Brief report

Distinguishable live erythroid and myeloid cells in β-globin ECFP x lysozyme EGFP mice

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We previously described a mouse line that contains green myelomonocytic cells due to the knock-in of enhanced green fluorescence protein (EGFP) into the lysozyme M gene.1 We have now created a transgenic line with fluorescent erythroid cells using a β-globin locus control region driving the enhanced cyan fluorescence protein (ECFP) gene. These mice exhibit cyan fluorescent cells specifically in the erythroid compartment and in megakaryocyte-erythroid progenitors. Crossing the animals with lysozyme EGFP mice yielded a line in which live erythroid and myeloid cells can readily be distinguished by fluorescence microscopy and by fluorescence-activated cell-sorter scanner. This cross allowed unambiguous identification of unstained mixed erythroid-myeloid colonies for the first time. The new mouse lines should become useful tools to dissect the branching between erythroid and myelomonocytic cells during in vitro differentiation of definitive multipotent progenitors. (Blood. 2003;101:903-906)

Study design

β-globin construct and development of transgenic lines

The ECFP gene was excised from pECFP-1 (Clontech, Palo Alto, CA) with Ncol and BsrGI. The resulting fragment was subcloned in a vector containing a 63-bp fragment that encodes a farnesylated peptide that directs the fusion protein to the membrane.6 The farnesylated ECFP (ECFP-far) was polymerase chain reaction (PCR) amplified from this vector, and an Ncol and BamHI restriction site was introduced for further subcloning. To generate the transgene construct, a 4.1-kb Clal/Sall fragment containing the human β major globin gene was digested with Ncol and BamHI, removing exon 1, intron 1 and part of exon 2, and ECFP-far was inserted. This intermediate was ligated to a 21.7 kilobase (kb) Sau3AclI fragment containing the entire β major globin locus control region (LCR).7 After linearization with Sall, a 26.1-kb fragment was generated (Figure 1A), which was injected into Fvb/N oocytes that were subsequently implanted into foster mothers. Of 19 pups born, 3 contained ECFP-positive cells in their peripheral blood.

Colony assays, immunofluorescence, and benzidine staining

Bone marrow cells of a β-globin ECFP mouse were seeded in 2 mL Methocult 3434 (Stem Cell Technologies, Vancouver, BC, Canada) and incubated at 37°C, 5% CO2. Images were first taken under brightfield and then under fluorescent illumination using a specific ECFP filter (excitation at 436 nm, emission 480 nm, dichroic 455 nm; Chroma no. 41044) and an enhanced yellow fluorescent protein (EYFP)-specific filter because it does not detect ECFP, while still detecting EYFP at a 20% efficiency. Acid benzidine staining was as described.

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FACS analyses

Separation of ECFP and EGFP was done according to published procedures on a FACS Vantage (Becton Dickinson, Mountainview, CA) using a 488-nm UV laser. ECFP was detected in Channel 1, filter 480/30 nm band-pass filter, and EGFP was detected in channel 2 (530/30 nm BP). A 457-nm long-pass Laser block was inserted in front of each band pass filter, and a 525-nm short-pass dichroic filter was inserted into the light path. Methods described earlier were used for the analysis of the progenitors.11 Hematopoietic stem cells (HSCs) were sorted as interleukin (IL–7Rα Lin–Sca–1c–Kit+; common myeloid progenitors (CMPs) as IL–7Rα Lin–Sca–1c–Kit–CD34+; FcγRII; granulocyte-macrophage progenitors (GMPs) as IL–7Rα Lin–Sca–1c–Kit–; CD34+ FcγRI; megakaryocyte-erythrocyte progenitors (MEPs) as IL–7Rα Lin–Sca–1c–Kit–; CD34+ FcγRI; common lymphoid progenitors (CLPs) as IL–7Rα Lin–Sca–1c–Kit–; pro–T cells as CD4+CD8+CD25+c–Kit+, and pro–B cells as B220+ IgM+CD43+ populations. All progenitor populations were first sorted using a double laser (488 nm/530 nm Enterprise II + 647 nm; Spectrum) high-speed cell sorter (MoFlo-MLS, Cytomation, Fort Collins, CO). Purified cells were then analyzed by a single laser (457 nm, Larger Argon) fluorescence-activated cell-sorter scanner (FACS) (Epics-Elite; Coulter, Miami, FL) to evaluate ECFP and fluorescein isothiocyanate (FITC) levels.

