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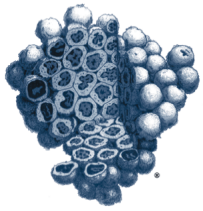

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Coexpression of Kit and the Receptors for Erythropoietin, Interleukin 6 and GM-CSF on Hemopoietic Cells

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Key Words. *SCF · EPO · IL-6 · GM-CSF · Receptor · Hemopoiesis · Flow cytometry · Biotin · Digoxigenin*

ABSTRACT

The detection of functional growth factor (GF) receptors on subpopulations of hemopoietic cells may provide a further dissection of immature cell subsets. Since little information is available about coexpression of different GF receptors at the level of single hemopoietic cells, we studied the feasibility of simultaneous cell staining with a combination of biotin- and digoxigenin-labeled GFs for flow cytometric detection of functional receptors. Using this methodology, coexpression of Kit and receptors for erythropoietin (EPO), interleukin 6 (IL-6), and GM-CSF on hemopoietic cells was studied by triple-staining of rhesus monkey bone marrow (BM) cells with labeled GFs and antibodies against other cell surface markers. Most of the immature, $CD34^{++}$ cells were Kit^{+} but did not display detectable levels of EPO-receptors (EPO-Rs) or GM-CSF-R. Approximately 60% of these $CD34^{++}/Kit^{+}$ cells coexpressed the IL-6-R, demonstrating that immature cells are heterogeneous with respect to IL-6-R expression. Maturation of monomyeloid progenitors, as demonstrated by decreasing CD34 and increasing CD11b expression, is accompanied by a decline of Kit and an increase in GM-CSF-R expression in such a way that $Kit^{+}/GM\text{-}CSF\text{-}R^{+}$ cells are hardly detectable. IL-6-R expression is maintained or even increased during monomyeloid differentiation. IL-6-R and GM-CSF-R were not identified on most $CD71^{++}$ cells, which indicated that these receptors are probably not expressed during erythroid differentiation. Together with previous results, our data show that both Kit and CD71 are upregulated with erythroid commitment of immature progenitors. Upon further differentiation, $Kit^{+}/EPO\text{-}R^{-}$ cells lose CD34 and acquire EPO-R. Maturing erythroid cells eventually lose CD71 and Kit expression but retain the EPO-R. In conclusion, this approach enables further characterization of the specificity of GFs for different bone marrow subpopulations. Apart from insight into the differentiation stages on which individual GFs may act, information about receptor coexpression may be used to identify individual cells that can respond to multiple GFs, and allows for further characterization of the regulation of lineage-specific differentiation. *Stem Cells 1997;15:275-285*

INTRODUCTION

Survival, growth, and differentiation of hemopoietic cells depend on binding of growth factors (GFs) to specific cell surface receptors. Studies with monoclonal antibodies (mAbs) against GF receptors (GF-Rs) or with labeled GF molecules have enabled analysis of the distribution of GF-Rs on hemopoietic cells by means of multiparameter flow cytometry [1]. Such approaches enable analysis of receptor expression in relation to the presence of other cell-surface antigens.

To detect receptor-expressing cells, the use of labeled GFs is preferable to that of antireceptor mAbs, because mAbs recognize only one epitope of a receptor, whereas high-affinity GF-Rs for many of the GFs active in hemopoiesis are complexes of more than one subunit [2-4]. Moreover, some of these subunits can be shared by receptors for different GFs [5-9]. Therefore, detection of receptor-expressing cells based on binding of the physiological ligand itself is more indicative of the presence of functional receptors than of the binding of antibodies.

Previously, we have used biotin-labeled GFs to study expression of one GF-R in relation to other parameters, such as the presence of markers for early hemopoietic cells, in

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particular CD34 [10, 11] and markers that identify activated or differentiating cells, (e.g., CD11b for monocytes and granulocytes, and CD71 for erythroid cells [12, 13]). This approach has provided information on the expression of receptors for interleukin 3 (IL-3), IL-6, GM-CSF, stem cell factor (SCF), and erythropoietin (EPO) on normal hemopoietic cells, and allowed us to compare the receptor phenotype of immature normal cells with that of their malignant counterparts in patients with chronic myeloid leukemia [14-19].

The possibility to simultaneously detect two or more GF-Rs on the same cells could help explain functional and physiological interactions between these GFs on hemopoietic cells. This would also be helpful to further delineate differential expression of these GF-Rs during differentiation of stem cells into mature cells of distinct lineages.

As an alternative to biotinylation, GFs can be labeled with the 391 D molecule digoxigenin (DIG) [20, 21], which can be detected using anti-DIG antibodies. This approach has recently been used to identify binding and internalization of DIG-labeled basic fibroblast GF by target cells [21]. In the present study, we have examined the feasibility of directly studying the coexpression of two GF-Rs on hemopoietic cell subsets by staining rhesus monkey bone marrow (BM) cells with combinations of biotin- and DIG-labeled GFs. By combining these labeled GF molecules with mAbs against CD34, CD11b, and CD71, we have examined receptor coexpression patterns during development of stem cells to mature blood cells of myelomonocytic and erythroid lineages to demonstrate that such an approach is feasible and yields results that are consistent with existing functional data. For these studies, we used rhesus monkey BM cells, since extensive data on single receptor staining were already available [15-18].

Materials and Methods

Labeling of GFs with DIG or Biotin

Recombinant human SCF (a gift of *Dr. S. Gillis* of Immunex, Seattle, WA) [22] was labeled with DIG using Digoxigenin-3-O-methylcarbonyl-ε-aminocaproic acid-*N*-hydroxy-succinimide ester (NHS-DIG, Boehringer Mannheim; Mannheim, Germany) under conditions similar to those described previously for SCF labeling with biotin-N-hydroxy-succinimide ester (NHS-Biotin) [17]. Briefly, NHS-DIG dissolved in dimethylsulfoxide was added to 5 µg aliquots of SCF in 0.05 M phosphate-buffered saline, pH 8.5, containing 0.02% (vol/vol) Tween-20, at molar DIG:Protein (D:P) ratios of 10:1, 50:1, or 100:1. A control sample was incubated with dimethylsulfoxide without DIG (D:P 0). After 3 h incubation at room temperature in the dark, DIG-SCF molecules were separated from the remaining free DIG molecules in the samples by size exclusion chromatography on a 1 ml Sephadex G-25 column (Pharmacia; Uppsala, Sweden), equilibrated in phosphate-buffered saline, pH 7.4, containing 0.02% (wt/vol) Tween-20.

