hTAF$_{II}$32 coding sequence and then inserting the sequence into the pGEX2TK vector.
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28. Transcriptional activation was assayed with fractionated HeLa extracts and a template without G's containing the adenovirus major late promoter with five upstream Gal4 sites (*25*) [M. Sawadoga and R. G. Roeder, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 4394 (1985)]. We incubated 100 ng of template with purified activator for 5 min on ice. HeLa cell fractions P.1/D.3(IIA) and P.5 were added and incubated at 30°C for 30 min. Transcription was initiated by addition of nucleotides [500 mM adenosine triphosphate and cytidine triphosphate and 25 mM [α-^{32}P]uridine triphosphate (UTP); 5 mCi per reaction] and allowed to proceed for 15 min. After separation on a denaturing polyacrylamide gel, [α-^{32}P]UTP incorporation was quantitated by phosphoimager (Molecular Dynamics, Sunnyvale, CA).
29. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
30. Partial complex assembly and in vitro transcription were performed essentially as described (*16*). Briefly, HA-hTAF$_{II}$250 bound to HA antibody beads served as the basis of the complex assembly. Purified, recombinant proteins were added in the order of dTBP, dTAF$_{II}$60, and dTAF$_{II}$40, with extensive washing between each addition. The complexes were eluted from the beads in 60 μl of buffer containing 1 mg/ml of HA peptide at room temperature for 30 min. In vitro transcription was performed with the G5BCAT template (*25*). Reaction products were detected by primer extension (*25*).
31. We thank R. Li of M. Botchan's laboratory for providing the GST-p53(1–73) plasmid; S. Ruppert for providing recombinant hTAF$_{II}$70; K. Yokomori for providing recombinant dTFIIA; M. Maxon, E. Wang, H. Rye, D. Rio, G. Gill, and J. Oliner for critical comments on the manuscript; J. Lin for helpful discussions; S. Albright and G. Cutler for excellent graphics advice; and J. Goodrich for providing HeLa nuclear extracts, a pET 3a vector derivative, helpful discussions, and encouragement. Supported by an NSF graduate fellowship (C.J.T.) and in part by a grant from NIH (R.T.).

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Functional Isolation and Characterization of Human Hematopoietic Stem Cells

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Hematopoietic cells differentiate in steps marked by the acquisition or loss of specific phenotypic characteristics. Human bone marrow cells that were responsive to the early-acting cytokines Kit ligand and interleukin-3 were forced to a metabolic death. The subfraction remaining represented 1 in 10^5 bone marrow mononuclear cells, was determined to be quiescent by cell cycle analysis, and had a stem cell immunophenotype. The cells were highly enriched for long-term culture-initiating cells, were capable of secondary colony formation, and produced both myeloid and lymphoid progeny. Thus, this technically simple strategy led to the efficient purification of cells with characteristics of hematopoietic stem cells.

The efficient isolation of hematopoietic stem cells would enhance both the investigation of the processes of lineage commitment and self-renewal and the clinical strategies of bone marrow transplantation and gene therapy with hematopoietic cells. The fraction of human bone marrow that is CD34$^+$, CD33$^-$, CD38$^-$, human lymphocyte antigen (HLA) DR$^-$, Thy-1^{lo}, negative for lineage-specific antigens, and stains lightly with the dye rhodamine-123 is enriched for repopulating stem cells (*1*, *2*). However, methods that depend on cell sorting require considerable mechanical manipulation of the cells, and antibody binding to cell surface structures may induce perturbations of the cells' physiology. Other enrichment methods have been based on functional characteristics of cells, including soybean agglutinin binding (*3*) and resistance to either the alkylating agent 4-hydroxycyclophosphamide (4-HC) (*4*) or the antimetabolite 5-fluorouracil (5-FU) (*5*). However, these methods result in persistent heterogeneity within the selected cell population.