Results and discussion

Generation and characterization of β-globin–ECFP transgenic mouse lines

To generate a mouse line in which erythroid cells are fluorescently labeled, we used a large fragment of the regulatory region of the β-globin gene to drive expression of ECFP. Because we wanted to detect the fluorescent protein also in mature red blood cells, ECFP was targeted to the plasma membrane through fusion with a farnesylation signal, generating ecfp-far δ. We then inserted ecfp-far δ at the β-globin ATG start site to produce a construct that contained a complete β-globin LCR, 815 bp of 5′ flanking sequence, the β-globin 5′ untranslated region, and a 2.8-kb region containing part of exon 2, intron 2, and exon 3 (Figure 1A). After verifying that a cytomegalovirus enhancer–driven construct is capable of directing the expression of membrane-bound ECFP in fibroblasts, a linearized version of the transgenic construct was injected into oocytes. Of 19 pups whose blood was examined under the fluorescence microscope, 3 showed a significant proportion of ECFP+ cells. These cells had the morphology of erythrocytes and exhibited membrane fluorescence, while no positive cells were seen in wild-type controls (Figure 1B). As determined by FACS, the positive cells were small in size and exhibited low side scatter, wild-type controls (Figure 1B). As determined by FACS, the positive cells were small in size and exhibited low side scatter, while no positive cells were seen in wild-type controls.

Qualitatively similar results were obtained by FACS analyses of bone marrow and spleen cell suspensions: while there was a significant overlap between Ter119+ cells and ECFP+ cells, little or no overlap was seen between Mac-1+ and B220+ cells on the one hand and ECFP+ cells on the other (data not shown). To determine whether ECFP is expressed in hematopoietic progenitors, bone marrow cells from a transgenic globin–ECFP mouse were depleted from lineage-antigen–positive cells and stained with FITC-conjugated anti-CD34, phycoerythrin (PE)–conjugated with lineage-specific antibodies. Cells were then evaluated by fluorescence microscopy. The results obtained with bone marrow from 2 transgenic mice showed that 84% of the Ter119+ cells were ECFP-positive (1176 total fluorescent cells counted). In contrast, there was essentially no overlap (1% or less) between Mac-1+ and B220+ cells on the one hand and ECFP+ cells on the other (of a total of 990 and 530 fluorescent cells counted, respectively). Qualitatively similar results were obtained by FACS analyses of bone marrow and spleen cell suspensions: while there was a significant overlap between Ter119+ cells and ECFP+ cells, little or no overlap was seen between Mac-1+ and B220+ cells on the one hand and ECFP+ cells on the other (data not shown). To determine whether ECFP is expressed in hematopoietic progenitors, bone marrow cells from a transgenic globin–ECFP mouse were depleted from lineage-antigen–positive cells and stained with FITC-conjugated anti-CD34, phycoerythrin (PE)–conjugated

ECFP is specifically expressed in Ter119+ erythroid cells and in megakaryocyte-erythrocyte progenitors

To determine whether the ECFP transgene is specifically expressed in the erythroid lineage in vivo, bone marrow and spleen cells were harvested from several 6-week-old transgenic animals and stained

Figure 1. Development and expression specificity of β-globin transgenic mice.

(A) Diagram of construct used to make the transgenic line. HS indicates DNase-1 hypersensitive sites within the β-globin LCR; pβ, β-globin promoter. (B) Peripheral blood from a β-globin transgenic (Tg) mouse (line no. 3) and a wild-type (WT) control, viewed under brightfield or fluorescence microscopy. Original magnification, ×20.

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anti-FcγRII/III, biotinylated anti–Sca-1, and allophycocyanin (APC)–conjugated anti–c–Kit monoclonal antibodies. Populations corresponding to HSCs, CMPs, GMPs, MEPs, CLPs, as well as pro–T cells and pro–B cells were sorted by FACS. As shown in Figure 1D, the only fraction that contained a significant number of ECFP+ cells were MEPs (63%); a small proportion of the HSC fraction also was weakly positive (0.84%), but none (0.07%) were seen in the subpopulation known to have long-term repopulation potential. It remains to be determined whether the ECFP-positive subfraction of MEPs yields only erythroid or also megakaryocyte colonies.

To test whether there is a correlation between ECFP-positive colonies and hemoglobin-expressing colonies, we analyzed day-8 methylcellulose colonies obtained from bone marrow cells of beta globin ECFP mice. Of a total of 500 colonies scored, a large proportion of the colonies resembling morphologically erythroid colony-forming units, erythroid burst-forming units, and mixed colony-forming cells contained cyan fluorescence–positive cells. The position of 21 colonies was marked, each colony photographed first under brightfield and then under fluorescence illumination, overlayed with a benzidine solution, and then photographed under brightfield again. Of 8 colonies that were ECFP-positive, all stained with benzidine, while none of the 13 ECFP-negative colonies did (Figure 2). There is therefore a strict correlation between ECFP and hemoglobin expression in individual colonies.