Human glycosylated recombinant IL-6 (kindly provided by Ares-Serono; Geneva, Switzerland) and human recombinant GM-CSF (a gift from Genetics Institute; Cambridge MA) were biotinylated using biotin-*N*-hydroxy-succinimide ester (Sigma; St. Louis, MO) under conditions similar to the NHS-DIG labeling of SCF as described previously [15, 16].

Human recombinant EPO (supplied by Behringwerke Aktiengesellschaft; Marburg, Germany) was biotinylated using biotin-aminocaproyl-hydrazide (Calbiochem; La Jolla, CA) as described previously [23].

Labeled GFs were stored at -80°C in the presence of 0.02% (wt/vol) sodium azide.

Cell Lines and Biological Activity Assay

Before adding sodium azide, the ability of the labeled GF preparations to stimulate cell proliferation was assayed using the appropriate GF-dependent cell lines, as described [15-17]. Briefly, the proliferative response of the human factor-dependent cell line MO7e was used to determine the activity of SCF or GM-CSF [24, 25], the human multifactor-responsive myeloid cell line TF-1 was used for EPO [26], and the IL-6-dependent murine plasmacytoma cell line T1165 for IL-6 [27]. Cells ($1\text{-}5 \times 10^4$ per well of 96-well flat-bottom microtiter plates) were cultured in 200 µl culture medium containing serial dilutions of GF. After 48 h, 0.25 µCi ^{3}H-Thymidine was added to each well. The cells were harvested after 16-18 h of thymidine incorporation, and the radioactivity was measured in a liquid scintillation counter. Alternatively, to determine the viable cell content of each well, a commercial proliferation kit (CellTiter 96 AQ_{ueous} nonradioactive cell proliferation assay; Promega; Madison, WI) was used, based on the colorimetric assay described by *Mosmann* [28]. Therefore, the cells were cultured for three days, after which a mixture of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate was added to the wells according to the manufacturer's instructions. After 4-18 h, the quantity of formazan formed by conversion of MTS by dehydrogenase enzymes in metabolically active cells was measured as the amount of 450 nm absorbance in a Bio-Rad model 450 microplate reader (Bio-Rad; Hercules, CA).

Low-Density BM Cell Preparation

Young adult rhesus monkeys (*Macaca mulatta*) from the TNO Primate Center, Rijswijk, The Netherlands, were used as BM donors. BM was collected by piercing the head of the humeral shaft with a pediatric spinal needle, or by extrusion of the cells in a tissue press from the cleansed

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bones of a monkey that was sacrificed for collection of BM for various experiments and transplantation purposes [29]. All animal experiments were performed in accordance with national ethical regulations. BM was collected in Hank's Hepes buffered salt solution (HH) with heparin and DNAse (0.5 mg/ml). A buffy coat fraction was obtained by centrifuging the cells for 15 min at 2,500 rpm at room temperature. To enrich for progenitor cells, buffy coat cells were subjected to centrifugation for 30 min at 2,000 rpm at room temperature over a discontinuous bovine serum albumin density gradient [30] consisting of 25%, 23%, 22%, 21%, and 17% (wt/vol) bovine serum albumin in 0.2 M Tris-buffer/phosphate buffer, pH 7.2. Fractions were collected and washed in HH. Erythrocytes were lysed using 10 mM potassium bicarbonate, 155 mM ammonium chloride, pH 7.4, containing 0.1 mM EDTA.

Immunocytochemical Staining

Cells were incubated overnight on ice with DIG-SCF and either biotin-EPO, biotin-IL-6, or biotin-GM-CSF in HH, containing 2% (vol/vol) fetal calf serum (FCS), 0.05% (wt/vol) sodium azide, and DNAse (0.5 mg/ml). Specificity of binding of the labeled GF samples was determined by incubating the cells in the presence of a 100-fold molar excess of unlabeled GF. After each incubation the samples were washed in HH with FCS and azide. The cells were stained for 30 min on ice with mouse anti-DIG antibodies (Boehringer Mannheim), followed by a mixture of fluorescein isothiocyanate- (Dako A/S; Glostrup, Denmark) or phycoerythrin (PE)-labeled goat antimouse antibodies (Sigma) and PE- (Molecular Probes; Eugene, OR) or TriColor-labeled streptavidin (Caltag; San Francisco, CA). Indirect staining of the DIG-SCF gave better fluorescence signals than direct staining methods using FITC-labeled sheep anti-DIG antibodies (Boehringer Mannheim). Attempts to amplify the FITC-labeled sheep anti-DIG signal using FITC-labeled rabbit antisheep antibodies (Nordic Immunological Laboratories B.V.; Tilburg, The Netherlands) resulted in high background. Fluorescence signals of biotin-GF labeled cells were amplified by incubating the cells for 30 min on ice with biotinylated antistreptavidin antibodies and fluorescently tagged streptavidin, as described earlier [31]. For three-color analysis, cells were stained in a final incubation step with fluorescently tagged antibodies against CD34, CD71 or CD11b. This was done by resuspending the cells in 50 µl HH with FCS and azide containing 4% (vol/vol) normal rhesus serum and 10% (vol/vol) normal mouse serum. After 15 min incubation on ice, 50 µl of the respective antibodies diluted in HH with FCS and azide were added, and the cells were incubated for another 30 min. We used an anti-CD34 antibody (antibody 566, kindly provided by *Dr. T. Egeland*, Institute of Transplantation Immunology, Rikshospitalet, Oslo, Norway) that was labeled with FITC (Sigma) using standard procedures. CD71-FITC and CD11b-PE mAbs were from Becton Dickinson (Mountain View, CA).

To distinguish between living and dead cells, in some of the experiments, propidium iodide ([PI], final concentration 0.6 µg/ml; Molecular Probes) was added prior to analysis to samples that were stained with FITC and PE, or Hoechst 33258 ([H58], final concentration 1 µg/ml; Molecular Probes) was added to samples that were also stained with TriColor.

