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P2Y-like receptor, GPR105 ($P2Y_{14}$), identifies and mediates chemotaxis of bone-marrow hematopoietic stem cells

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Hematopoiesis in mammals undergoes a developmental shift in location from fetal liver to bone marrow accompanied by a gradual transition from highly proliferative to deeply quiescent stem cell populations. P2Y receptors are G-protein-coupled nucleotide receptors participating in vascular and immune responses to injury. We identified a P2Y-like receptor for UDP-conjugated sugars, GPR105 ($P2Y_{14}$), with restricted expression on primitive cells in the hematopoietic lineage. Anti-GPR105 antibody selectively isolated a subset of hematopoietic cells within the fetal bone marrow, but not in the fetal liver, that was enriched for G0 cell cycle status and for in vitro stem-cell-like multipotential long-term culture capability. Conditioned media from bone marrow stroma induced receptor activation and chemotaxis that was sensitive to Gαi and anti-receptor antibody inhibition. GPR105 is a G-protein-coupled receptor identifying a quiescent, primitive population of hematopoietic cells restricted to bone marrow. It mediates primitive cell responses to specific hematopoietic microenvironments and extends the known immune system functions of P2Y receptors to the stem cell level. These data suggest a new class of receptors participating in the regulation of the stem cell compartment.

[*Keywords*: Stem cells; hematopoiesis; chemotaxis; G-protein-coupled receptors; P2Y receptors]

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G-protein-coupled receptors (GPRs) have a broad repertoire of activating ligands ranging from photons to chemokines and induce an array of cellular events in virtually every physiologic system. Yet there remains a large proportion of GPR without known ligands or with limited known functions. A group of GPRs responsive to nucleotides (termed P2Y receptors) has been defined mediating cell–cell communication in the nervous system and in modulating vascular tone (Di Virgilio et al. 2001). A well-defined effect of nucleotides on platelet activation and vascular smooth muscle migration and growth has suggested participation of P2Y receptors in the response to injury. More recently, these receptors have been noted to affect cellular constituents of the innate immune system altering functional characteristics of monocytes, eosinophils, and dendritic cells and to play a critical role in terminating the inflammatory response in vivo (Mutini et al. 1999; Ferrari et al. 2000; Idzko et al. 2001; Santiago-Perez et al. 2001; Warny et al. 2001; Wilkin et al. 2001). The P2Y receptor specificity originally thought to be restricted to purine (adenine) nucleotides has been extended to pyrimidine nucleotides (uridine) and more recently to a receptor with specificity for UDP, but only when conjugated to glucose or related sugars (Chambers et al. 2000). This receptor, GPR105 (recently designated $P2Y_{14}$; Abbracchio et al. 2003), was originally noted to be expressed in rat brain tegmentum, but has no known function apart from being the presumed basis for UDP-glucose to induce diaphragmatic contraction or neural action potentials (Pastoris et al. 1979, 1981). We provide evidence that this receptor participates in regulation of hematopoietic cells with stem cell characteristics.

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Bone-marrrow-derived hematopoietic stem cells have multipotential capability (Till et al. 1975; Morrison and Weissman 1994; Krause et al. 2001) and have a functional phenotype of relative quiescence and relative insensitivity to cytokine stimulation (Traycoff et al. 1995, 1996; Abkowitz et al. 1996; Bradford et al. 1997; Veena et al. 1998). The altered cell cycling of stem cells in the absence of the cyclin-dependent kinase inhibitor $p21^{cip1/waf1}$ has suggested that dominance of inhibitory tone may dictate the limited cycling of cells in the adult mammal (Cheng et al. 2000a,b). However, the quiescence of the stem cell compartment is developmentally determined and acquired as hematopoiesis shifts from the fetal liver to the bone marrow in the second trimester of gestation. The association of cycling phenotype with anatomic location suggested that microenvironmentally determined cues may affect the relative quiescence of the stem cell compartment, leading us to seek cell surface receptors preferentially present in conditions of stem cell quiescence.

Results

We used a strategy of antimetabolite treatment combined with cytokines known to drive human progenitor populations into cell cycle to selectively eliminate actively cycling cells (Berardi et al. 1995), thereby exploiting the known relative quiescence and cytokine unresponsiveness of the stem cell compartment. This method yields a rare population of human bone-marrow-derived cells (1 in 10^6 mononuclear cells) that were previously defined to be small in size, quiescent, and produce progeny only after prolonged exposure to bone marrow stroma (Berardi et al. 1995). A similar strategy has been used by others to isolate cells shown to have efficient in vivo stem-cell-like characteristics including repopulation of lethally irradiated hosts (Bertolini et al. 1997a,b). The rarity of these cells has required the use of PCR-based strategies for characterization of the cellular gene expression profile (Berardi et al. 1995; Cheng et al. 1996). Based on analysis of individual cells, the population of cells isolated by the directed suicide technique has been shown to have relative molecularly homogeneity within the constraints of the assays used (Berardi et al. 1995; Cheng et al. 1996).

Using this cell population as a reagent for identification of novel regulatory genes, we generated cDNA from ~20 human cells using a poly(dT)-primed reverse transcriptase PCR (RT–PCR) technique that has been shown to preserve fidelity in both complexity and relative abundance of input mRNAs (Brady et al. 1995; Cheng et al. 1996). This cDNA was then sequentially subtracted against cDNA from more mature $CD34^+$ $CD38^+$ cells using biotinylated nucleotides incorporated into the subtractant population and avidin-mediated removal of common sequences (Fig. 1A). Approximately 200 clones with increased expression in the G0 population were isolated and sequenced. Among these, approximately one-third were considered recombination artifacts, one-third were novel, and those with homology to transmembrane molecules were considered for further evaluation. One encoded a 7-transmembrane G-protein-coupled receptor corresponding to a gene in GenBank (KIAA0001; accession no. D13626) originally cloned from a human $CD34^+$ hematopoietic progenitor line, KG1, and subsequently identified in rat brain, but for which no function has been defined (Nomura et al. 1994; Charlton et al. 1997). This clone was used to probe cDNA from unsubtracted suicide-selected G0 cells, cDNA from suicide-selected G0 cells sequentially subtracted against $CD34^+/CD38^+$ cell cDNA, and $CD34^+/CD38^+$ cell cDNA. The receptor was expressed in quiescent, suicide-selected $CD34^+/CD38^-$ cells, but not in $CD34^+/CD38^+$ cells (Fig. 1B).

The KIAA0001 G-protein-coupled receptor gene is predicted to encode a 338-amino-acid protein named GPR105 and noted to have specificity for UDP conjugated to glucose, galactose, glucuronic acid, or *N*-acetylglucosamine, but not UDP, ADP, ATP, or 29 other ligands tested (Chambers et al. 2000). Closely related genes are H963, GPR34, and EB12, GPR of unknown function, and platelet-activating factor receptor (PAF-R), known to affect cell motility (Boccellino et al. 2000). GPR105 has remote (20%–30% amino acid) similarity to chemokine receptors and does have the sequence motif DRYYKIV, located at the end of transmembrane III, similar to the DRYLAIV motif, a signature sequence for chemokine receptors (Youn et al. 1997). The chromosomal location of *GPR105* coincides with the GPR receptor cluster on Chromosome 3 (Napolitano et al. 1996). Isolation and sequencing of the murine *GPR105* cDNA demonstrated high cross-species homology, with a human:murine similarity of 90% and an identity of 81% at the amino acid level (Fig. 1C).

