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In Vitro Generation of Long-Term Repopulating Hematopoietic Stem Cells by Fibroblast Growth Factor-1

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Summary

The role of fibroblast growth factors and their receptors (FGFRs) in the regulation of normal hematopoietic stem cells is unknown. Here we show that, in mouse bone marrow, long-term repopulating stem cells are found exclusively in the FGFR$^+$ cell fraction. During differentiation toward committed progenitors, stem cells show loss of FGFR expression. Prolonged culture of bone marrow cells in serum-free medium supplemented with only FGF-1 resulted in robust expansion of multilineage, serially transplantable, long-term repopulating hematopoietic stem cells. Thus, we have identified a simple method of generating large numbers of rapidly engrafting stem cells that have not been genetically manipulated. Our results show that the multipotential properties of stem cells are dependent on signaling through FGF receptors and that FGF-1 plays an important role in hematopoietic stem cell homeostasis.

Introduction

Fibroblast growth factors (FGFs) belong to a large family of proteins encoded by more than 20 genes that share substantial sequence identity (Ornitz and Itoh, 2001). To date only four distinct fibroblast growth factor receptor (FGFR) tyrosine kinase genes have been identified, but alternative splicing results in the presence of multiple isoforms of each receptor. Some of these isoforms show tissue-restricted expression patterns, indicating distinct physiological roles (Beer et al., 2000; De Moerlooze et al., 2000). Most FGFs are capable of binding and signaling through multiple receptors, but some FGFs may actually never leave the cell and instead act as nuclear transcription-like factors (Goldfarb, 2001). The FGF-FGFR system is a highly complex and partially redundant signaling network that has been shown to be involved in the development and maintenance of a wide variety of tissues. Particularly, there exists a wealth of studies linking the temporal and spatial expression of specific FGF and FGFR genes to the emergence of certain cell types during embryogenesis, indicating that FGFs and FGFRs are associated with fate decisions of primitive cell subsets (Vasiliauskas and Stern, 2001). Mice deficient for FGFR show highly variable phenotypes. FGFR-1- and FGFR-2-deficient embryos die during embryogenesis (Arman et al., 1998; Deng et al., 1994). FGFR-3$^{-/-}$ mice demonstrate aberrant skeletal growth and deafness (Colvin et al., 1996; Wang et al., 1999), whereas FGFR-4-deficient mice fail to display any overt phenotype (Weinstein et al., 1998). Similarly, the phenotypes of FGF-deficient mice vary widely (see http://tbase.jax.org/ for a full description of phenotypes).

Whether FGFs play a role in the development and maintenance of the normal adult hematopoietic stem cell compartment is unknown. In vitro studies have produced ambiguous results on the effects of FGF-1 and FGF-2 on the growth of clonogenic progenitors (Gabbianelli et al., 1990; Ratajczak et al., 1996). Human CD34$^+$ cells have been shown to be unresponsive to FGF-2, and FGF receptors were not detected on these cells, as evaluated by single-cell reverse transcription PCR (Berardi et al., 1995). However, a recent study has documented the presence of a small population of CD34$^+$ FGFR-1$^+$ cells in human marrow, showing early endothelial progenitor cell characteristics (Burger et al., 2002). In addition, several studies have recently shown that FGF-2 may promote primitive erythropoiesis from embryonic cells (Bartunek et al., 2002; Yuen et al., 1998). Also, it has been shown that FGFR-1$^{-/-}$ embryonic stem cells are hampered in their differentiation into hematopoietic cells in vitro (Faloon et al., 2000). FGFs have been shown to have a stimulatory effect on bone marrow stromal cells in vitro (Quito et al., 1996; Wilson et al., 1991), which may also indirectly affect hematopoietic cell growth. Interestingly, several myeloproliferative disorders have recently been described in leukemic patients in which the FGFR-1 gene on chromosome 8 is translocated to various fusion partners on chromosome 6, 13, or 19, resulting in the constitutive (i.e., ligand-independent) activation of this receptor (Guasch et al., 2001; Mugneret et al., 2000; Ollendorff et al., 1999; Popovici et al., 1999; Xiao et al., 2000). These clinical data suggest that FGFR-1 plays an important role in homeostasis of primitive cell subsets.