Flow Cytometry

Samples were analyzed using a FACScan or a FACS Vantage flow cytometer (Becton Dickinson; San Jose, CA). Cells were illuminated with the 488-nm line of an argon ion laser (Coherent Innova 90; Palo Alto, CA). Green FITC fluorescence was measured through a 530-nm/30-nm bandpass filter, orange PE fluorescence through a 575-nm/26-nm or a 585-nm/42-nm bandpass filter, and red TriColor or PI fluorescence was measured through a 620-nm or a 650-nm longpass filter. H58 fluorescence was measured through a KV408 cut-off filter using UV light (351/364 nm) from the second argon ion laser (Series 2000, Spectra Physics; Mountain View, CA) of the FACS Vantage. Data of 10,000-20,000 cells per sample were stored in list mode using Lysys software (Becton Dickinson) on an HP340 computer. Data were analyzed using Spyro data analysis software developed by *R.R. Jonker* (TNO; Delft, The Netherlands) on an Apple Macintosh computer. Viable H58- or PI-negative cells were analyzed in a light scatter window to include cells with intermediate to high forward light scatter and low to intermediate perpendicular light scatter properties, and to exclude granulocytes, dead cells, and cellular debris.

Statistics

Results are given as means ± standard deviation if appropriate. Standard deviations were calculated on the assumption of a normal distribution.

Results

Biological Activity and Cell Staining

SCF was labeled with DIG at molar DIG:protein (D:P) ratios of 10:1, 50:1 or 100:1. The ability of these DIG-SCF preparations to stimulate cell proliferation was assayed using the human-factor-dependent megakaryocyte cell line, MO7e [24, 25], which expresses 35,000-120,000 high-affinity cell surface SCF receptors per cell [32, 33]. As shown in Figure 1, SCF retained all of its biological activity at a D:P ratio of 10:1. At D:P 50, approximately 67% of the

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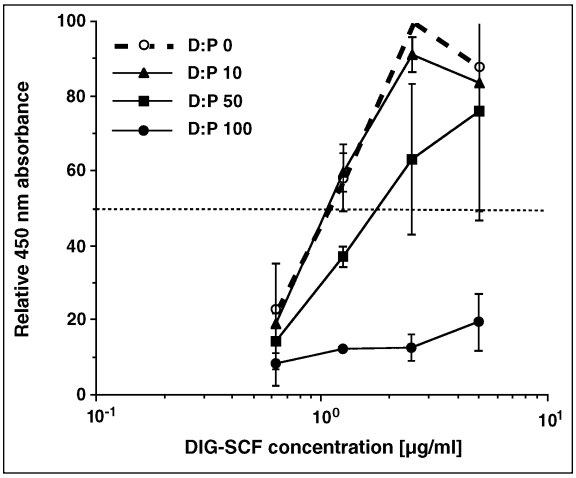

Figure 1. DIG-SCF was prepared at different D:P ratios. *A control sample (D:P 0) was mock-labeled without adding DIG. MO7e cells were incubated with serial dilutions of DIG-SCF for three days in 96-well microtiter plates, after which a mixture of MTS and phenazine methosulfate was added to the wells (see* ***Materials and Methods****). The amount of 450 nm absorbance of each well was measured, indicating the number of metabolically active cells. Open circles represent mock-labeled control SCF; triangles, squares, and closed circles represent DIG-SCF at D:P 10, 50, and 100, respectively. Each data point represents the mean value of three different wells. Standard deviations are indicated. Dotted horizontal line indicates 50% maximal stimulation of the cells.*

biological activity of SCF remained, whereas at D:P 100 most of the biological activity was lost. All three preparations of DIG-SCF bound to MO7e cells, as was examined by flow cytometry (Fig. 2). Binding of DIG-SCF was specific, since the fluorescence signal of cells incubated with DIG-SCF in the presence of an excess of unlabeled SCF was almost identical to that of control cells incubated without DIG-SCF (Fig. 2). Despite the partial loss of biological activity, the DIG-SCF sample prepared at a D:P ratio of 50 yielded a stronger fluorescence signal than the other samples. This preparation was used in further experiments.

Correlation of Kit Expression and EPO-, IL-6-, and GM-CSF-R Expression on BM Cells

The availability of DIG-SCF enabled us to examine coexpression of Kit and receptors for other growth factors, using biotinylated preparations of these growth factors. Cell surface expression of Kit on low-density rhesus monkey BM cells was studied in relation to the expression of EPO, IL-6-, and GM-CSF-R, respectively, using DIG-labeled SCF in combination with biotinylated preparations of each of these GFs. In Figure 3, the results of one of five separate experiments are shown. Staining with the labeled GFs was specific, as demonstrated by incubation with DIG-SCF and biotin-GF in the presence of a 100-fold molar excess of the relevant GF.

Combination of DIG-labeled SCF with biotinylated EPO (Fig. 3B) showed that more than half of the Kit^+ cells also expressed the EPO-R. This $Kit^+/EPO\text{-}R^+$ fraction contained approximately 11% of the low-density cells (Table 1) and included cells with high, as well as cells with low or intermediate, Kit expression. A subset of $EPO\text{-}R^+$ cells did not express Kit at detectable levels. This population represented approximately 6% of the BM cells and appeared to be contiguous to the Kitlow/$EPO\text{-}R^+$ subset. A separate cluster of Kit^+ cells did not express the EPO-R, and contained ~9% of the low-density BM cells. Most of these cells showed intermediate Kit expression.

Staining with DIG-SCF and biotin-IL-6 (Fig. 3C) indicated that most cells with high Kit expression were $IL\text{-}6\text{-}R^-$, whereas most cells with high IL-6-R expression did not express Kit. The $Kit^+/IL\text{-}6\text{-}R^-$ subset contained approximately 10% of the low-density BM cells; the $Kit^-/IL\text{-}6\text{-}R^+$ fraction represented ~22% of the cells (Table 1). A small population containing approximately 7% of the cells expressed both receptors, but mostly at intermediate levels.