Northern blots of human tissues demonstrated abundant signal in the heart, placenta, and smooth muscle, with minimal detectable signal in spleen, lymph node, and thymus (Fig. 2A). Of note, two bands were seen in placenta, although the basis for this is unknown. Further characterization of expression within human hematopoietic cells used immunophenotypic populations sorted to high purity by FACS or, in the case of G0 cells, by the selected suicide strategy of $CD34^+$ cells noted above. Probing cDNA from subpopulations of bone-marrow-derived stem or progenitor populations or fully mature blood cells demonstrated that the expression of GPR105 is highly restricted to the suicide-selected G0 $CD34^+$ $CD38^-$ bone marrow cells (Fig. 2B).

To further assess subpopulations of cells expressing GPR105, we generated rabbit anti-peptide antiserum against the deduced sequence of the first extracellular domain of human and mouse GPR105. Reactivity of the antiserum to native protein was confirmed by immunoprecipitation of in vitro translated HA-tagged GPR105 protein using affinity-purified antiserum (Fig. 3A). The ability of the antiserum to selectively recognize GPR105 expressed on the cell surface was demonstrated by flow cytometric analysis of NIH3T3 cells transfected with a retroviral expression construct (MSCV-GFP) of GPR105 versus an empty vector control (Fig. 3B). The specificity of the antibody was further validated by selecting pri-

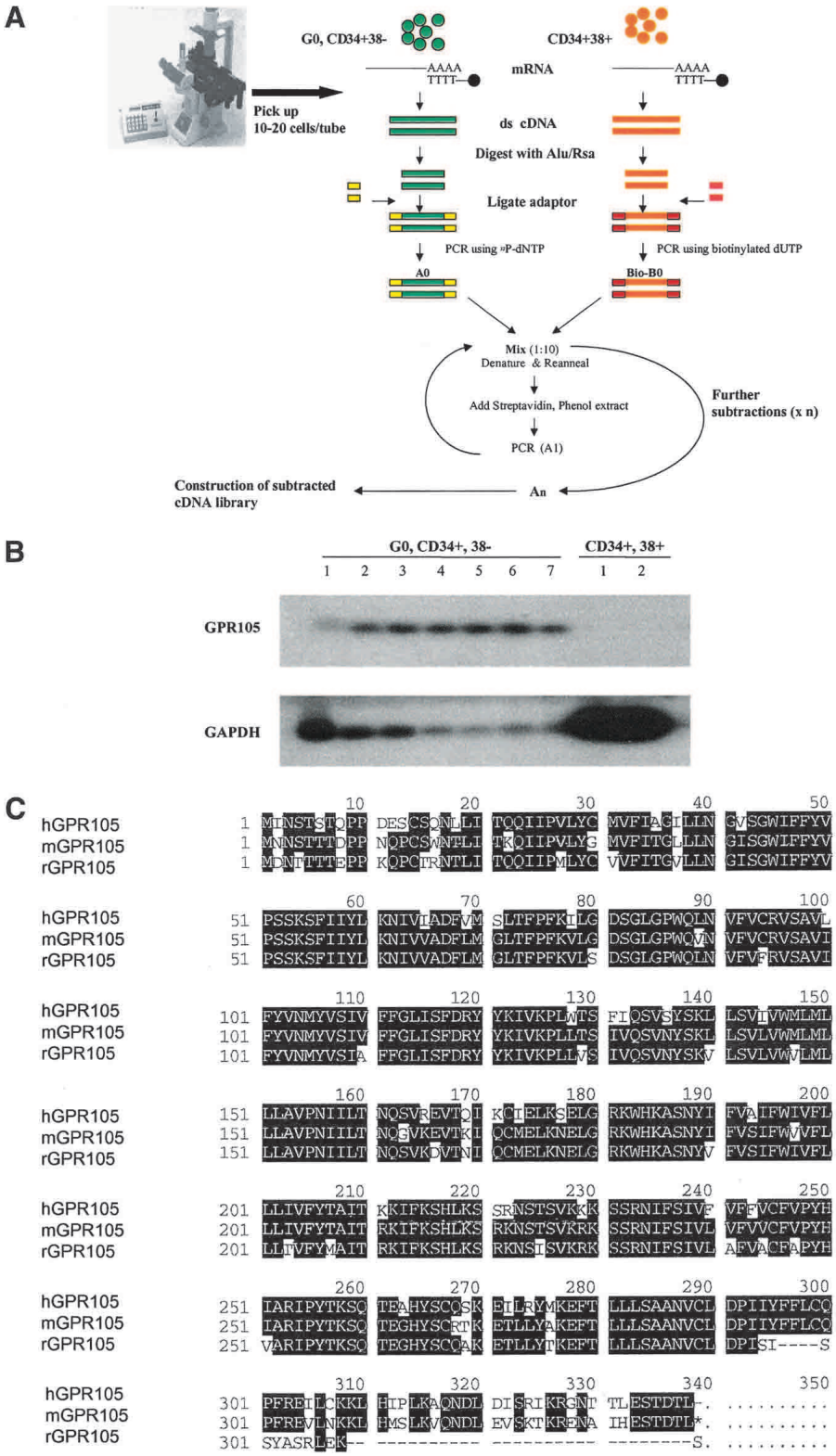


Figure 1. (*A*) Schema for subtractive cDNA cloning of G0 CD34$^+$ CD38$^-$ expressed sequences against CD34$^+$ CD38$^+$ cell sequences. (*B*) cDNA from each round of subtractive hybridization probed with GPR105 (*top* panel) or GAPDH (*bottom* panel) demonstrates progressive enrichment of GPR105 and depletion of GAPDH. The lane numbers in the G0 CD34$^+$ CD38$^-$ bracket represent the round of subtraction; the lanes in the CD34$^+$ CD38$^+$ bracket represent independent samples. (*C*) Sequence alignment of human, mouse, and rat GPR105; the amino acid number is indicated. The mouse GPR105 sequence was submitted to the NCBI GenBank (accession no. AF177211).