We reasoned that by combining cytokine stimulation with antimetabolite treatment, we could induce cell death in responding cells and therefore select for cells resistant to the proliferative effects of the cytokines. The earliest identifiably committed progenitor cells respond to Kit ligand (KL) and interleukin-3 (IL-3) and have been identified as primitive cells capable of forming high proliferative capacity colonies type 1 (HPP-CFC-1), an in vitro marker of a very primitive myeloid cell (*6*). Because the IL-3 receptor is not thought to be present on the hematopoietic stem cell (*7*), we incubated these cells with KL and IL-3 in the presence of serum plus 5-FU for a prolonged period to functionally select for primitive stem cells.

We assessed the selection technique by flow cytometric analysis with DNA staining dyes (Fig. 1). When either bulk bone marrow mononuclear preparations or bone marrow cells selected with CD34$^+$ immunobeads were used, the selection strategy efficiently eliminated cells in the S or G_2-M phase. Remaining cells were shown to be in the G_0 phase by staining with the ribosomal RNA dye Pyronin Y (Polysciences, Warrington, Pennsylvania). Testing a number of different selection strategies, we determined that the maximal efficiency was attained when high concentrations of 5-FU were used (>200 μg/ml) and when selection was continued for at least 7 days. At high concentrations, 5-FU inhibits RNA splicing as well as DNA synthesis and may therefore act to more effectively eliminate metabolically activated cells (*8*).

We evaluated whether our selection had isolated cells resistant to the proliferative effects of KL and IL-3 by use of the fluorescent membrane dye PKH26, which is stable

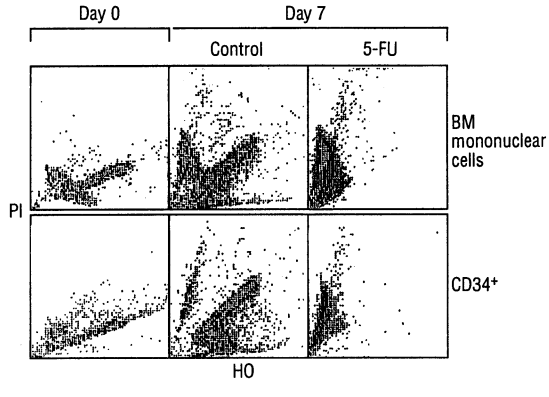

Fig. 1. Cell cycle status of CD34$^+$-selected or unselected bone marrow (BM) mononuclear cells cultured under control or 5-FU–selected conditions (*23*). Cells were stained with propidium iodide (PI) and Hoechst 33342 (HO) (Sigma) (*24*) and then analyzed by flow cytometry on days 0 and 7. HO staining intensity varies with DNA content and increases as cells transit from G_0-G_1 into the S and G_2-M phases of the cell cycle. PI staining increases with either loss of viability ($PI_{mod,hi}$, HO_{lo}) or as cells undergo the S, G_2, and M phases ($PI_{mod,hi}$, $HO_{mod,hi}$). Under control conditions, the full spectrum of the cell cycle was represented as indicated by variable HO staining, with more nonviable cells ($PI_{mod,hi}$, HO_{lo}) present after the 7-day incubation period, which results from terminally differentiated cells in liquid culture. However, the 5-FU–treated cells are restricted to HO_{lo} staining and had an increased proportion of nonviable cells. A remaining small subpopulation of viable cells (PI_{lo}, HO_{lo}) in the 5-FU–treated cultures was confirmed by trypan blue exclusion analysis. Similar results were obtained with either CD34$^+$-selected (bottom) or bulk preparations of bone marrow mononuclear cells (top).

in cell membranes. As cells divide, the dye partitions in daughter cells, yielding an exponential decrease in the fluorescence intensity per cell. During the selection process, cells with high PKH26 fluorescence were enriched, and unlike control cells, their peak fluorescence intensity did not decline (Fig. 2).

Morphologically, the residual cells were small in size (6 μm) with dense chromatin and a faint halo of cytoplasm (Fig. 3B). Immunophenotypic analysis was performed by fluorescence microscopy on cytocentrifuged preparations by two independent readers (9). No cells stained with markers for potentially contaminating quiescent lymphoid cells (CD19 or CD45Ra) or for more differentiated progenitor cells (CD38, CD33, or HLA-DR). In contrast, all cells scored positively for CD34 and c-Kit. These latter markers are present on cells capable of providing multilineage human hematopoietic tissue in a chimeric fetal sheep model (*10*). The particular combination of markers present on the selected cells matches that found by a number of other investigators to identify a subset of very primitive hematopoietic cells (*1*). The yield of cells selected by our method was approximately 1 in every 10^5 bone marrow mononuclear cells.