In the present study we have evaluated the hematopoietic progenitor and stem cell potential of normal mouse bone marrow cells that express FGFRs. We show that the expression of FGFRs critically distinguishes long-term repopulating stem cells from short-term repopulating progenitors. Furthermore, we were able to generate large numbers of transplantable, long-term repopulating hematopoietic stem cells in vitro, using FGF-1 as a single stimulatory growth factor in serum-free medium.

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Results

FGFR Expression Separates Long-Term Repopulating Stem Cells from Committed Progenitors

We used a fusion protein, containing a portion of the Fc domain of human IgG linked to human FGF-1, to identify, isolate, and functionally characterize cells in the bone marrow that lack expression of hematopoietic lineage-specific markers and that bind FGF-1. Previous studies have shown that this fusion protein is a physiological ligand for FGFR-1 and retains FGF-1 mitogenic properties (Dikov et al., 1998). Limited background staining was observed when Lin^- bone marrow cells were stained with the secondary PE-labeled anti-human-IgG alone (Figure 1A). However, a large percentage of Lin^- bone marrow cells were able to specifically bind the Fc-FGF fusion protein, although there was considerable variation in the fluorescence intensity of stained cells (Figure 1B). We confirmed specificity of the binding of the fusion protein by isolating Lin^- $FGFR^-$ cells and Lin^- $FGFR^{++/+++}$ cells and staining cytospin preparations of both cell populations using a fluorescently labeled anti-FGFR-1 antibody that recognizes an intracellular epitope of the FGFR-1 protein (Figure 1C). To further verify the specificity of Fc-FGF to detect FGFR-expressing cells, we sorted Lin^- $FGFR^-$ cells and Lin^- $FGFR^{++/+++}$ cells and assessed expression levels of FGFR-1 by RT-PCR. Lin^- $FGFR^{++/+++}$ cells indeed showed strong FGFR-1 expression, whereas the Lin^- $FGFR^-$ population showed only weak expression levels (Figure 1D).

To functionally characterize the hematopoietic potential of cells detected by the fusion protein, we fractionated Lin^- cells in four subpopulations, on the basis of relative FGFR expression levels (Figure 1B). The four cell fractions were deposited in a limiting dilution setup in 96-well plates containing a preestablished confluent stromal cell layer, as described previously (de Haan et al., 2000). It has repeatedly been shown that the time point of appearance of cobblestone area-like colonies in such long-term bone marrow cultures strongly correlates with primitiveness of the hematopoietic cells (Ploemacher et al., 1991). In the mouse system, committed progenitors form colonies after 7–14 days (hence, they are referred to as cobblestone area-forming cells [CAFCs] day 7 and day 14), whereas more primitive stem cells start proliferating only after 4–5 weeks (CAFCs day 28 and day 35). We observed a striking correlation between the extent of FGFR expression by the Lin^- cells and the time-dependent clonogenic activity: whereas Lin^- $FGFR^-$ cells were highly enriched for CAFC day 7 activity ($\sim$17,000 CAFCs day 7/10^5 sorted cells), this population of cells was completely devoid of CAFCs day 35 (Figure 1E). Conversely, the small Lin^- $FGFR^{+++}$ cell fraction showed barely detectable CAFC day 7 activity but, instead, contained most primitive CAFCs day 35 ($\sim$550 CAFCs day 35/10^5 sorted cells). Although virtually all CAFCs day 35 are present in the Lin^- $FGFR^{++/+++}$ cell fraction, the majority of the Lin^- $FGFR^{++/+++}$ bone marrow cells did not read out in our assay and, thus, may not be committed to hematopoiesis.

To confirm the in vitro CAFC data, we performed in vivo transplantation assays in which 500, 1000, or 5000 Lin^- $FGFR^{++/+++}$ cells or equal numbers of Lin^- $FGFR^-$ cells were cotransplanted with 2×10^5 unfractionated congenic competitor bone marrow cells and injected in lethally irradiated recipient mice. Whereas Lin^- $FGFR^-$ cells provided some short-term engraftment 3 weeks after transplant, these cells failed to reconstitute the recipients at later time points. In contrast, Lin^- $FGFR^{++/+++}$ cells, at all doses tested, did provide long-term engraftment (Figure 1F). Although average engraftment levels remained modest, as mentioned above, most Lin^- $FGFR^{++/+++}$ cells appear to be nonhematopoietic, and we estimate that transplants of 500 Lin^- $FGFR^{++/+++}$ cells contain only $\sim$2.5 CAFCs day 35 (compare Figure 1E). In addition, engraftment levels varied and, in some animals, reached values of 40%. Engraftment levels in all recipients transplanted with Lin^- $FGFR^{++/+++}$ cells were equal in lymphoid and myeloid compartments (data not shown).