Similar to the distribution of Kit and IL-6-R, analysis of Kit and GM-CSF-R expression (Fig. 3D) showed a subset of $Kit^+/GM\text{-}CSF\text{-}R^-$ cells which comprised approximately 11% of the cells, as well as a cluster of $Kit^-/GM\text{-}CSF\text{-}R^+$

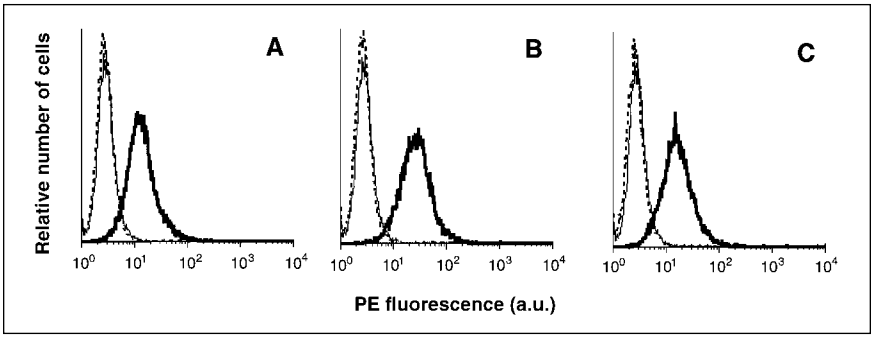

Figure 2. Fluorescence histogram of Kit expression on MO7e cells. *MO7e cells were sequentially stained with DIG-SCF, anti-DIG mouse antibodies, and PE-conjugated antimouse goat antibodies (thick solid lines). Control cells were incubated in the presence of a 100-fold molar excess of unlabeled SCF (broken lines) or stained only with anti-DIG mouse antibodies and PE-conjugated antimouse goat antibodies without DIG-SCF (thin solid lines). A) DIG-SCF labeled at a D:P ratio of 10:1; B) DIG-SCF labeled at a D:P ratio of 50:1; C) DIG-SCF labeled at a D:P ratio of 100:1.*

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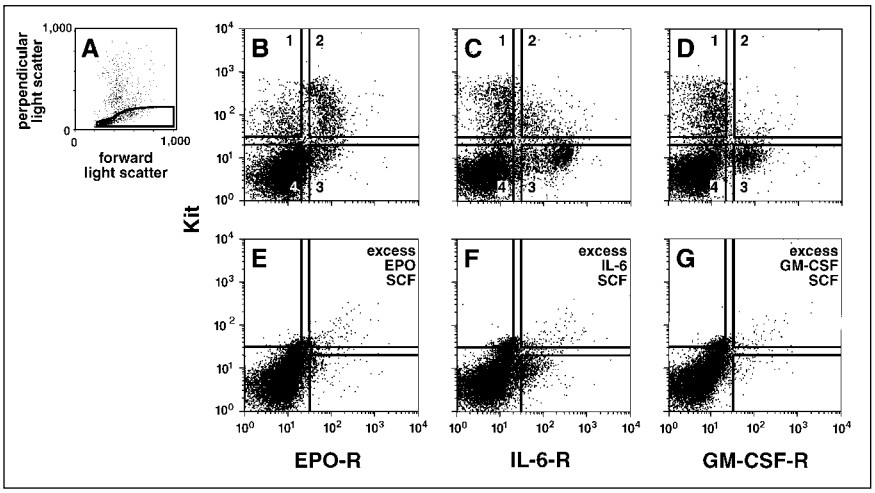

Figure 3. Light scatter properties and GF-R expression of low-density rhesus monkey BM cells. *A) Perpendicular versus forward light scatter. The scatter window that includes cells with low to high forward and low to intermediate perpendicular light scatter properties was used to exclude granulocytes, dead cells, and cellular debris. B), C), and D) Coexpression of Kit and receptors for EPO, IL-6, or GM-CSF, respectively. Cells were stained with DIG-SCF combined with biotinylated EPO, IL-6, or GM-CSF. The windows used to analyze the subsets coexpressing the different GF-Rs are indicated. E), F), and G) Control cells were incubated in the presence of a 100-fold molar excess of the appropriate unlabeled GF.*

cells which represented approximately 6% of the low-density cells (Table 1). Unlike Kit and IL-6-R, very few, if any, cells expressing both Kit and GM-CSF-R were found.

Phenotypic Characterization of BM Subsets Based on Expression of Kit and EPO-R

To examine the nature of the different subsets of GF-R-expressing cells, phenotypic analysis of low-density rhesus monkey BM cells was performed after triple-staining with labeled GFs and mAbs against different cell-surface markers (Figs. 4, 5, 6, and Table 2). Consistent with previous findings [14], very few cells that expressed CD34 at high levels characteristic for immature hemopoietic cells were EPO-R$^+$ (Fig. 4A and Table 2). Most of the CD34^{++} cells were Kit$^+$/EPO-R$^-$ (Fig. 4A). The majority of the Kit$^+$/EPO-R$^+$ cells showed low CD34 expression, demonstrating that these cells were more differentiated than the CD34^{++}/Kit$^+$/EPO-R$^-$ cells. The average level of CD34 expression on the Kit$^-$/EPO-R$^+$ cells was even lower, indicating that these cells were more mature than the Kit$^+$/EPO-R$^+$ cells. Consistent with earlier results showing Kit$^+$ as well as Kit$^-$ cells within the CD34^{++} subset of rhesus monkey BM [17], some CD34^{++} cells were also found in the Kit$^-$/EPO-R$^-$ subset.

Almost all erythroid cells, identified by high CD71 expression [12, 13], were Kit$^+$ (Fig. 4B) [17], and most of these (~80%) expressed the EPO-R (Table 2). These CD71^{++} cells comprised one-third of the cells in the Kit$^+$/EPO-R$^-$ fraction and the majority of cells in the Kit$^+$/EPO-R$^+$ fraction. Most of the Kit$^-$/EPO-R$^+$ cells were CD71$^-$. These CD71$^-$/Kit$^-$/EPO-R$^+$ cells displayed the low forward and perpendicular light scatter pattern that is characteristic of normoblasts (not shown), which are known to have lost CD71 expression [13]. The combined data are consistent with a model in which Kit expression on erythroid progenitors precedes expression of the transferrin receptor and the EPO-R. The data also demonstrate that during terminal erythroid maturation, the EPO-R is retained until a later differentiation stage than both Kit and the transferrin receptor.