Functional characterization of human hematopoietic progenitor cells depends on in vitro assays. Cells selected in KL, IL-3, and 5-FU were uniformly incapable of forming colonies in methylcellulose upon completion of the selection process, despite supplementation with multiple combinations of early- and late-acting cytokines. However, when cultivated with irradiated bone marrow stromal feeder cells in a long-term, culture-initiating cell (LTC-IC) assay (*11*), the cells generated colonies of mixed myeloid lineages beginning at 3 weeks. To quantitatively assess the ability of the selected cells to generate more differentiated cells over time, we used a limiting dilution, 96-well-plate LTC-IC assay (*12*). Cells were plated with the Becton Dickinson cell deposition unit (CDU), which has an accuracy of plating of 0.1% (*13*), to provide 24 wells each of 0, 1, 5, 10, or 100 cells per well. The fraction of samples with one cell per well that yielded colonies after 5 weeks of cultivation with stroma, then 2 weeks in methylcellulose, in five independent experiments was 73% (95% confidence interval, 64 to 80%) (Table 1). When normalized for the maximal number of wells scoring positive in the samples with 5, 10, or 100 cells per well (which is thought to reflect the supportive capacity of the irradiated feeder layer), the percent positive was 89% (95% confidence interval, 82 to 95%). This result compares favorably with the highest yield reported with any other subselection technique for bone marrow cells (*12*) and was significantly better than the results obtained with $CD34^+$ bone marrow cells in our assay ($P < 0.001$ by Fisher's exact test). Stroma-alone control cultures were uniformly negative.

Individual subselected cells were capable of inducing many areas of cobblestonelike proliferation on the stromal feeder layers and, after 5 weeks of cultivation with stroma, multiple colonies in methylcellulose (mean, 5.2 colonies per cell; 95% confidence interval, 4.4 to 6.0 colonies per cell; range, 1 to 17 colonies per cell) (Fig. 4). Dispersed cells from these colonies were capable of forming secondary cultures on replating in methylcellulose with an efficiency of 40%. The colony morphology varied between compact and loosely associated clusters. The latter morphology predominated in the cultures and is consistent with that of a colony-forming unit (CFU) blast.

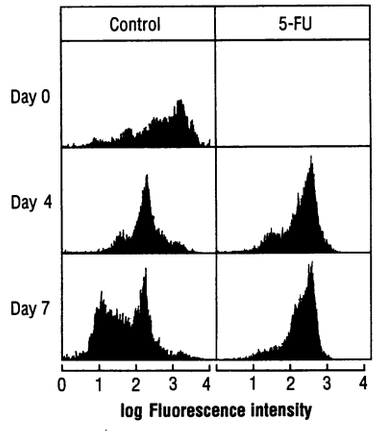

Fig. 2. Lack of cell division in cytokine plus 5-FU–selected cells. We evaluated bone marrow mononuclear cell proliferation by staining with the stable membrane dye PKH26 (Zynaxis, Malvern, Pennsylvania) and evaluated it by flow cytometry (FACScan, Becton Dickinson, San Jose, California) using the FL2 channel. PKH26 stains cells differentially on the basis of cell type, which is thought to be the basis for the range of overlapping peaks when unselected bone marrow mononuclear cells are used (top, left).