Most, if not all, hematopoietic stem cells have been shown to express both c-kit and Sca-1 (Okada et al., 1992). All CAFCs day 35 have a Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ phenotype, although a sizeable fraction of CAFCs day 7 are contained in this population, as well (de Haan et al., 2000). Using four-color FACS analysis, we assessed the extent of FGFR expression by Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ stem cells, which constitute $\sim$0.1% of total bone marrow cells (Figures 2A and 2B). Clearly, virtually all Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ cells bound the Fc-FGF fusion protein efficiently, indicating extensive expression levels of FGFRs on stem cells (Figure 2D). However, even in this relatively homogenous population, FGFR expression varied significantly, with a fraction of Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ cells showing very high levels of FGFRs. In the mouse, and possibly in human, during normal steady-state hematopoiesis, the most primitive hematopoietic stem cell subsets are $CD34^-$ (Ogawa, 2002; Osawa et al., 1996; Sato et al., 1999). We compared FGFR expression in Lin^- $CD34^-$ and Lin^- $CD34^+$ cell compartments. Virtually all $FGFR^+$ cells were contained in the Lin^- $CD34^-$ population, whereas Lin^- $CD34^+$ cells failed to bind the Fc-FGF fusion protein (Figure 2C).

As the fusion protein contains the FGF-1 protein and FGF-1 is a potential ligand for FGFR-1, -2, -3, and -4, we next determined which of these four receptors were expressed by Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ cells. To this end cDNA was prepared from purified Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ cells, and the presence of FGFR-1, -2, -3, and -4 transcripts was determined by RT-PCR. We were consistently able to detect transcripts of FGFR-1, -3, and -4 in Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ cells, whereas FGFR-2 transcripts were below detection levels (Figure 2E). FGFR-1 was the most prominently expressed.

Primitive Hematopoietic Subsets Are Expanded by FGF-1 In Vitro

To further document the physiological relevance for stem cells of signaling through FGFRs, we cultured unfractionated bone marrow cells in heparin-supplemented serum-free medium containing FGF-1 as a single stimulating growth factor and quantified the number of hematopoietic progenitors that were produced by stem cells present in the cell inoculum. Initially, the number of nonadherent cells in these cultures dropped strongly (data not shown), and very few progenitors