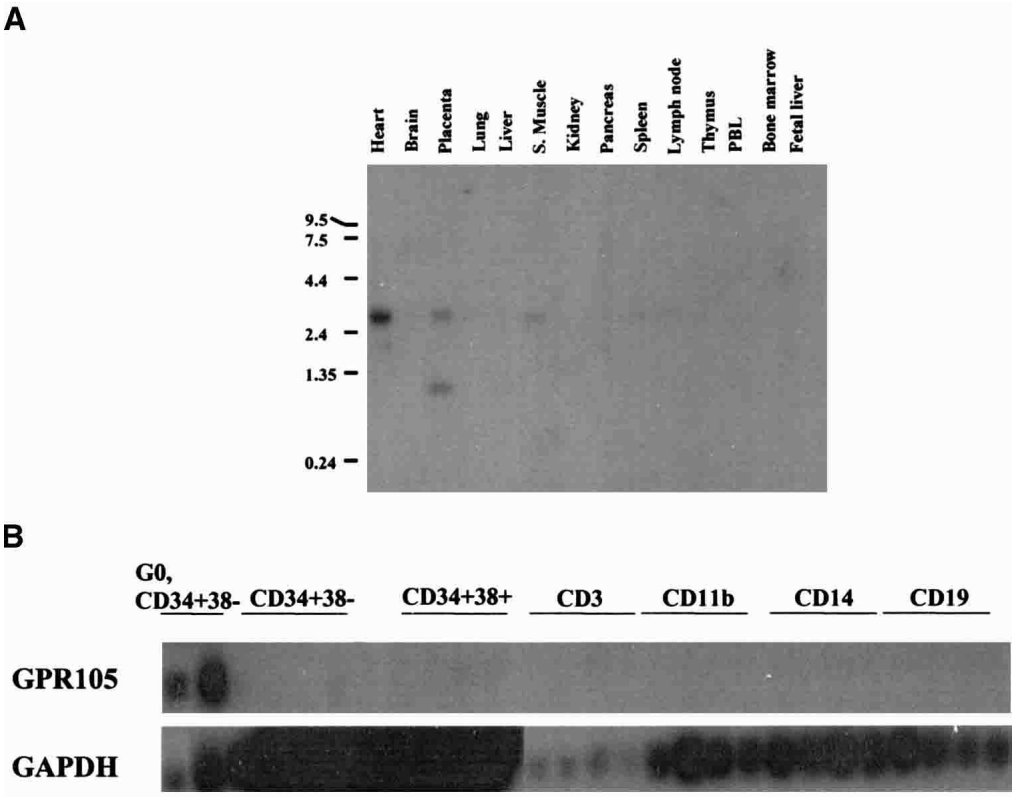


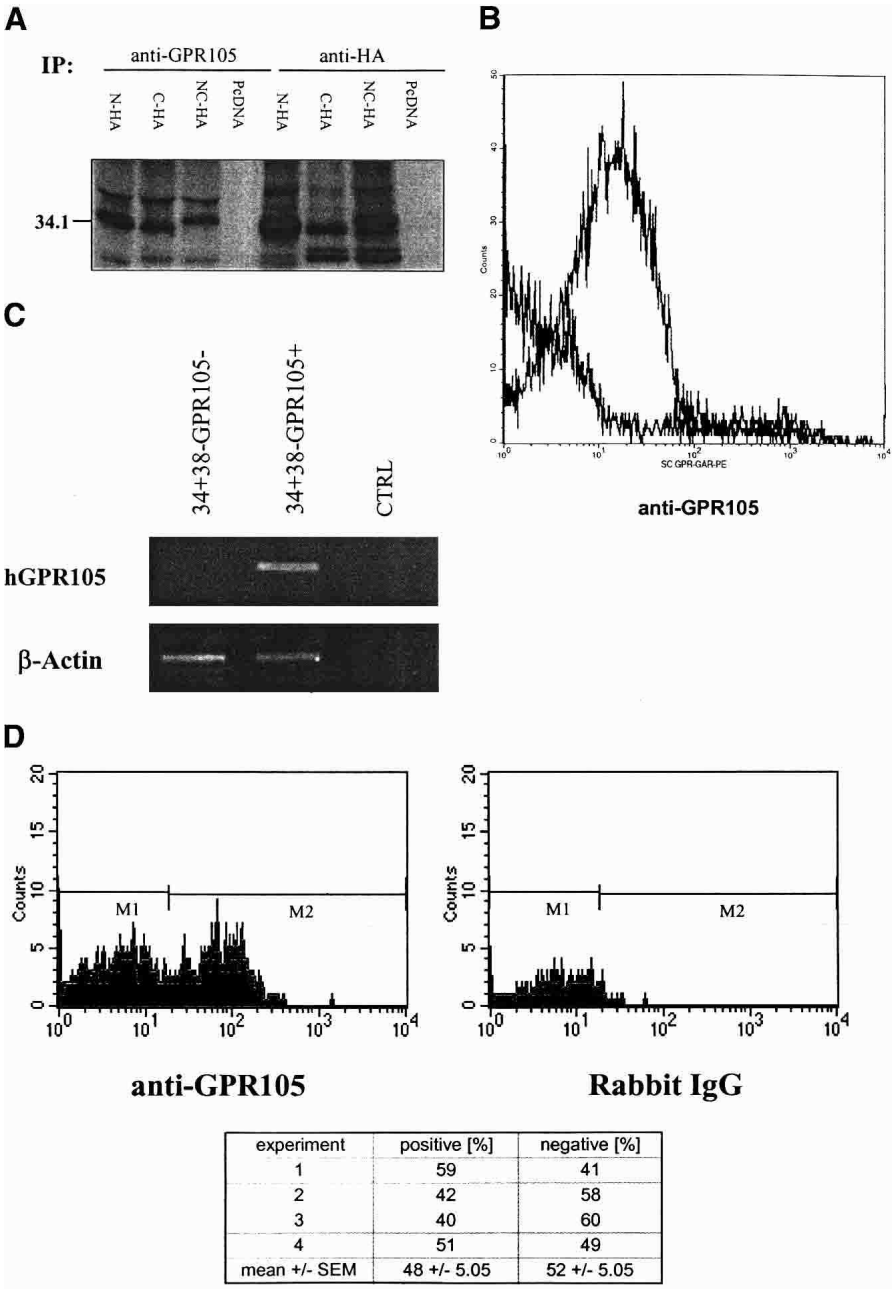
Figure 2. GPR105 is restricted in tissue expression and within hematopoietic cells is limited to primitive cells. (*A*) A human tissue blot with indicated mRNAs was probed with radiolabeled *GPR105* cDNA and hybridization determined by autoradiography. Size markers are indicated in kilobases. (*B*) Adult human cell mRNA from cells bearing the indicated phenotype was assessed by poly(A)-primed RT–PCR and the resulting cDNA was probed with either GPR105 or GAPDH sequences. Cells labeled G0 $CD34^+$ $CD38^-$ were derived using the directed metabolic suicide method previously described (Berardi et al. 1995) and represent <0.1% of $CD34^+$ $CD38^-$ cells isolated by cell sorting.

mary human fetal bone marrow cells based on antibody binding and evaluating them for expression of *GPR105* mRNA by semiquantitative RT–PCR. GPR105 message was detected in antibody staining *GPR105*$^+$ cells but not in equivalent numbers of *GPR105*$^-$ cells (Fig. 3C).

GPR105 expression in primary human adult hematopoietic populations was examined by flow cytometry; ~1% of CD34 dim cells from bone marrow were positive, whereas no CD34 bright cells stained positively. Reasoning that a very primitive population of cells may be in too low abundance in adult bone marrow to accurately detect by flow cytometry, we evaluated human fetal bone marrow cells and observed 48% ± 5% positivity in the $CD34^+$ $CD38^-$ cells in multiple independent samples (Fig. 3D). In contrast to bone marrow, no anti-GPR105 staining cells were identified in human fetal liver, although a faint signal could be detected by RT–PCR for GPR105. A similar restriction of staining fetal bone marrow, but not fetal liver cells, was noted in the mouse. Analyzing another source of human stem cells, neither did umbilical cord blood reveal anti-GPR105 staining cells. These data indicate a link of GPR105 expression with bone marrow localization, highly unusual among other stem-cell-associated markers.

Evaluation of the cell cycle within human fetal bone marrow $CD34^+$ $CD38^-$ cells by DNA and RNA analysis (Gothot et al. 1997) revealed that only 2%–3% were in the G2–M + S phase, with little difference noted between the $GPR105^+$ and $GPR105^-$ populations. Among those cells in G0/G1, however, cells not expressing GPR105 were predominantly in G1 (95%; Fig. 4A), contrasting markedly with $GPR105^+$ cells, in which 57% were in G1, with the remaining 43% in G0. Thus, quiescent cells are preferentially found within the GPR105-expressing population.