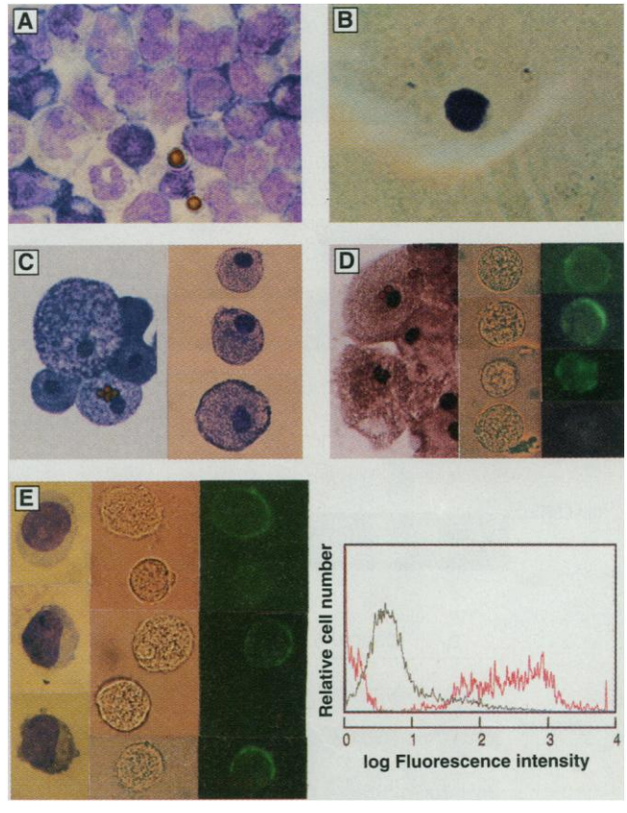

Fig. 3. Morphology of $CD34^+$ bone marrow mononuclear cells cultured in the presence of IL-3 and KL (**A**) or in the presence of IL-3, KL, and 5-FU (**B**) for 7 days. Control cells (original magnification ×400) were large with evidence of active mitosis, whereas 5-FU–selected cells (original magnification ×1000) were uniformly small with dense nuclei and only a faint rim of cytoplasm. After long-term bone marrow culture of single cells, cytospin preparations revealed cells of variable size with large, macrophage-like cells that were often clustered (**C**) (original magnification ×400) and that stained positively for α-naphthyl esterase [(**D**), left] or CD11b antibody [(D), right]. Dispersed cells and some clustered larger cells were negative for α-naphthyl esterase; stroma-alone cultures revealed rare α-naphthyl esterase–positive cells. Isotype control antibody staining was negative [(D), right, bottom]. Smaller, dispersed cells (magnification ×1000) were lymphoid in appearance [(**E**), left], and some stained positively for B cell–specific CD19 [(E), right]. The middle panels of (D) and (E) are phase-contrast micrographs of the cells shown in the panels to their right. (Lower right panel) Pooled nonadherent cells were stained with CD19 antibody (red) or isotype control antibody (black) and analyzed by flow cytometry. Images of cell morphology shown were obtained after Wright-Giemsa staining.

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Less than 20% of colonies were of the HPP-CFC or mixed colony-forming unit (CFU-mix) morphologic type.

We next sought to determine the differentiative capacity of the selected cells—specifically, whether the selected cells were capable of differentiating along lymphoid as well as myeloid lineages. First, we confirmed that individually selected cells plated under standard Dexter culture conditions (*14*) produced progeny that histologically resembled macrophages, histochemically stained positively for the enzyme α-naphthyl esterase, and were cell surface–positive for CD11b by immunofluorescence. The selected cells were thereby considered capable of maturing along myeloid lines, specifically producing monocytoid cells in liquid culture. Single-cell preparations were then cultivated under Dexter culture conditions for 4 weeks and then switched to culture conditions adapted from the lymphoid-supporting Whitlock-Witte cultivation system; this modification in murine systems results in the production of both myeloid and lymphoid cell elements (*15*). A sample of cells derived from these culture conditions was stained for the B cell–specific marker CD19 and myeloid markers CD11b and esterase. Single cells yielded both myeloid (esterase$^+$, CD11b$^+$) (Fig. 3, C and D) and lymphoid (CD19$^+$) (Fig. 3E) progeny. Pooled nonadherent cells stained with CD19 and analyzed with flow cytometry were 57% lymphoid (Fig. 3). These data are consistent with the conclusion that the selected subpopulation of bone marrow cells had multilineage potential in addition to having LTC-IC functional capacity.