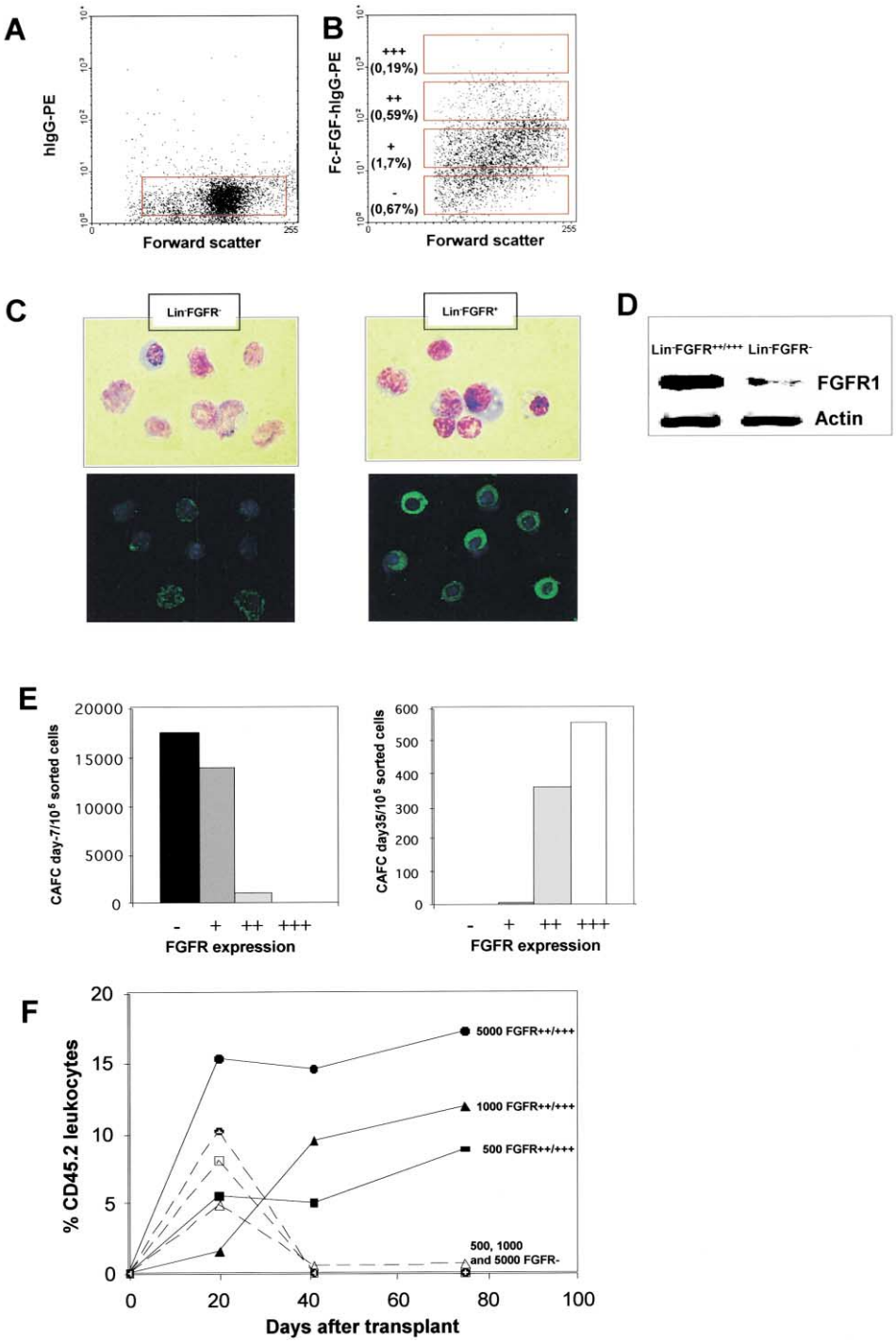

Figure 1. FGFR Expression Levels in Lin$^-$ Bone Marrow Cells Correlate with In Vitro and In Vivo Stem Cell Activity

Lineage-depleted bone marrow cells were incubated with PE-labeled goat F(ab')$_2$ anti-human-IgG alone to detect background staining (A). A large fraction of Lin$^-$ bone marrow cells specifically binds the Fc-FGF fusion protein (B). Cells were fractionated in four subpopulations, on the basis of relative FGFR expression levels. The percentage of total bone marrow cells showing a Lin$^-$ FGFR$^-$, Lin$^-$ FGFR$^+$, Lin$^-$ FGFR^{++}, or Lin$^-$ FGFR^{+++} phenotype is indicated. To confirm specificity of Fc-FGF cell staining, we isolated, cytospinned, and stained Lin$^-$ FGFR$^-$ and Lin$^-$ FGFR$^{++/+++}$ cell fractions with May-Gruenwald-Giemsa or an anti-FGFR-1 antibody (C). In addition, RT-PCR analysis with cDNA isolated from purified Lin$^-$ FGFR$^{++/+++}$ and Lin$^-$ FGFR$^-$ cells confirmed specificity of the Fc-FGF fusion protein to detect cells expressing FGFR-1 transcripts (D). Lin$^-$ bone marrow cells were fractionated on the basis of the levels of FGFR expression (see sorting gates in [B]), and their in vitro cobblestone area-forming potential was determined (E). CAFCs day 7 were exclusively present in the Lin$^-$ FGFR$^-$ and Lin$^-$ FGFR$^+$ cells (left panel). In contrast, primitive CAFCs day 35 were highly enriched in the Lin$^-$ FGFR^{++} and Lin$^-$ FGFR^{+++} population (right panel). Seven independent experiments were performed with very similar results. Lin$^-$ FGFR$^-$ and Lin$^-$ FGFR$^{++/+++}$ CD45.2 bone marrow cells were purified, and 500, 1000, or 5000 cells were transplanted in lethally irradiated recipients together with 2×10^5 unfractionated, genetically distinct (CD45.1) competitor cells (F). The percentage of total leukocytes derived from the sorted Lin$^-$ FGFR$^-$ or Lin$^-$ FGFR$^{++/+++}$ CD45.2 cell fractions is shown. Data represent mean values from five recipient mice per group. Levels of chimerism did not change significantly after day 80.

