cell transplantation studies identified only 20–40% of CD34$^-$KSL cells as long-term repopulating cells [10,13,18–20]. Because the seeding efficiency is thought to be more than 50% [10], CD34$^-$KSL cells are still not a pure population of HSCs. In the present study, we demonstrated that the fluorescence intensity of FucciG$_1$(red) can be used for further purification of CD34$^-$KSL HSCs, and probably other HSC populations such as CD150$^+$CD48$^-$KSL and CD34$^-$CD150$^+$KSL cells. Adult HSC-specific properties, including cell cycle quiescence, are altered during ontogeny and aging [2,21,22]. In addition, recent studies have identified functionally distinct HSC subtypes, including myeloid-biased, lymphoid-biased, and balanced HSCs [23]. Thus, Fucci transgenic mice are expected to facilitate further characterization of HSCs with distinct properties in terms of their cell cycle status.

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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.2014.12.074.

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