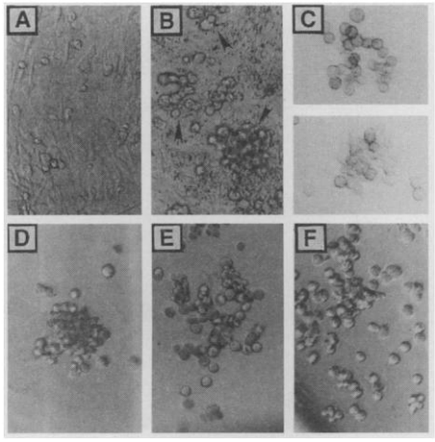

Fig. 4. Functionally selected bone marrow mononuclear cells (G_0 cells) could generate cobblestonelike areas when cultivated with human stroma. Control stromal cultures (**A**) appeared distinct on phase microscopy from the cobblestonelike cultivation cultures (**B**), where cobblestonelike areas are marked by arrowheads and in which clusters of cells extended vertically into the supernatant (photographed at a different focal plane) (**C**). After cultivation with irradiated bone marrow stroma feeder layers, colonies in methylcellulose were evident (**D** through **F**). Original magnification, ×400.

To evaluate the characteristics of the selected cells, we isolated individual cells from various stages of differentiation in microtiter wells and generated complementary DNA (cDNA) that was nonspecifically amplified by a modified polymerase chain reaction method (*15*, *25*). Panels of cDNAs were then probed for expression of cytokine receptors. To confirm that the panels of