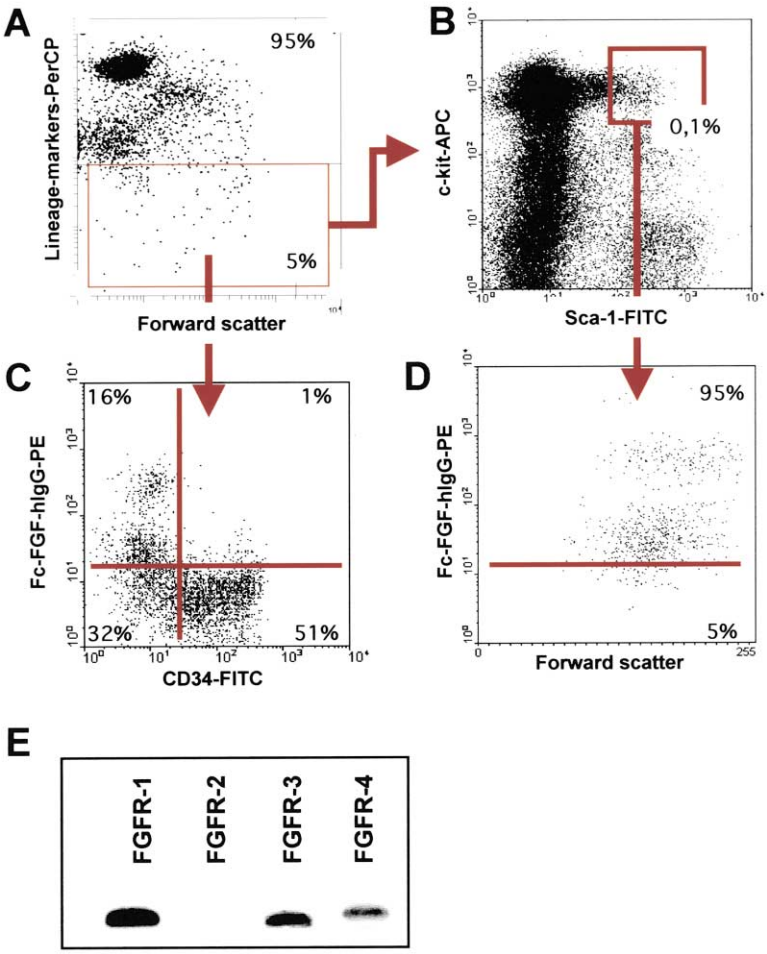

Figure 2. FGFR Expression by Lin$^-$ Sca-1$^+$ c-kit$^+$ and Lin$^-$ CD34$^-$ Stem Cells

Total bone marrow cells were stained with a cocktail of lineage-specific antibodies (A), and the 5% most negative cells were gated to identify the Lin$^-$ Sca-1$^+$ c-kit$^+$ population (B) and the CD34$^+$ population (C). FGF receptors were virtually exclusively expressed by Lin$^-$ CD34$^-$ bone cells, whereas Lin$^-$ CD34$^+$ cells were FGFR$^-$. More than 95% of all Lin$^-$ Sca-1$^+$ c-kit$^+$ cells expressed FGFRs (D). Lin$^-$ Sca-1$^+$ c-kit$^+$ cells were purified, and the expression of FGFR-1, -2, -3, and -4 transcripts was determined by RT-PCR (E). FGFR-1 transcripts were most abundant, but FGFR-3 and FGFR-4 transcripts were also easily detectable. FGFR-2 expression was below detection level.

could be harvested from the culture during the first 2 weeks. However, after more than 3 weeks of culture, the number of progenitors increased dramatically (Figure 3A). Cell cultures contained some adherent stromal cells, but most cells were nonadherent (Figure 3B) and had a blast-like morphology (Figure 3C). This delayed growth pattern is highly divergent from similar in vitro studies with hematopoietic cytokines, which invariably have shown strong progenitor expansion early after culture initiation, but rapid exhaustion after 2–3 weeks (McNiece and Briddell, 2001; Srour et al., 1999). In parallel cultures with SCF, instead of FGF-1, we also found the latter growth kinetics. Maximal SCF-induced progenitor cell expansion was observed at 2 weeks, and numbers declined rapidly thereafter (Figure 3A).