We next assessed the functional phenotype of $GPR105^+$ versus $GPR105^-$ $CD34^+$ $CD38^-$ subpopulations isolated by cell sorting from human fetal bone marrow and assayed for lineage-committed progenitor cell function by measuring colony-forming cells (CFCs) in methylcellulose. Stem cell function was measured in parallel by long-term culture cobblestone area formation (CAFC) or long-term culture-initiating cell (LTC-IC) assays on bone marrow stroma. Among $CD34^+$ $CD38^-$ cells, $GPR105^+$ cells performed poorly compared with $GPR105^-$ cells in the CFC assays (mean 4.2 vs. 42.0 in 6 independent experiments, $p = 0.00003$; Fig. 4B). Poor colony production could indicate either that $GPR105^+$ cells are simply unable to grow in methylcellulose, are postmitotic, terminally differentiated cells, or are a more primitive, rela-



experiment	positive [%]	negative [%]
1	59	41
2	42	58
3	40	60
4	51	49
mean +/- SEM	48 +/- 5.05	52 +/- 5.05

Figure 3. Anti-GPR105 recognizes GPR105 and identifies a subset of $CD34^+$ $CD38^-$ fetal bone marrow cells. (*A*) HA-epitope-tagged *GPR105* cDNA was in vitro transcribed and translated. (*Left*) In vitro translated protein was immunoprecipitated with anti-GPR105 antibody. Note that the increase of the size in NC-tagged HA is caused by an additional tagging. (*Right*) Similar results were obtained using anti-HA tag monoclonal antibody for immunoprecipitation. PcDNA without GPR105 was used as a negative control. N-HA, N terminus-tagged; C-HA, C terminus-tagged; NC-HA, N and C terminus-tagged. The position of a molecular weight marker (size in kilodaltons) is indicated to the *left*. (*B*) Flow cytometry of cells transduced with either MSCV control (blue line) or MSCV-GPR105 (red line) vectors and stained with affinity-purified anti-GPR105. (*C*) $CD34^+$ $CD38^-$ $GPR105^+$ human fetal bone marrow cells express *GPR105* mRNA as demonstrated by semiquantitative RT–PCR. Cells isolated by cell sorting were assessed using 3×10^3 cells of each type and analyzed for *GPR105* or *actin* mRNA; control refers to a no-cDNA template. (*D*) $CD34^+$ $CD38^-$ fetal bone marrow cells stained with anti-GPR105-FITC or control antiserum from a single experiment (*top* panels) or composite data from four independent experiments (table).

tively cytokine-unresponsive stem cell subset. Long-term assays performed using limit dilutions of cells cultured on primary human marrow stroma discriminated between the latter two. Three independent experiments scored weekly demonstrated low cobblestone area production with the human $GPR105^+$ cells (Fig. 4C). However, human $GPR105^+$ cells demonstrated a marked difference in capacity to provide a sustained output of

hematopoietic colonies ($p = 0.008$). In contrast to human GPR105$^-$ cells, with which CAFC production declined over time as would be expected from a predominantly progenitor population (91% decline by week 8), GPR105$^+$ cells consistently demonstrated sustained cobblestone formation over the same interval (47% decline; Fig. 4C). When the CAFC cultures were overlaid with cytokine-supplemented methylcellulose at week 5 (LTC-IC assay), colonies emerged, which were micropippetted and evaluated morphologically. Cells with myeloid cell histologic appearance were observed (Fig. 4D). Thus, low-level mature cell production was ongoing for prolonged intervals, a characteristic consistent with a stem cell phenotype. Although ectopic expression experiments need to be interpreted with caution, when we transduced primary human CD34$^+$ cells with either a GPR105-encoding or a control retroviral vector with a bicistronic GFP, we noted a shift in the functional phenotype. Progenitor function as measured by CFC declined ($p = 0.02$; Fig. 4E), whereas stem cell function as measured by LTC-IC or CAFC increased ($p = 0.03$; Fig. 4F). This was accompanied by a decrease in the fraction of cells actively cycling ($p = 0.04$; Fig. 4G). These data suggest that GPR105 may play a direct role in the stem cell phenotype, although the physiologic role of GPR105 cannot be definitively demonstrated by these studies.

Transplantation of human umbilical cord blood into immunodeficient mice provides an additional model for assessing stem cell function. However, this model is poorly efficient for engraftment by other stem cell sources presumably because of species-specific molecular interactions (Noort et al. 1998; Bonnet et al. 1999; Holyoake et al. 1999; van Hennik et al. 1999). Others have found that human fetal bone marrow engrafts multi-immunodeficient mice very poorly with efficiencies (1 in 8 to 1 in 12 animals) at even massive cell doses that preclude comparative analysis with rare populations (Turner et al. 1996, 1998). Similarly, in our work, fetal bone marrow (CD34$^+$ CD38$^-$, GPR105$^+$ or GPR105$^-$) did not engraft in NOD/SCID mice with a frequency permissive of comparison between groups despite high-level engraftment of cord blood controls. More broadly useful xenogeneic models are needed to assess stem cell function of GPR105$^+$ human cells in vivo. In assessing comparable populations in the mouse, the GPR105$^+$ cells were within the lineage marker negative, Sca-1$^+$, c-kit$^-$ populations (Fig. 4H). This population has been defined as a population of cells deeply quiescent and highly resistant to cytokine stimulation (Randall and Weissman 1998; Ortiz et al. 1999). The function of these cells is controversial. However, murine lin$^-$, Sca-1$^+$, c-kit$^-$ cells have been shown to provide mature, multilineage descendent cells posttransplantation (Ortiz et al. 1999) and a comparable c-kit$^-$ population of human cells has been defined as enriched for a stem cell phenotype (Laver et al. 1995; Sakabe et al. 1998). Of note, in the murine transplant studies, an interval of 10–12 mo posttransplant was required for mature cell emergence, perhaps reflecting the deep quiescence of the population.

To further assess whether the GPR105$^+$-expressing cells had multilineage potential as would be expected of a stem cell population, we cultured cells ex vivo under conditions permissive of lymphoid differentiation in addition to the myeloid differentiation shown above. Lin$^-$ GPR105$^+$ murine cells were cultured on the murine bone marrow stromal cell line 14F1.1 (a kind gift of Dov Zipori, Weizmann Institute, Rehovot, Israel), shown to permit B-cell differentiation by others (Zipori and Lee 1988). Methylcellulose containing cytokines permissive of either myeloid or lymphoid differentiation was added, and the cells were assessed by morphology and RT–PCR. Cells produced cobblestones consistent with a primitive phenotype (Fig. 4I), and colony formation was observed under both myeloid-specific and lymphoid-specific conditions. Cells from the myeloid culture were of characteristic myeloid morphology, whereas cells from the lymphoid culture were documented to express the lymphoid-specific genes *RAG1* and *RAG2* by RT–PCR (Fig. 4J). Therefore, cells expressing GPR105 are capable of undergoing multilineage differentiation consistent with a hematopoietic stem cell phenotype.

GPR activation by ligand binding may result in intracellular calcium flux. COS-7 cells were transiently transfected with GPR105 or control vector and surface GPR105 was confirmed by fluorescence microscopy (Fig. 5A). Cells expressing GPR105 were loaded with Fura-2 and monitored by fluorimetry following exposure to conditioned medium from primary human fetal bone marrow stroma compared with conditioned medium from other fetal hematopoietic organ stroma sources (Fig. 5B). Fetal bone marrow, but not spleen or thymic stroma-conditioned medium, induced a calcium flux. Thus, GPR105 and a ligand source colocalize to the bone marrow.