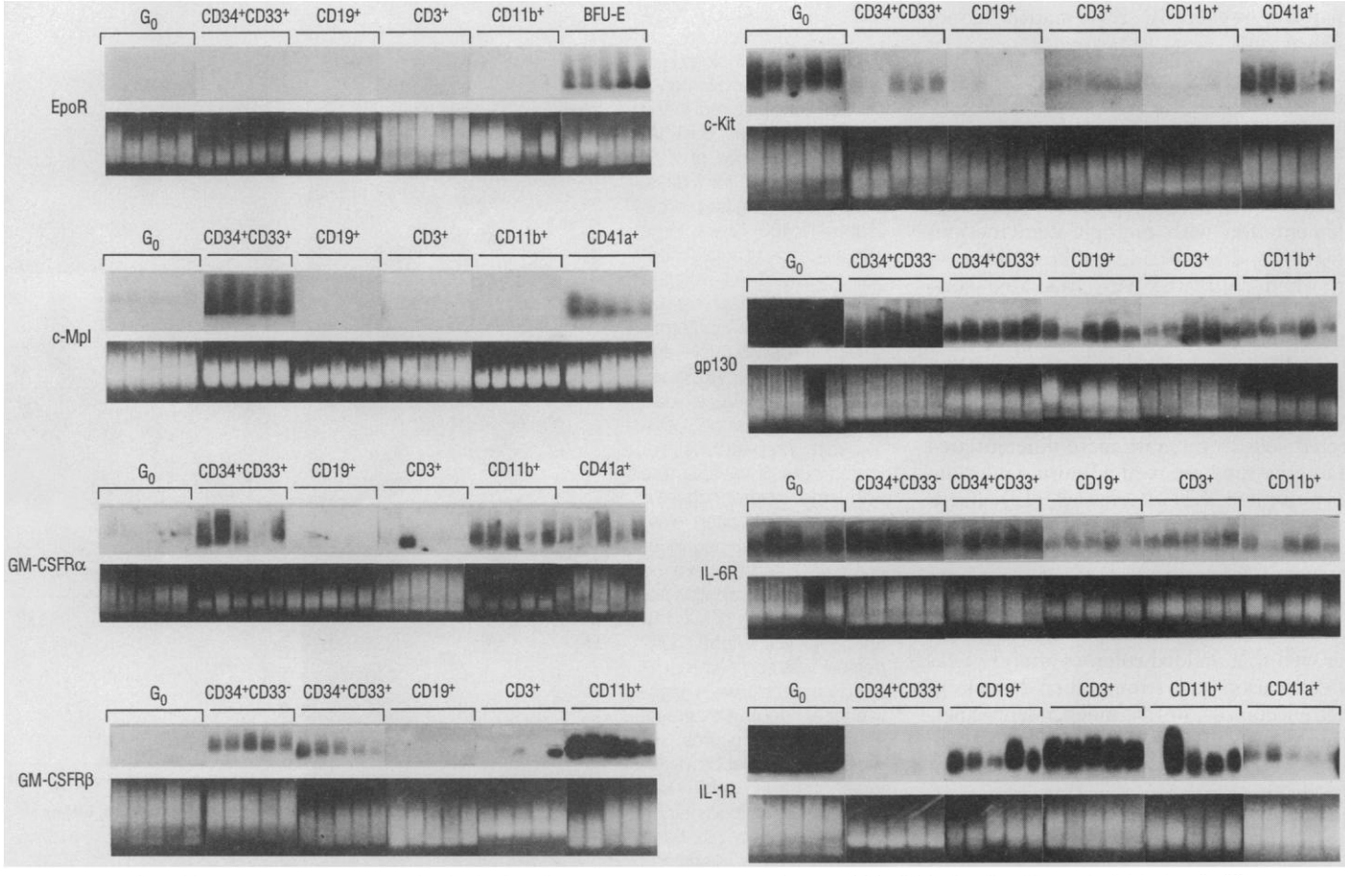

Fig. 5. Analysis of cytokine receptor gene expression in single cells representing various stages of hematopoietic differentiation. The lower panel of each receptor set is the ethidium bromide–stained cellular cDNA subsequently transferred to a nylon filter, probed with the indicated receptor cDNA, and subjected to autoradiography (upper panels). Panels of five cells of each type were used to avoid individual cell artifacts; individual cell differences may represent heterogeneity in the cell subset or contamination by extraneous cells. The amplification schema results in variable length cDNAs from any given transcript, resulting in smears rather than bands when they are hybridized to a corresponding probe (*17*).

Table 1. Limiting dilution LTC-IC assay results from the wells plated at one cell per well in 24 replicates in five independent experiments. Wells yielding colonies in methylcellulose were scored as positive. Data from all wells (mean, 73%; 95% confidence interval, 64 to 80%) or data normalized to the maximum number of wells positive of the samples with 5, 10, or 100 cells per well (mean, 89%; 95% confidence interval, 82 to 95%) are shown. No colonies were seen in any control wells (0 cells per well); no statistically significant differences between the samples with 5, 10, or 100 cells per well were detected (mean, 79%, 81%, and 81%, respectively). Control $CD34^+$ bone marrow mononuclear cells similarly plated yielded an estimated LTC-IC frequency of 4.5 to 8.6% when evaluated by Poisson analysis (*25*).

Experiment	Normalized		All wells	
	Positive/total	% positive	Positive/total	% positive
1	17/19	89	17/24	71
2	19/21	90	19/24	79
3	19/21	90	19/24	79
4	17/18	94	17/24	71
5	15/18	83	15/24	63

cDNAs represented differentiation-specific subsets of cells, we initially probed for receptors whose expression is known to be cell type–specific (Fig. 5). The erythropoietin receptor was selectively present in cDNA derived from the dispersed cells of an early burst-forming unit erythroid (BFU-E), as would be predicted (*18*). Similarly, the transcript for c-Mpl (*19*), associated with megakaryocytic differentiation, was detected in CD41a-selected cells (megakaryocytes) and the early myeloid committed $CD34^+CD33^+$ cells from which megakaryocytes descend. Unexpectedly, the subselected stem cells also expressed c-Mpl. The granulocyte-macrophage colony-stimulating factor α receptor (GM-CSFRα) was expressed on mature $CD11b^+$ (granulocytic and monocytic) and $CD41a^+$ (megakaryocytic) cells as well as on the common precursor to these cells, $CD34^+CD33^+$, an observation consistent with the known responsiveness of these cell types to GM-CSF (*20*).

Other receptors had different patterns of expression. The gp130 subunit shared by multiple cytokine receptors such as IL-6, IL-11, leukemia inhibitory factor, oncostatin M, and ciliary neurotrophic factor was in all cell types tested. Multiple hematopoietic cell types, including primitive cells, responded to IL-1 and IL-6 (*21*), and cDNAs for both were detectable in most cells tested, except the $CD34^+CD33^+$ subset.