We wished to assess whether in vitro culture of bone

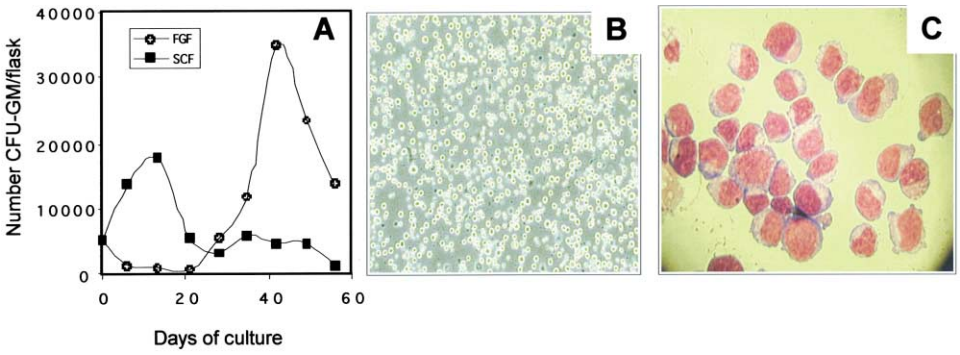

Figure 3. FGF-1 Stimulates Production of Large Numbers of Progenitors Ex Vivo

Serum-free cultures were initiated by incubating 5 $\times$ 10^6 unfractionated bone marrow cells with FGF-1 or SCF in the presence of heparin, and progenitor cell numbers were evaluated for 60 days (A). The absolute number of CFU-GM colonies per flask at each time point is indicated. Most of the FGF-1-stimulated bone marrow cells were nonadherent 4 weeks after initiating the culture (B) and had a blast-like morphology (C).

marrow cells in the presence of FGF-1 resulted in expansion not only of progenitors, but also of long-term repopulating multilineage stem cells. To this end unfractionated bone marrow cells harvested from C57BL/6.SJL-CD45.1 congenic mice were cultured for 4 weeks in the presence of FGF-1, without the addition of other growth factors or serum. Weekly cell counts were performed, and the number of CFU-GM colonies was determined. After 4 weeks the cultures were harvested, and the cells were used in various stem cell assays. First, the fraction of Sca-1$^+$ c-kit$^+$ cells was quantified by FACS analysis. Whereas the percentage of putative stem cells expressing the Sca-1$^+$ c-kit$^+$ markers in unfractionated bone marrow is ~0.3%, this fraction had increased substantially, to ~20%, after FGF-1 culture (Figure 4A). Cobblestone area-forming cell assays were performed to quantify stem cell activity in the starting cell population (i.e., normal unfractionated bone marrow cells) and in cells recovered from the expansion culture after 4 weeks (Figure 4B). The absolute number of early appearing CAFCs day 7 present in the FGF-1-stimulated culture was increased ~2-fold. However, a dramatic expansion of more-primitive subsets was observed, as CAFC day 28 and day 35 numbers were increased more than 100-fold (Figure 4C). This selective expansion of late-appearing CAFC subsets was in full agreement with the expansion of Sca-1$^+$ c-kit$^+$ cells and strongly suggested the in vitro amplification of long-term repopulating stem cells.

In Vitro FGF-1-Expanded Bone Marrow Cells Generate Multilineage Hematopoiesis In Vivo

To demonstrate that the in vitro-generated cells indeed contained in vivo repopulating ability, we performed several additional experiments. First, CD45.1 congenic bone marrow cells were expanded for 4 weeks in FGF-1, mixed in various ratios with 2 × 10^5 wild-type C57BL/6-CD45.2 competitor cells, and transplanted into lethally irradiated recipients. The fraction of cells derived from the expansion culture was quantified at various time points after transplant, on the basis of the expression of CD45.1 or CD45.2 markers and characteristic forward/sideward scatter profiles to distinguish between granulocytes and lymphocytes (Figure 5A, left panels).