We next evaluated whether the receptor on transduced primary cells responded to the putative ligand, UDP-glucose. Using CD34$^+$ cord blood cells transduced with GPR105, intracellular calcium flux was observed following exposure to 10 mM UDP-glucose (Supplementary Fig. 1), similar to reports from others using GPR105-expressing cell lines (Chambers et al. 2000).

To test whether GPR105 participates in cell localization, primary human fetal bone marrow CD34$^+$ CD38$^-$ cells were assessed in trans-well assays, scoring chemotaxis toward fetal bone marrow stroma-conditioned medium. Chemotaxis was observed and was inhibited by anti-GPR105 or anti-CXCR4 antibodies or pertussis toxin, but only anti-GPR105 antibody was specific in its inhibition (Fig. 5C). Therefore, endogenously expressing human GPR105$^+$ cells are responsive to GPR105 agonist(s) derived from the bone marrow microenvironment, responding with physiologic events strongly associated with tissue localization. The extent of inhibition inducted by anti-GPR105 antibody was unanticipated, given that only ~50% of cells would be expected to express GPR105 on their cell surface. It is not clear why more than 50% of cells were inhibited from transmigration, but cooperative effects on cell migration among diverse populations of cells have been noted and may be an explanation (Adams et al. 2003).

To further assess whether chemotaxis was specific to activation of GPR105, primary human umbilical cord blood CD34$^+$ cells were transduced with GPR105 or empty vector and GFP$^+$ cells were evaluated in transwell and, independently, in Boyden chamber assays. Fetal bone marrow stroma-conditioned medium induced chemotaxis that was also specifically inhibited by anti-GPR105 antibody (Fig. 5D). Of note, no chemotaxis was seen in response to UDP-glucose (data not shown), possibly reflecting a distinct ligand (multiple ligands being common among some GPRs) or the inability to sufficiently maintain a gradient of a readily diffusible molecule like UDP-glucose.

Evaluation for secondary effects of GPR105 potentially affecting cell localization was performed using flow cytometry. GPR105-transduced human CD34$^+$ cells did not alter surface CXCR4 or L-selectin expression ($p = 0.91$ and $p = 0.65$, respectively; $n = 3$), but did have higher levels of VLA-4 ($p = 0.04$; $n = 4$), a recognized mediator of stem cell bone marrow localization. Therefore, GPR105 may affect stem cell localization by two mechanisms: directly through chemotaxis and indirectly through enhanced integrin expression.

Discussion

We conclude that GPR105 is a chemoattractant receptor highly restricted in expression within the hematopoietic cascade that colocalizes with its ligand to the bone marrow compartment. It may be a marker for hematopoietic stem cells, yet it was not observed in the gene array analyses of stem cells reported by others (Phillips et al. 2000; Terskikh et al. 2001; Ivanova et al. 2002; Ramalho-Santos et al. 2002). However, variable cell sources have been used, and shared sequences among these analyses are remarkably few. The sensitivity and specificity of these methods may be suboptimal for the GPR105 and potentially other comparably expressed transcripts. The absence of GPR105 in the fetal liver stem cell libraries, our inability to detect GPR105$^+$ cells in fetal liver or cord blood, and the responsiveness of GPR105 cells to bone marrow stromal products underscore the potential for this chemoattractant receptor to participate in bone marrow localization of stem cells. This may be particular to the developmental movement of hematopoiesis from fetal liver to bone marrow. Whether GPR105 is relevant for adult stem cell localization to bone marrow is less clear, because of the rare expression in the adult. Why the bone marrow should have preferential effects on UDP-sugar receptor (GPR105)-bearing cells is unknown.

Recent evidence that osteopontin, secreted at sites of bone remodeling and a known immune modulator, directly alters other P2Y-receptor-induced cell migration suggests a possible mechanism (Chaulet et al. 2001). It has also recently been reported that UDP-galactose:ceramide galactosyltransferase is expressed in hematopoietic cells, and inhibition of this enzyme in stromal cells specifically has been associated with marked alteration of hematopoietic cell differentiation (P. Frenette, pers. comm.). Release of intracellular nucleotide sugars may therefore occur through specific modulation of stromal elements or may be an indication of injury. The data we provide demonstrate a novel association of GPR105 to stem cells and a new function for this receptor in cell localization. To the extent that the nucleotide and nucleotide-sugar receptors may be grouped together, the evidence presented here suggest that P2Y and related receptors may influence host response to injury on multiple levels including the most basic element of repair, the stem cell.

Materials and methods

Construction and screening of a subtracted cDNA library

Human bone marrow cells were obtained from healthy volunteers who provided written informed consent to a protocol approved by the Massachusetts General Hospital Institutional Review Board (IRB). Cord blood was obtained from St. Louis Uni-

Figure 4. CD34$^+$ CD38$^-$ cells expressing GPR105 are disproportionately in G0 and are enriched for a stem cell functional phenotype. (*A*) Flow cytometric analysis of PY and Ho staining of human fetal bone marrow cells sorted for CD34$^+$ CD38$^-$ and GPR105; note that events are clustered at the upper limit of the PY axis with the gain set to enhance discrimination of ±PY. The percentage of G0 and G1 cells is indicated, respectively. These data are a representative of two independent experiments with comparable results. (*B*) CFC of GPR105$^+$ versus GPR105$^-$ CD34$^+$ CD38$^-$ cells ($n = 6$; $p = 0.00003$). (*C*) CAFC over time of GPR105$^+$ versus GPR105$^-$ CD34$^+$ CD38$^-$ cells calculated as the ratio relative to week 2 (*top* panel). The fractional proportion of week 2 colonies is provided in the accompanying table. Cells were plated at 3–6 twofold dilutions in replicate wells and scored each week. Data shown are one representative of three independent experiments with comparable results. (*D*) Morphology of cells derived from LTC-IC of GPR105$^+$ CD34$^+$ CD38$^-$ cells is consistent with mature myeloid lineage cells. Cells were isolated by micropippetting, stained with May-Grunwald-Giemsa, and assessed by photo-light microscopy. (*E*) Primary cord blood-derived CD34$^+$ cells generate reduced CFC with GPR105 expression. CD34$^+$ cells transduced with MSCV-GPF or MSCV-GFP-GPR105 vector were sorted for GFP expression and assessed in a standard CFC assay. (*F*) Limit dilution CAFC is increased with GPR105 expression in transduced primary CD34$^+$ cells. CD34$^+$ GFP-positive cells as above were evaluated in either CAFC (shown) or LTC-IC assays (data not shown). (*G*) Cell cycle analysis of transduced cord blood CD34$^+$ cells. GFP$^+$ cells transduced as above were assessed for cell cycle status by Hoechst 33342 staining. (*H*) Cells from murine fetal bone marrow and liver were stained for surface markers of lineage, Sca-1, c-Kit receptor, and GPR105. Lin$^-$ Sca-1$^+$ cells were then gated, and the expression of c-Kit and GPR105 was analyzed. The percentages in the *upper left* quadrant represent the proportion of the lin$^-$ Sca-1$^+$ cells that are GPR105$^+$. Data represent one of three experiments with comparable results. (*I*) Cobblestone area formation on 14F1.1 cells (*left*) and myeloid morphology of cells derived from culture of murine lin$^-$ GPR105$^+$ cells under myeloid growth conditions (*right*). (*J*) Rag1 and Rag2 expression of murine lin$^-$ GPR105$^+$ cells cultured on 14F1.1 cells and under lymphoid cytokine containing methylcellulose. (Lane *1*) Colonies with and without RT. (Lane *2*) 14F1.1 stroma cells with and without RT. (Lane *3*) No template control.