Analysis of expression of CD11b, which is a marker for differentiating precursors of the granulocyte and monocyte lineages, showed that most of these cells were Kit$^-$ and EPO-R$^-$ (Fig. 4C and Table 2). This is consistent with the absence of

Table 1. Distribution of low-density rhesus monkey BM cells over different fractions, based on expression of GF receptors

	Fraction[a]	%[b]
1	Kit$^+$/EPO-R$^-$	8.5 ± 1.9
2	Kit$^+$/EPO-R$^+$	10.6 ± 5.3
3	Kit$^-$/EPO-R$^+$	5.5 ± 2.5
4	Kit$^-$/EPO-R$^-$	49.8 ± 4.8
1	Kit$^+$/IL-6-R$^-$	10.2 ± 1.3
2	Kit$^+$/IL-6-R$^+$	6.8 ± 0.4
3	Kit$^-$/IL-6-R$^+$	22.2 ± 0.6
4	Kit$^-$/IL-6-R$^-$	38.7 ± 0.8
1	Kit$^+$/GM-CSF-R$^-$	18.4 ± 4.7
2	Kit$^+$/GM-CSF-R$^+$	ND[c]
3	Kit$^-$/GM-CSF-R$^+$	11.3 ± 1.3
4	Kit$^-$/GM-CSF-R$^-$	41.7 ± 3.9

[a]Cells were stained with DIG-SCF and biotinylated EPO, IL-6, or GM-CSF.

[b]Data represent the distribution of low-density BM cells over the receptor-expressing regions as indicated in Figures 3B-3D (mean ± SD of five separate samples), analyzed inside a light scatter window as shown in Figure 3A. Note that percentages do not add up to 100% since events outside the four receptor-expression windows were left out of the analysis.

[c]ND = not detectable.

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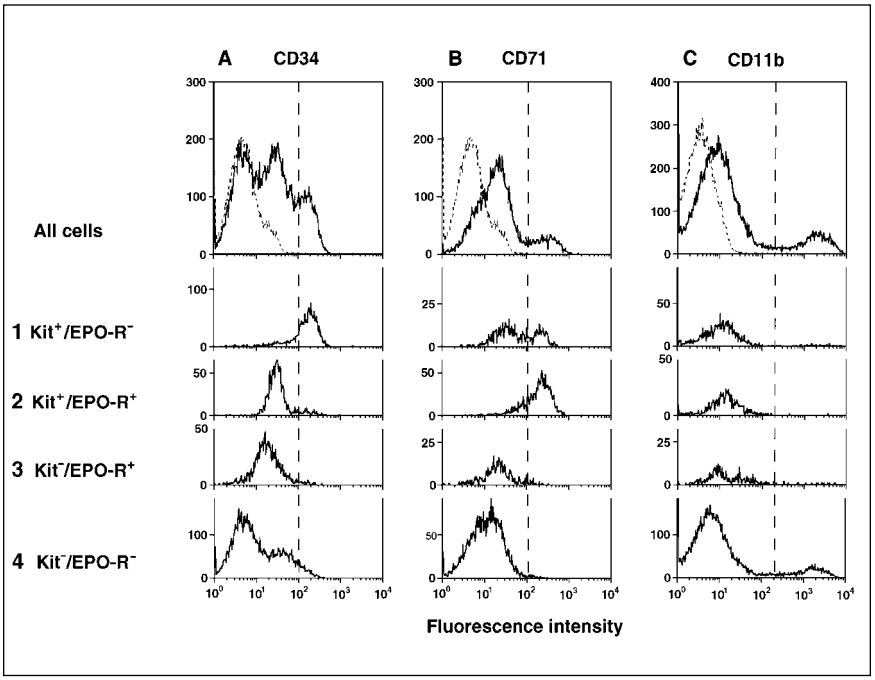

Figure 4. Expression of CD34, CD71, and CD11b on different fractions of low-density rhesus monkey BM cells, based on expression of Kit and EPO-R. *Cells were stained with labeled GFs and A) anti-CD34, B) anti-CD71, or C) anti-CD11b mAbs, and analyzed inside a light scatter window as shown in Figure 3A, in the regions that are indicated in Figure 3B. From top to bottom are shown: all cells; 1) Kit$^+$/EPO-R$^-$ cells; 2) Kit$^+$/EPO-R$^+$ cells; 3) Kit$^-$/EPO-R$^+$ cells; and 4) Kit$^-$/EPO-R$^-$ cells. Dotted histograms in the upper panels show background fluorescence of control cells. Vertical broken lines indicate the threshold values that were used to discriminate between cells with low or no fluorescence signal and brightly stained cells. Results are representative for three independently performed experiments.*

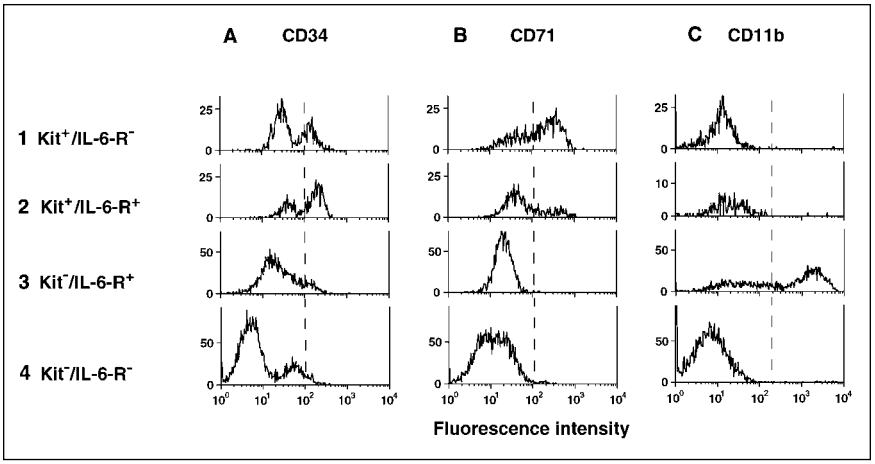

Figure 5. Expression of CD34, CD71, and CD11b on different fractions of low-density rhesus monkey BM cells, based on expression of Kit and IL-6-R. *Cells were incubated with DIG-SCF combined with biotinylated IL-6 and the same antibodies as in Figure 4. Cells were analyzed inside a light scatter window as shown in Figure 3A in the regions that are indicated in Figure 3C. 1) Kit$^+$/IL-6-R$^-$ cells; 2) Kit$^+$/IL-6-R$^+$ cells; 3) Kit$^-$/IL-6-R$^+$ cells; 4) Kit$^-$/IL-6-R$^-$ cells.*