To confirm that our KL- and IL-3–selected population was not responsive to these cytokines, we probed for their receptors. We detected expression of c-Kit, but no expression of the β chain shared between GM-CSF and IL-3. Thus, because KL induces proliferative responses only in the presence of a second cytokine and may act alone as a survival factor (*22*), these data are consistent with the functional isolation strategy we used.

This system may be useful for characterizing the cytokine stimuli and adhesive interactions that will trigger the quiescent stem cell population into the cell cycle. Our method exploits the functional characteristics of cells proceeding along hematologic differentiation programs in order to isolate subsets of primitive cells. This technique avoids the mechanical trauma of multiple rounds of cell sorting and the potential perturbation of cell function induced by antibody binding. Further, this strategy is potentially applicable to clinical situations, because the mechanical handling of the cells is minimal. The output of this isolation strategy is a highly enriched subfraction of multipotent cells with in vitro characteristics that are consistent with those of repopulating stem cells. If stem cell gene transduction is desired, this method may augment the specific targeting of such cells, particularly important when virus-based transduction strategies are involved. Further, the relative purity of this population lends itself to the molecular study of the events regulating the quiescent stem cell.

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9. Cells were incubated at 4°C with fluorescein isothiocyanate–conjugated CD34, CD38, CD33, CD45, CD19, c-Kit, or HLA-DR antibodies (Amac, Westbrook, ME) for 30 min, washed three times with cold phosphate-buffered saline (PBS), and fixed in 0.1% paraformaldehyde. The fixed cells were examined by fluorescence microscopy, and 50 cells each were scored qualitatively.
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17. Single cells were plated in a 96-well plate by either the CDU [based on forward and side scatter (G_0) or antibody staining properties] or by limiting dilutions of BFU-E cells manually aspirated from methylcellulose assays by micropipette directly into 4 μl of lysis buffer [50 mM tris HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, deoxyribonucleotide triphosphate (2 μM) (Pharmacia), 100 ng/ml of oligo$(dT)_{24}$, Inhibit Ace 100 U/ml (5′-3′, Boulder, CO), RNAguard (2000 U/ml) (Pharmacia), and 0.5% NP-40] as described (*16*). The samples were heated to 65°C for 1 min, cooled to 22°C for 3 min, and put on ice. One hundred units of Moloney (Gibco-BRL) and 2 units of avian reverse transcriptase (Promega, Madison, WI) were added and the samples incubated at 37°C for 15 min before heat inactivation at 65°C for 10 min. Resultant cDNA was then subjected to polyadenylate tailing in 200 mM potassium cacodylate, 4 mM $CoCl_2$, 0.4 mM dithiotheitol, 200 μM deoxyadenosine triphosphate, and 10 units of terminal transferase (Boehringer Mannheim, Indianapolis, IN) at 37°C for 30 min. Polymerase chain reaction (PCR) was then performed

with 10 mM tris HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM deoxyribonucleotide triphosphate, 0.05% Triton X-100, 5 μM $(dT)_{24}X$ primer (ATG TCG TCC AGG CCG CTC TGG ACA AAA TAT GAA TTC dT_{24}), and 5 units of Taq polymerase (Perkin-Elmer Cetus, Newton Centre, MA). Cell-free and reverse transcriptase–free samples were used as negative controls. The PCR product was electrophoresed through 1% agarose, stained with ethidium bromide and photographed, transferred to a nylon membrane, and hybridized to the indicated radiolabeled probes with the use of either cDNA clones [human erythropoietin receptor (EpoR), GM-CSFRα, and GM-CSFRβ chains and human c-Kit] or oligodeoxyribonucleotides derived from the cDNA sequence [human c-Mpl (CAGATCAGCTGGGAGGAGCCAAGCACTGAACTTCACCGTCGC), the human IL-1 receptor (IL-1R) (ATAGCAGCCCAGGGCACTTCAGAGTAAGAGGGCTTGGGAAGATCTTTTTAAAA), human IL-6R (CTTACTTAGGTGTGGGGGAAGCACCATAACTTTGTTTAGCCCAAAACCAAG), or human gp130 (CTGTACGGCAAGGCGGCTACATGCCTCAGTGAAGGACTAGTAGTT)]. Final wash conditions for the membranes were in 0.2× saline sodium citrate and 0.5% SDS at 40°C.