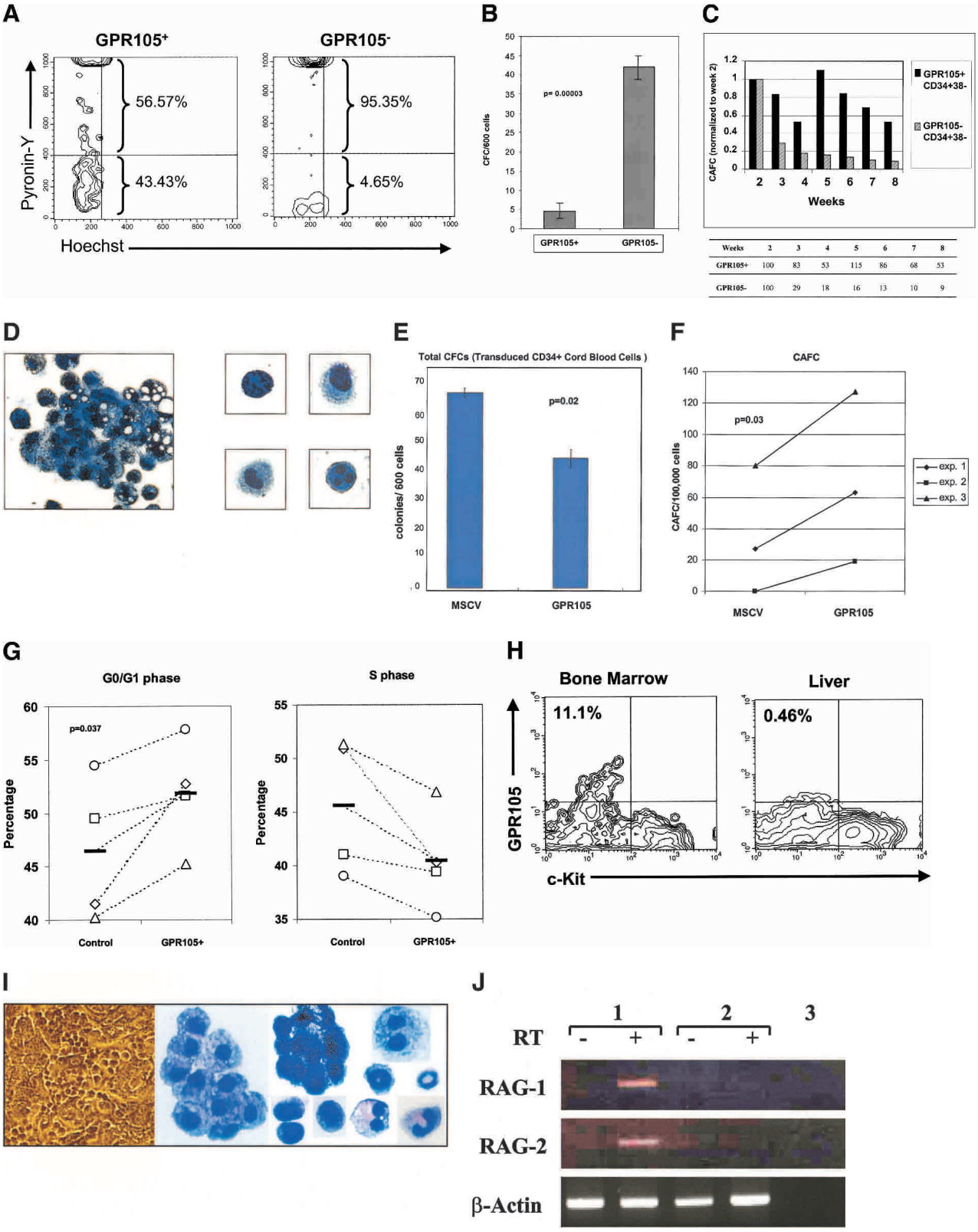


Figure 4. (*Figure 4 legend on facing page*)