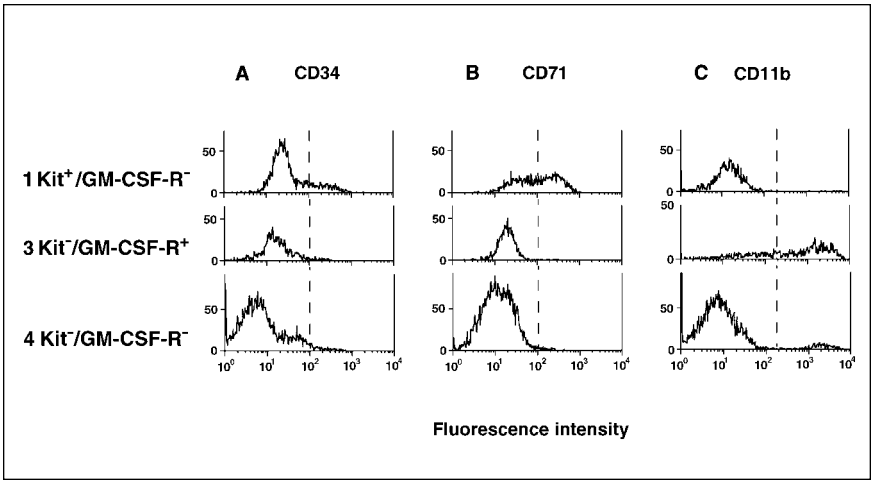

Figure 6. Expression of CD34, CD71, and CD11b on different fractions of low-density rhesus monkey BM cells, based on expression of Kit and GM-CSF-R. *Cells were incubated with DIG-SCF combined with biotinylated GM-CSF and the same antibodies as in Figure 4. Cells were analyzed inside a light scatter window as shown in Figure 3A, in the regions that are indicated in Figure 3D. 1) Kit$^+$/GM-CSF-R$^-$ cells; 3) Kit$^+$/GM-CSF-R$^+$ cells; 4) Kit$^-$/GM-CSF-R$^-$ cells (note that the Kit$^+$/GM-CSF-R$^+$ fraction 2) contained too few cells for analysis).*

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Table 2. Distribution of low-density rhesus monkey BM cells over different fractions, based on expression of GF receptors and other cell surface markers

	fraction[a]	CD34^{++} %[b]		CD71^{++} %[b]		CD11b^{+} %[b]	
1	Kit^{+}/EPO-R^{-}	8.7	(56.0)	2.5	(14.6)	0.2	(1.9)
2	Kit^{+}/EPO-R^{+}	0.7	(4.2)	13.2	(78.9)	0.1	(1.0)
3	Kit^{-}/EPO-R^{+}	0.2	(1.4)	0.3	(1.5)	0.4	(3.2)
4	Kit^{-}/EPO-R^{-}	2.5	(16.2)	0.4	(2.3)	6.0	(46.3)
1	Kit^{+}/IL-6-R^{-}	2.8	(20.7)	7.4	(58.9)	0.0	(0.2)
2	Kit^{+}/IL-6-R^{+}	4.4	(33.4)	1.8	(14.1)	0.8	(4.7)
3	Kit^{-}/IL-6-R^{+}	2.5	(18.8)	0.2	(1.4)	12.5	(75.2)
4	Kit^{-}/IL-6-R^{-}	1.2	(9.3)	0.5	(3.7)	0.2	(1.3)
1	Kit^{+}/GM-CSF-R^{-}	4.1	(53.3)	8.1	(66.7)	0.0	(0.6)
2	Kit^{+}/GM-CSF-R^{+}	ND[c]		ND[c]		ND[c]	
3	Kit^{-}/GM-CSF-R^{+}	0.4	(5.0)	0.1	(0.7)	6.3	(37.3)
4	Kit^{-}/GM-CSF-R^{-}	1.2	(16.5)	0.4	(3.4)	2.0	(11.8)

[a]Cells were stained with labeled GFs and anti-CD34, anti-CD71 or anti-CD11b mAbs. Data represent results of one experiment, which was representative of three independently performed experiments. Cells were analyzed inside a light scatter window as shown in Figure 3A, in the receptor-expressing regions as indicated in Figures 3B-3D.

[b]Data represent the percentages of BM cells that were found in the receptor-expression windows and that expressed CD34, CD71, or CD11b, respectively. Between parentheses are the same data, relative to the total number of cells expressing these markers (normalized to 100%; note that percentages do not add up to 100% since events outside the four receptor-expression windows were left out of the analysis). For example, 8.7% of the BM cells were CD34^{++}/Kit^{+}/EPO-R^{-}, which means that 56.0% of all CD34^{++} cells were found inside the Kit^{+}/EPO-R^{-} region.

[c]ND = not detectable.

the EPO-R on myelomonocytic cells and the disappearance of Kit during later stages of myeloid differentiation (see below).

Phenotypic Characterization of BM Subsets Based on Expression of Kit and IL-6-R

In parallel with the examination of Kit and EPO-R expression on hemopoietic subsets, we also studied coexpression of Kit with the IL-6-R and GM-CSF-R, respectively. The immature cells were heterogeneous with respect to IL-6-R expression, since high CD34 expression was found on Kit^{+}/IL-6-R^{+} as well as Kit^{+}/IL-6-R^{-} cells (Fig. 5A and Table 2). Average CD34 expression was higher on Kit^{+}/IL-6-R^{+} than on Kit^{+}/IL-6-R^{-} cells, suggesting that on average the Kit^{+}/IL-6-R^{+} cells were more immature than the Kit^{+}/IL-6-R^{-} cells.