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23. Heparinized bone marrow was obtained from normal volunteers who provided written informed consent to a protocol approved by the Deaconess Hospital Institutional Review Board. The low-density, adherence-depleted mononuclear cells were either selected by immunomagnetic beads (Dynal; Great Neck, NY) for CD34 expression (Amac) or not before selection by incubation at 37°C with 5% CO_2 for 7 days in Iscove's modified Dulbecco's medium (Gibco) containing 10% fetal calf serum (HyClone, Logan, UT) supplemented with (100 ng/ml) KL and (100 ng/ml) IL-3 with or without 5-FU (0.6 mg/ml) (Solo Pack, Elk Grove Village, IL).
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Association of Protein Kinase A and Protein Phosphatase 2B with a Common Anchoring Protein

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Specificity of protein kinases and phosphatases may be achieved through compartmentalization with preferred substrates. In neurons, adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase (PKA) is localized at postsynaptic densities by association of its regulatory subunit with an A kinase anchor protein, AKAP79. Interaction cloning experiments demonstrated that AKAP79 also binds protein phosphatase 2B, or calcineurin (CaN). A ternary complex of PKA, AKAP, and CaN was isolated from bovine brain, and colocalization of the kinase and the phosphatase was established in neurites of cultured hippocampal neurons. The putative CaN-binding domain of AKAP79 is similar to that of the immunophilin FKBP-12, and AKAP79 inhibited CaN phosphatase activity. These results suggest that both PKA and CaN are targeted to subcellular sites by association with a common anchor protein and thereby regulate the phosphorylation state of key neuronal substrates.

Protein phosphorylation is a primary means of mediating signal transduction events that control cellular processes. Accordingly, the activities of protein kinases and phosphoprotein phosphatases are highly regulated. One level of regulation is reflected by restriction of the subcellular distribution of several kinases and phosphatases by association with targeting proteins or subunits (*1*), which promotes rapid and preferential modulation of specific targets within a defined microenvironment in response to diffusible second messengers. For example, the type II PKA is targeted by association of its regulatory subunit (RII) with AKAPs (*2*), and disruption of this interaction in neurons affects the modulation of glutamate receptor channels (*3*). In accordance with the targeting subunit hypothesis, AKAPs associate with other cellular components to adapt the kinase for specific roles; however, these additional protein-protein interactions are poorly understood.

To identify AKAP-binding proteins, we used the yeast two-hybrid system (*4*) to isolate complementary DNAs (cDNAs) that encode proteins that associate with AKAP79, a human neuronal anchor protein (*5*, *6*). One positive clone, termed 11.1, contained cDNA for a murine β isoform of the CaN A subunit (*7*). Control experiments with dihybrid crosses showed that CaN specifically interacted with AKAP79; matings of yeast containing CaN cDNA with those expressing Gal4 fusions of RII, casein kinase 1, or phosphodiesterase, or the Gal4 DNA-binding domain alone (pAS1), were negative (Fig. 1). The two-hybrid system also positively identified interactions between RII and itself (dimerization) and between RII and AKAPs 79 or Ht31 (*8*) (Fig. 1). These observations provide evidence for association of AKAP79 with CaN and, because AKAP79 also binds RII, suggest the occurrence of a ternary complex between type II PKA, AKAP79, and CaN.

We used biochemical methods to examine whether PKA and CaN are associated in mammalian brain. Calmodulin-binding proteins were isolated from bovine brain extracts by affinity chromatography, and CaN was immunoprecipitated with affinity-purified antibodies to the CaN A subunit (*9*). Immunoprecipitates were incubated with cAMP, and the resulting eluate was assayed for PKA activity by addition of adenosine triphosphate (ATP) and kemptide substrate. The specific activity of protein kinase was increased by purification of a 30 to 60% saturated ammonium sulfate fraction of the extract on calmodulin-agarose (28 ± 6-fold; mean ± SD, $n = 3$) and by immunoprecipitation with specific antibodies to CaN (123 ± 3.6-fold) (Fig. 2A).

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