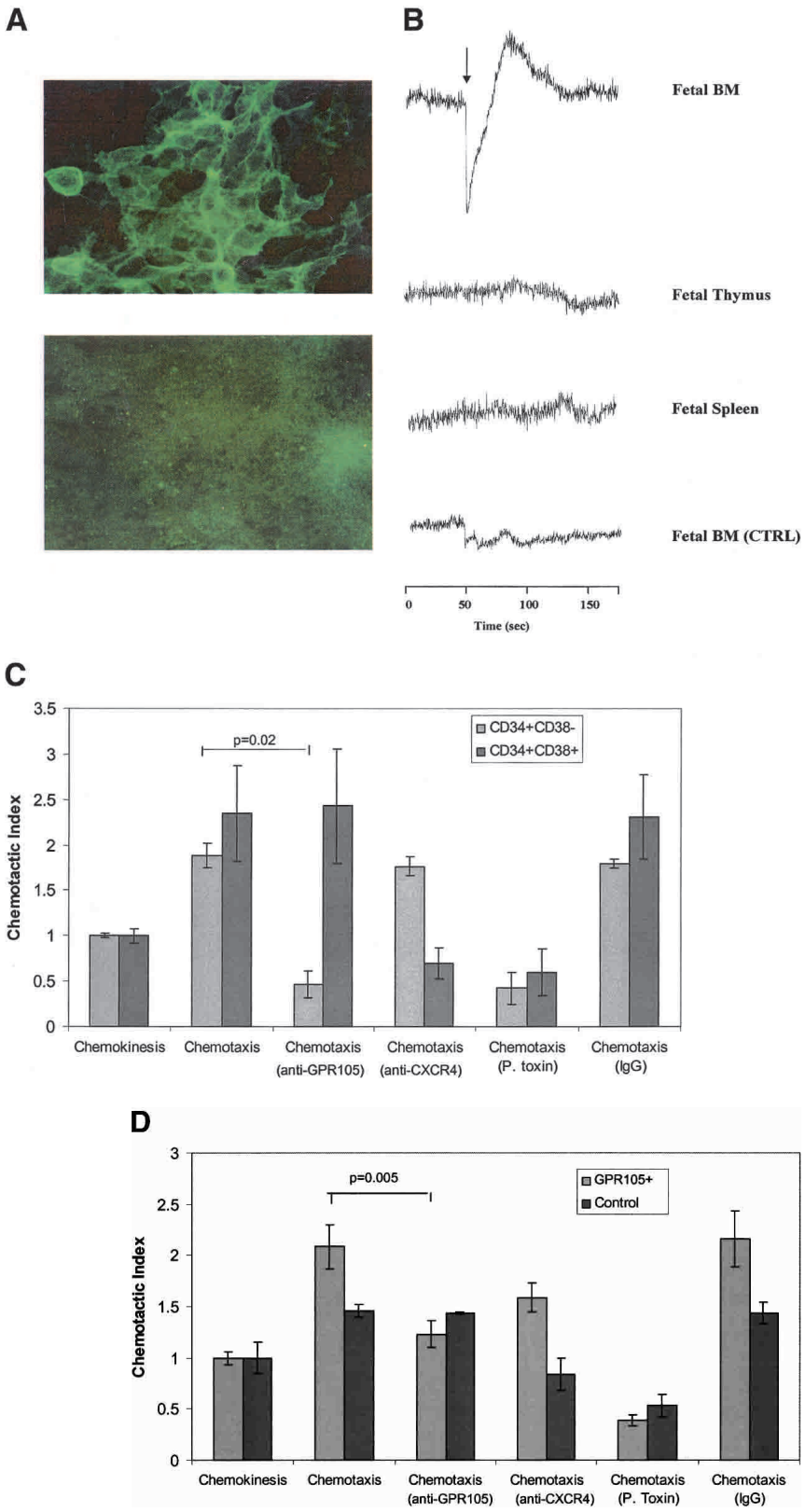


Figure 5. Bone-marrow-conditioned medium activates GPR105 altering GPR105$^+$ cell function. (*A*) GPR105 is expressed on the cell surface of transduced cells. COS-7 cells were transiently transfected with HA-epitope-tagged GPR105 (*top* panel) or vector alone (*bottom* panel) and stained with anti-HA monoclonal antibody without cell permeabilization. Panels are representative fluorophotomicrographs. (*B*) GPR105-transduced cells undergo calcium flux in response to selected conditioned media. GPR105-transfected COS-7 cells were loaded with Fura-2 and assayed using microscopic fluorimetry. Cells were stimulated with the indicated conditioned medium at points indicated by the arrow. Intracellular calcium concentration was monitored by the fluorescence ratio (F340/F380) plotted on the vertical axis. Similar results were obtained in three additional, independent experiments. (*C*) Primary human fetal bone marrow cells that express GPR105 transmigrate in response to bone-marrow-conditioned medium. Transmigration assays using GPR105-enriched CD34$^+$ CD38$^-$ cells or those cells that do not express GPR105 (CD34$^+$ CD38$^+$). Results represent the mean and S.E.M. of one of four independent experiments. Migration is expressed as a chemotactic index calculated from the percentage of cells in the test wells passing through a 5-μm filter over 3 h, divided by the percentage of migration in chemokinesis controls. To test for the specificity of transmigration, cells were pretreated with pertussis toxin (P. Toxin), anti-CXCR4 antibody (antiX4), anti-GPR105 antibody (antiGPR105), or control antibody (IgG). Comparison of chemotaxis with and without pretreatment with anti-GPR105 antibody is indicated with the *p* value following Student's *t*-test analysis. (*D*) GPR105-transduced primary cord blood CD34$^+$ cells transmigrate in response to conditioned medium from fetal bone marrow stroma. Data are represented as the mean chemotactic index and S.E.M. from six independent experiments with pretreatment conditions as indicated using GFP$^+$ cells in each group. Comparison of chemotaxis with and without pretreatment with anti-GPR105 antibody is indicated with the *p* value following Student's *t*-test analysis.

versity using IRB-approved protocols. Fetal cells were obtained from Advanced Bioscience Resources, Inc. Bone marrow cells from the long bones of 22-week-old fetuses were obtained by flushing with DMEM 10% FCS using a 20G needle attached to a 5-cc syringe. Donor cells of each type were derived from multiple independent donors and used to independently confirm

stated results. Within a single experiment, a single donor or pooled donors were generally used. $CD34^+$ cells were isolated with MACS (Miltenyi) according to the manufacture's instructions. Quiescent $CD34^+$ $CD38^-$ cells were prepared as described except using a higher concentration of 5-FU (Pharmacia Inc.; Berardi et al. 1995). Briefly, $CD34^+38^-$ cells were incubated at 37°C with 5% CO_2 in IMDM (GIBCO-BRL) containing 10% fetal calf serum (Sigma) supplemented with KL (100 ng/mL) and IL-3 (100 ng/mL) with 5-FU (2.5 mg/mL). Approximately 10–20 cells were picked by a Quixell micromanipulator (Stoelting Co.) and transferred to a PCR tube containing lysis/binding buffer (100 mM Tris-HCl at pH 8.0, 500 mM LiCL, 10 mM EDTA, 1% LiDS, 5 mM DTT). The mRNA was purified by adding 10 µL of oligo(dT)-linked magnetic beads (Dynal A.S.). After incubation at room temperature for 10 min, the magnetic beads were washed three times with 50 µL of RT buffer. RT–PCR was carried out in situ as described (Karrer et al. 1995). Further amplification of cDNA and subtractions were carried out as described (Sive and St John 1988). Briefly, cDNA was digested completely with *Alu*I and *Alu*I plus *Rsa*I separately to yield 200–600-bp cDNA fragments, which prevents disproportionate PCR amplification of smaller cDNAs. After seven rounds of subtractive hybridization against biotinylated cDNA generated from $CD34^+$ $CD38^+$ cells, subtracted cDNAs were cloned into the vector Topo2.1. DNA (Invitrogen). A mouse clone was identified from the NCBI dbest database. BLAST searches of the dbest database with a conserved region between GPR105 and VTR 15–20 retrieved an EST clone (GenBank accession no. AA139729). The clone generated by the IMAGE consortium was obtained from ATCC as an *Eco*RI–*Not*I insert in the pT7T3D-Pac vector (Pharmacia), and a full-length clone was identified and sequenced subsequently.

Antibody preparation

Anti-GPR105 antibody was generated using a peptide (named N-GPR105, MINSTSTQPPDESCSQN) that spans 17 amino acids of the first extracellular domain of the GPR105 protein. N-GPR105 was conjugated with a carrier protein, Keyhole Lymphet Hemocyanin (KLH), and injected into rabbits. After the second boost, the rabbits were bled, and the serum was isolated and used for affinity purification. GPR105 peptide was immobilized to SulfoLink Coupling Gel (Pierce). Antiserum was mixed with GPR105-peptide-conjugated affinity gels and gently rocked at room temperature for 1 h. After extensive washing with PBS, antibody was eluted with 3 M KSCN and used for FACS analysis and immunoprecipitation.

cDNA and transfection

The GPR105 coding region was generated by PCR using the human fetal thymus cDNA (Clontech) as the template and the primers GPR105-sBam and GPR105-aXho. The primer GPR105-sBam, 5′-CGGGATCCCGAAGTTACAAGATGATCAATTCA ACC-3′, and GPR105-aXho, 5′-CCGCTCGAGCGGAAGAGG GTAGGAACTCA-3′, correspond to positions 346 and 1444 in the published sequence and span the entire coding region for the GPR105 of 338 amino acids. Products of the expected size (in base pairs) were cloned into the *Bam*HI–*Xho*I polylinker sites of PcDNA3 (Invitrogen). To create an HA-tagged (YPYDVPDYA) GPR105, untagged vector was used as a template for PCR. The HA tag was inserted into either the N or the C terminus of a GPR105-coding sequence. Sequence analysis of the final expression plasmids confirmed that there were no PCR-generated mutations. COS-7 cells were transfected with an HA-tagged GPR105 expression plasmid using Geneporter (GTS) according to the manufacturer's instructions. Retroviral transduction was performed as previously described using an MSCV-GPR105 constructed by cloning the full-length GPR105 upstream of the IRES (Carlesso et al. 1999). The mean transduction efficiencies were GPR105, 28.4% (range 7.7%–53.8%); control, 28.8% (range 10.7%–63.5%). Within individual experiments, the transduction efficiencies were highly comparable with <10% difference between groups. GFP-positive cells were used for subsequent cell cycle or functional analyses.