Of the approximately 20%-25% of CD34^{++} cells that did not express Kit, approximately two-thirds were IL-6-R^{+} (Fig. 5A and Table 2), which suggests that there are also immature cells that express IL-6-R but not Kit. Almost all CD11b^{+} cells were also found in the Kit^{-}/IL-6-R^{+} subset (Fig. 5C), which is consistent with the expression of IL-6-R during differentiation of monocytes and granulocytes [15]. Therefore, the CD34^{++}/Kit^{-}/IL-6-R^{+} cells may represent differentiating myelomonocytic progenitors that have lost Kit expression. The CD11b^{-} cells in the Kit^{-}/IL-6-R^{+} population probably represent mature T cells, which have been shown to express IL-6-R at high levels [15, 34, 35].

Most of the Kit^{+} cells with low CD34 expression, which include erythroid colony-forming cells (CFU-E) and (pro)erythroblasts [17], were found in the IL-6-R^{-} fraction (Fig. 5A). Similarly, IL-6-R was not expressed on the majority of the Kit^{+}/CD71^{++} erythroid cells (Fig. 5B and Table 2). However, the presence of approximately 14% of the CD71^{++} cells in the Kit^{+}/IL-6-R^{+} fraction indicated that IL-6-R may be present on a subset of erythroid cells.

In conclusion, these results are consistent with a model in which Kit and IL-6-R are coexpressed on a subset of progenitors which includes the most immature cells identified by high CD34 levels. During maturation of erythroid cells, Kit expression increases and IL-6-R expression decreases. In contrast, myeloid differentiation is accompanied by a decrease in Kit expression and an increase in IL-6-R expression.

Phenotypic Characterization of BM Subsets Based on Expression of Kit and GM-CSF-R

As shown in Figure 3, Kit^{+} and GM-CSF-R^{+} populations showed little overlap. Kit^{+}/GM-CSF-R^{-} populations included primitive cells with the highest CD34 expression and erythroid cells with high CD71 expression (Figs. 6A and 6B). The Kit^{-}/GM-CSF-R^{+} population, on the other hand, contained most of the CD11b^{+} cells (Fig. 6C and Table 2), which is consistent with the expression of GM-CSF-R on monocytic and granulocytic cells [16, 36]. As shown in Figure 6C, this GM-CSF-R^{+} population covered a broad range of CD11b

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expression, which indicates that GM-CSF-Rs are expressed from immature ($CD11b^{low}$) to mature ($CD11b^{bright}$) monomyeloid cells. Additionally, some $CD11b^{+}/Kit^{-}$ cells that did not express detectable amounts of the GM-CSF-R were identified (Fig. 6C) which may represent a subset of natural killer (NK) cells or their precursors. These combined results are consistent with a model in which immature $Kit^{+}/CD11b^{-}$ cells lose Kit expression (and acquire GM-CSF-R) during monomyeloid differentiation and develop into $CD11b^{+}$ cells that express high levels of GM-CSF-R.

Discussion

In this paper, we show that DIG-SCF and biotinylated GFs can be used to study coexpression of Kit and other GF-Rs on hemopoietic cells. As expected from previous studies using biotin [17], SCF could be labeled efficiently with DIG, with retention of most of its ability to stimulate cell proliferation. By combining DIG-SCF with biotinylated GFs and mAbs against differentiation markers, we were able to examine coexpression of Kit and receptors for EPO, IL-6, and GM-CSF on rhesus monkey BM cells at different stages of differentiation of various blood cell lineages. This allowed a better definition of the subpopulation structure of the immature hemopoietic cell compartment. A schematic representation of changes in GF-R expression during erythroid and monomyeloid differentiation is shown in Figure 7.

The majority of the immature $CD34^{++}$ hemopoietic cells were Kit^{+}. This is in agreement with previous studies using anti-Kit antibodies on human cells [37-41] and biotin-SCF on rhesus monkey BM cells [17]. In these reports, using antibodies against CD34, CD33, CD38, and HLA-DR, Kit expression was demonstrated on the most immature subsets of $CD34^{++}$ cells that can be detected in vitro [42-44], which probably contain pluripotent hemopoietic stem cells relevant to hemopoietic reconstitution [45]. In line with this, cells with long-term repopulating ability were also found in the Kit^{+} fraction of murine BM [19, 46-48].

GM-CSF-Rs were absent from or expressed at a very low level on immature cells. This is in agreement with previous studies using purified $CD34^{+}$ rhesus monkey BM cells, showing no GM-CSF-Rs on erythroid and myeloid progenitors with light scatter characteristics that were similar to those we used for analysis [16]. Similar findings were recently reported by *McKinstry et al.*, who found little or no binding of radiolabeled GM-CSF to purified mouse BM subsets enriched for hemopoietic stem cells [49]. Taken together, these results suggest that GM-CSF-Rs are not expressed on hemopoietic stem cells, or only at extremely low levels.

Within the population of $CD34^{++}/Kit^{+}$ immature hemopoietic cells, $IL\text{-}6\text{-}R^{+}$ as well as $IL\text{-}6\text{-}R^{-}$ cells were found, consistent with the previously demonstrated heterogeneity of immature hemopoietic cells with respect to IL-6-R expression [15]. The average CD34 expression of the $CD34^{++}/Kit^{+}/IL\text{-}6\text{-}R^{-}$ cells is lower than that of the $CD34^{++}/Kit^{+}/IL\text{-}6\text{-}R^{+}$ cells, which might indicate that the $IL\text{-}6\text{-}R^{+}$ cells are more immature. Previous three-color immunofluorescence experiments showed that IL-6-Rs are expressed on a large subset of very immature rhesus monkey or human cells with high CD34 and low HLA-DR and CD38 expression [1, 45]. In situ autoradiographic studies and reverse transcriptase polymerase chain reaction also showed binding of IL-6 and expression of mRNA for IL-6-R and its partner gp130 on highly enriched fractions of murine BM stem cells and progenitor cells [49, 50]. However, as some cells with a very high CD34 expression are also present in the $Kit^{+}/IL\text{-}6\text{-}R^{-}$ subset (Fig. 5), it is possible that the most immature cells lack the IL-6-R. This is supported by the finding that the majority of the long-term culture-initiating cells and mixed colony-forming cells have been found in the $CD34^{+}/IL\text{-}6\text{-}R^{-}$ fractions of human BM and cord blood [51]. This population did, however, express gp130 and could respond to IL-6 in the presence of soluble IL-6-R [51].

