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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$^{-/low}$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)^{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.