Immunocytochemistry

Immunocytochemistry was performed using the avidin–biotin system and anti-HA mouse monoclonal antibody. All incubations were done at room temperature unless otherwise stated. Briefly, cells were fixed in 4% (v/v) paraformaldehyde for 20 min. Slides were incubated with anti-HA antibody (Babco) at 4°C overnight, followed by incubation with a biotinylated goat anti-mouse secondary antibody (Sigma). Slides were then incubated with the ExtrAvidin-FITC conjugate (Sigma). Slides were mounted in Fluoromount-G (Southern Biotechnology Associates, Inc.) and examined using fluorescence microscopy.

Intracellular Ca^{2+} measurements

The calcium efflux assay was performed essentially as described (Klein et al. 1999). COS-7 cells transfected with PcDNA3 containing GPR105 were cultured on glass slides and loaded with 5 µM fura-2/AM (Molecular Probes) at 37°C for 60 min in the dark. Cells were washed twice with PBS and once with DMEM. A slide was placed onto a microscope stage (Nikon TE200) connected to a spectrofluorometer. The cells on the slide were submerged with loading buffer. Stroma-conditioned media from different hematopoietic organs were loaded onto the slide, and fluorescence was measured. Data are presented as the relative ratio of fluorescence at an emission frequency of 510 nm and excitation frequencies of 340 nm and 380 nm.

Transduced cord blood cells were incubated with 3 µg/mL Indo-1 (Molecular Probes) at 37°C for 45 min and then resuspended in Ca^{2+}-containing PBS. Calcium flux was measured by a ratio of 400/40 (short) to 510/20 (long) wavelengths with UV light from an He-Cad laser (325 nm) on an LSR cytometer (Becton Dickinson) after the addition of UDP-glucose. Data were analyzed using FlowJo software (Tree Star Inc.).

Chemotaxis assay

Cell migration was assessed using 24-well plate chambers with 5-µm pores (Corning Inc.). Human bone marrow stoma cells were cultured at confluence in the wells of the lower compartment. The medium was changed 3–4 d before the assay. Alternatively, for the $CD34^+$-transduced cells, conditioned serum-free medium was collected from human fetal bone-marrow-conditioned medium. GPR105-MSCV- or MSCV-infected Jurkat cells (2×10^6 cells/mL) or $CD34^+$ umbilical cord blood cells (3×10^5 cells/mL) were placed in the upper wells of the chamber. In some experiments, primary $CD34^+$ cells were pretreated with 10 µg/mL of anti-CXCR4 antibody (Pharmingen) or anti-GPR105 antiserum or 100 ng/mL of pertussis toxin (Sigma). The chamber was incubated at 37°C in humidified air containing 5% CO_2, for 3 h. After incubation, the filter was removed, and two independent investigators counted the number of migrated cells. All assays were done in triplicate.

Immunoprecipitation

Coupled in vitro transcription/translation was carried out using the TNT-coupled reticulocyte lysate system (Promega) accord-

ing to the manufacturer's instructions. The lysate was mixed with affinity-purified anti-GPR105 antibody and incubated at 4°C overnight. The immunoprecipitates were washed three times with RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris at pH 8.0) and boiled for 5 min in 30 µL of sample buffer. Then 20 µL was loaded into a Laemmli 10% SDS–polyacrylamide gel. To confirm the results, immunoprecipitation was repeated using anti-HA antibody. Both methods detected proteins of similar molecular weight.

CAFC/LTC-IC and CFC assays

GPR105-positive and -negative cells were plated in triplicate in 1 mL of methylcellulose media containing the following recombinant human cytokines: SCF (50 ng/mL), GM-CSF (20 ng/mL), IL-3 (20 ng/mL), IL-6 (20 ng/mL), G-CSF (20 ng/mL), and EPO (3 U/mL; Methocult GF+ H4435, Stem Cell Technologies). Colonies were scored under an inverted microscope at 10 d after inoculation.

CAFC cultures were established according to described methods (Sutherland et al. 1990). Sorted cells were plated at twofold dilutions (3–6 dilutions/sample) on irradiated (15 Gy) primary human bone marrow stromal layers established at 33°C and cultured in Human Long-term Bone Marrow Culture Media (Stem Cell Technology) at 37°C. Cultures were very gently refed with 50 µL of medium after semidepletion weekly, and the CAFCs and/or blast colonies were scored up to the eighth week. In some experiments, methycellulose was added to the well at 5 wk and cultured for an additional 10 d prior to scoring LTC-IC by phase contrast microscopy. The absolute number of CAFCs was calculated using Poisson statistics.

To evaluate myeloid and lymphoid potential, murine lin^- $GPR105^+$ and lin^- $GPR105^-$ cells were plated onto confluent 14F1.1 bone marrow stromal cells. Myelopoiesis and pre-B lymphopoiesis were induced as described by Zipori and coworkers (Zipori and Lee 1988). After 48 h of incubation with 14F1.1, methylcellulose containing cytokines (Methocult M3434 for myelopoiesis, Methocult M3630 for pre-B lymphopoiesis; Stem Cell Technologies) was overlaid onto cocultured cells. After 7–10 d, colonies were collected and stained by Giemsa (for myeloid colonies) or subjected to RT–PCR analysis using Rag1 and Rag2 primers (for lymphoid colonies; Hikida et al. 1996).

RT–PCR analysis

Messenger RNA was isolated using oligo(dT) magnetic beads according to the manufacturer's protocol (Dynabeads mRNA DIRECT Micro kit; Dynal). Reverse transcription was performed using the magnetic bead oligo(dT) for priming and Superscript II (Invitrogen) according to the manufacturer's protocol.

PCR was performed using 1 µM primers, 1.25 U of Taq Gold (Perkin Elmer), and 2.5 mM $MgCl_2$. For PCR, the magnetic beads were subjected to 94°C for 5 min followed by two rounds of amplification at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Subsequently, the reactions were incubated at 72°C for 5 min and 94°C for 2 min. The magnetic beads were isolated, and the supernatant was subjected to 30 rounds of PCR at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min. Then 10 µL was removed and added to a total of 50 µL of PCR reaction with fresh primers and Taq enzyme. Twenty additional rounds of PCR were carried out, with 10 µL removed every 5 rounds and resolved on a 2% agarose gel. The bands presented were achieved after the first five rounds of amplification, or a total of 35 cycles. Additional controls were included, and rounds of amplification were performed to confirm that the products observed were in the exponential phase of PCR (data not shown). Magnetic beads were washed using Tris-HCl according to the manufacturer's directions and used as template for additional PCR reactions.

For Rag-1, Rag-2, and β-actin amplification, 35 cycles of PCR were performed, and 10 µL of product was resolved on a 2% agarose gel. The PCR primers used were Rag-1S-5′-ATGGCT GCCTCCTTGCCGTCT-3′, Rag-1A, 5′-GTATCTCCGGCTGT GCCCGTC-3′; Rag-2S, 5′-ATGTCCCTGCAGATGGTAACA 3′; Rag-2A, 5′-TAAATCTTATCGGAAAGCTCA-3′; hGPR105-1744S, 5′-AGGAAAAGCTGACACCCAGA-3′; hGPR105-2100A, 5′-CTTTCCCATTCGCCAGTAGA-3′; β-actin-S, 5′-CT GAACCCCAAGGCCAACCGCGAGAAGATG-3′; β-actin-A, 5′-GGTAGTTTCGTGGATGCCACAGGACTCCAT-3′.

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