Maturation of progenitors into monomyeloid cells is accompanied by a loss of Kit expression prior to acquisition of the CD11b antigen (Figs. 4-6). The decline of Kit expression occurs after the progenitor cell stage, since cells that form in vitro colonies (CFU-granulocyte/macrophage) were previously shown to be $CD34^{++}/Kit^{+}$ [17]. Expression of the

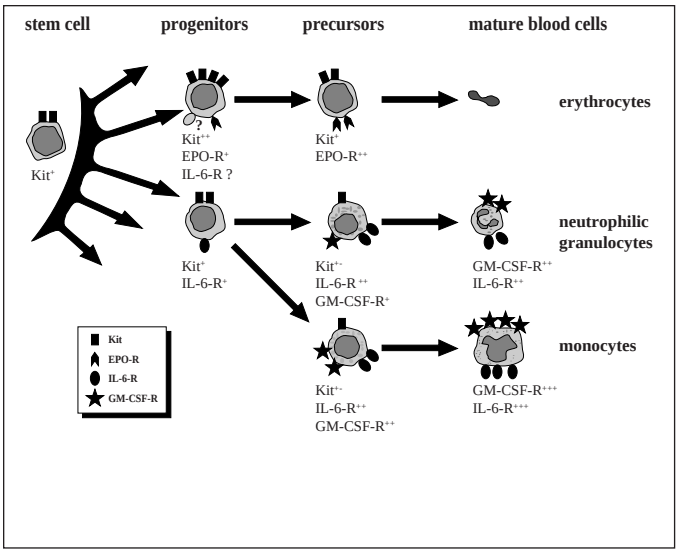

Figure 7. Schematic representation of erythroid and monomyeloid differentiation, displaying receptor expression for SCF, EPO, IL-6, and GM-CSF.

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IL-6-R increases during monomyeloid differentiation, as most $CD11b^+$ cells were IL-6-R^+, and high levels of IL-6-R expression have been identified on monocytes, macrophages, and granulocytes, and on the precursors of these cells (Fig. 5) [15, 49, 51-53]. As GM-CSF-Rs were present on the majority of the $CD11b^+$ cells but not on most of the immature $CD34^{++}$ cells (Fig. 6), expression of GM-CSF-R also increased during differentiation into mature granulocytes and monocytes. Similar results have been derived from binding studies using ^{125}I-GM-CSF or biotin-GM-CSF on cell lines and murine and rhesus monkey BM cells [16, 49, 54]. A small cluster of $CD11b^+$ cells was found in the Kit^-/GM-CSF-R^- fraction and may represent NK cells [55]. $CD11b^-$ cells with the scatter pattern of lymphocytes were found in the subsets that were Kit^-, EPO-R^- and GM-CSF-R^-. A fraction of these cells expressed IL-6-R (Fig. 5) as is in agreement with the presence of IL-6-R on T lymphocytes and absence on resting B lymphocytes [15, 34, 35].

Differentiation into $CD71^{++}$ erythroid cells is accompanied by a gradual loss of CD34 expression of the immature $CD34^{++}$/Kit^+ cells during the transition of BFU-Es, which are $CD34^+$ and express very high Kit levels, to CFU-Es, which have been found in the $CD34^{low}$/Kit^+ fraction [17]. Simultaneously, EPO-R, which is not detectable on most $CD34^{++}$ cells, is upregulated during erythroid differentiation, reaching maximal levels on $CD71^{++}$/Kit^+/EPO-R^+ CFU-E and pro-erythroblasts [14, 56]. Expression of EPO-R on a subset of cells that did not express Kit or CD71 and showed the light scatter pattern characteristic of normoblasts indicates that the EPO-R is retained on maturing erythroid cells even after the loss of the transferrin receptor and Kit.

The expression of IL-6-R on erythroid cells is less clear. Most of the $CD71^{++}$ cells were IL-6-R^-, which demonstrated that IL-6-R is not expressed on the majority of the erythroid cells. Moreover, most CFU-Es have been found in the $CD34^+$/IL-6-R^- fraction of human BM and cord blood [51]. A small subset of $CD71^{++}$ cells was identified in the Kit^+/IL-6-R^+ fraction, which may indicate that some erythroid cells expressed IL-6-R. This would be in line with the reported erythropoietic effect of IL-6, as demonstrated by an increase in BM normoblasts induced by in vivo administration of IL-6 in rats [57]. Alternatively, these cells might also represent activated nonerythroid cells that are Kit^+/IL-6-R^+, as CD71 is also upregulated on activated cells of other lineages [12, 58].

In conclusion, GFs that are labeled with DIG provide a useful extension of the existing cell-staining methods of detecting receptor-expressing cells by flow cytometry. It is relatively easy to construct biotin- or DIG-labeled GFs which retain the ability to bind to receptor expressing cells. By combining different labeled GFs with mAbs against cell-surface markers, it is possible to distinguish small subpopulations of BM cells which coexpress various high-affinity GF-Rs simultaneously. This approach is particularly useful to study changes in receptor expression patterns during differentiation of immature hemopoietic cells and to explain the stimulatory and inhibitory interactions of hemopoietic GFs on phenotypically defined hemopoietic cells. However, it is more difficult to exclude a direct action on a given cell type, because the detection limit of the method is approximately 50-100 receptor molecules per cell [15, 16, 31], whereas expression of only a few receptors can be sufficient for a biological response [1, 16, 18]. It is likely that neither radioactive labeling nor fluorochromes will yield even lower detection levels, which means that detection of such very low receptor levels can as yet only be achieved by sensitive reverse transcriptase polymerase chain reaction methods at the single-cell level.

Keeping these limitations in mind, simultaneous cell staining with DIG-GF and biotin-GF can be used to identify individual cells responding to multiple GFs. Since two GF-Rs can engage in physical interactions when coexpressed on the same cell, as has recently been demonstrated for Kit and EPO-R on transfected murine cell lines [59], the ability to detect the binding of different GFs to the same target cell can be used to examine whether similar interactions can take place on primary hemopoietic cells. Moreover, it provides an alternative approach to examination of the sharing of receptor subunits among different GF-Rs on rare hemopoietic cells, which are difficult to isolate in sufficient numbers to perform competition experiments using radioactively labeled GFs. A combination of various labeled GFs with a panel of cell-surface markers will allow further characterization of stem cells and progenitor cell subsets on the basis of GF-R expression.

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