A cross between globin ECFP and lysozyme EGFP mice contains cyan erythroid cells and green myeloid cells

To determine whether it is possible to develop a mouse line in which live erythroid and myeloid cells are distinguishable, we crossed the β-globin ECFP line with lysozyme EGFP mice. As illustrated in Figure 3A-B, in mice of this cross, cyan fluorescent cells can easily be distinguished from green fluorescent myelomonocytic cells in peripheral blood and bone marrow simply by overlaying the images obtained with ECFP and EYFP filters (there is some overlap: green myeloid cells also are detected with the ECFP filter and vice versa. However, the latter can be avoided by using a filter designed for optimal detection of the yellow variant, EYFP, with which ECFP+ cells are not detected). Of 793 cells from bone marrow scored, 13% were ECFP-positive and 48% were EGFP-positive, with 39% being fluorescence-negative. We also analyzed the blood and bone marrow cells from 4 double-transgenic mice by FACS. As shown in Figure 3C-D, although separation between the 2 populations was not perfect, essentially all of the ECFP-positive cells correspond to erythroid cells (small cells with low granularity, gate R2), while the EGFP-positive cells correspond to leukocytes (large cells with low to high granularity, gate R3). The proportion of fluorescence-positive cells in the bone marrow was 4.6% for ECFP and 24.5% for EGFP (n = 2). Staining of the same samples with APC-coupled antibodies revealed no overlap of B220+ cells with ECFP+ and EGFP+ cells, while essentially all the ECFP+ cells were contained in the Ter119+ fraction and the EGFP+ cells in the Mac-1+ fraction (data not shown). The fraction of EGFP-positive cells that also seems to be ECFP-positive (Figure 3D, gate R3) probably represents an artifact of compensation, since double-positive cells should have shown a

Figure 2. Methylcellulose colonies from β-globin ECFP mouse bone marrow. (A,C) Overlay between fluorescence and brightfield images. (B,D) Brightfield images of the same colonies after benzidine staining, with positive cells shown in black. The colony shown in panels A and B is erythroid, and the one in C and D is myeloid. Some cells or clusters within the colonies have shifted position after staining with benzidine. Original magnification, × 10.

Figure 3. Analysis of hematopoietic cells from a β-globin ECFP x lysozyme EGFP mouse. (A-B) Overlay of fluorescence microscopic images obtained with ECFP and EYFP filters from bone and bone marrow, respectively (in B, the brightfield image also was included). (C-D) FACS profiles of the same cells. Cells within the gates highlighted in the SSC/FSC plots were analyzed with a laser/filter configuration that allows to distinguish between ECFP and EGP fluorescence. SSC indicates side scatter; FSC, forward scatter; ECFP and EGFP, log fluorescence intensity in the respective channels. Numbers indicate percentages of cells within a given quadrant. (E) Mixed colony in methylcellulose culture, photographed as in panel B. In addition to numerous small cyan fluorescent (erythroid) and large green fluorescent (myeloid) cells, the colony contains a very large unlabeled cell, possibly a megakaryocyte. Original magnifications: × 20 (A-B, E).
different localization within the plot, and no clear double-positive cells were detected by microscopy. Finally, to determine whether mixed colonies of live cells can be visualized by fluorescence, live bone marrow cells of the ECFP/EGFP line were seeded in methylcellulose and day-10 colonies photographed with the filters described above. As illustrated in Figure 3D, mixed erythroid-megakaryocytic colonies could be detected by their content of cyan and green fluorescent cells.

Our results demonstrate that hematopoietic cells of the β-globin ECFP line are specifically labeled within erythroid lineage and erythroid/megakaryocytic precursors. This is perhaps surprising in view of the fact that β-globin can be detected by single-cell PCR not only in MEPs but also in a fraction of HSCs (as well as in CMPs but not in GMPs or lymphoid progenitors). Whether this is due to differences in the sensitivity of the 2 techniques, to the posttranslational regulation of β-globin expression, or to other reasons remains to be determined. Previously, an ε-globin EGFP transgenic line that exhibits green fluorescent embryonic erythroid cells and β-globin transgenic lines that exhibit LacZ-positive adult erythroid cells have been described. However, the β-globin ECFP transgenic line described here is the first in which live definitive erythroid cells can be observed. This line and its cross with lysozyme EGFP mice should become useful for the analysis of adult erythroid/myeloid cell differentiation as well as for the study of erythrocyte-macrophage interactions.

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