All animals transplanted with FGF-1-expanded marrow cells showed robust levels of expansion culture-derived myeloid cells early after engraftment. In animals transplanted with only 1000 expanded cells, in conjunction with a 200-fold excess of normal, freshly harvested bone marrow cells, more than 50% of the granulocytes in the peripheral blood were derived from the expanded cells 1 month after transplant. Lymphoid reconstitution in all these animals was also present, but, at early time points, engraftment levels were significantly lower. Animals transplanted with 5000 expanded cells showed early myeloid engraftment levels higher than 80% and lymphoid engraftment of ~30%. Grafts containing 2 × 10^4 expanded cells initially fully reconstituted the myeloid compartment and ~50% of the lymphoid compartment. At later time points average myeloid engraftment levels in all recipients decreased, but lymphoid reconstitution in the same animals increased, such that mean myeloid and lymphoid engraftment became equal and stable. Robust and stable engraftment was observed in recipients receiving 2 × 10^5 expanded cells, and little contribution from the cotransplanted competitor cells could be detected. To verify multipotential properties of

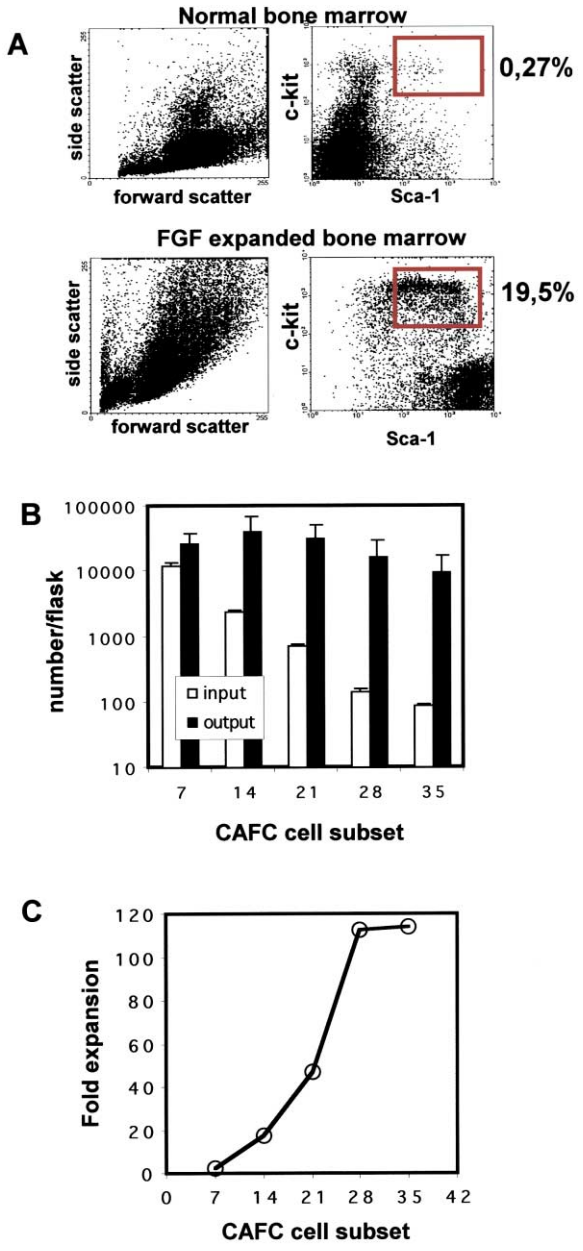

Figure 4. In Vitro Characterization of FGF-1-Expanded Bone Marrow Cells

The fraction of Sca-1$^+$ c-kit$^+$ cells in bone marrow cultures expanded for 4 weeks with FGF-1 was quantified by FACS analysis (A). For comparison Sca-1$^+$ c-kit$^+$ staining of freshly isolated bone marrow cells is also shown. Cobblestone area-forming cell assays were performed to quantify the absolute number of various CAFC subsets in the starting cell population (input; i.e., 5 × 10^6 normal unfractionated bone marrow cells) and the number of progenitor and stem cells recovered from the FGF-1-expansion culture (output). Error bars show standard deviations from five replicate experiments (B). The fold expansion of each CAFC subset (C) was calculated from the data as shown in (B).

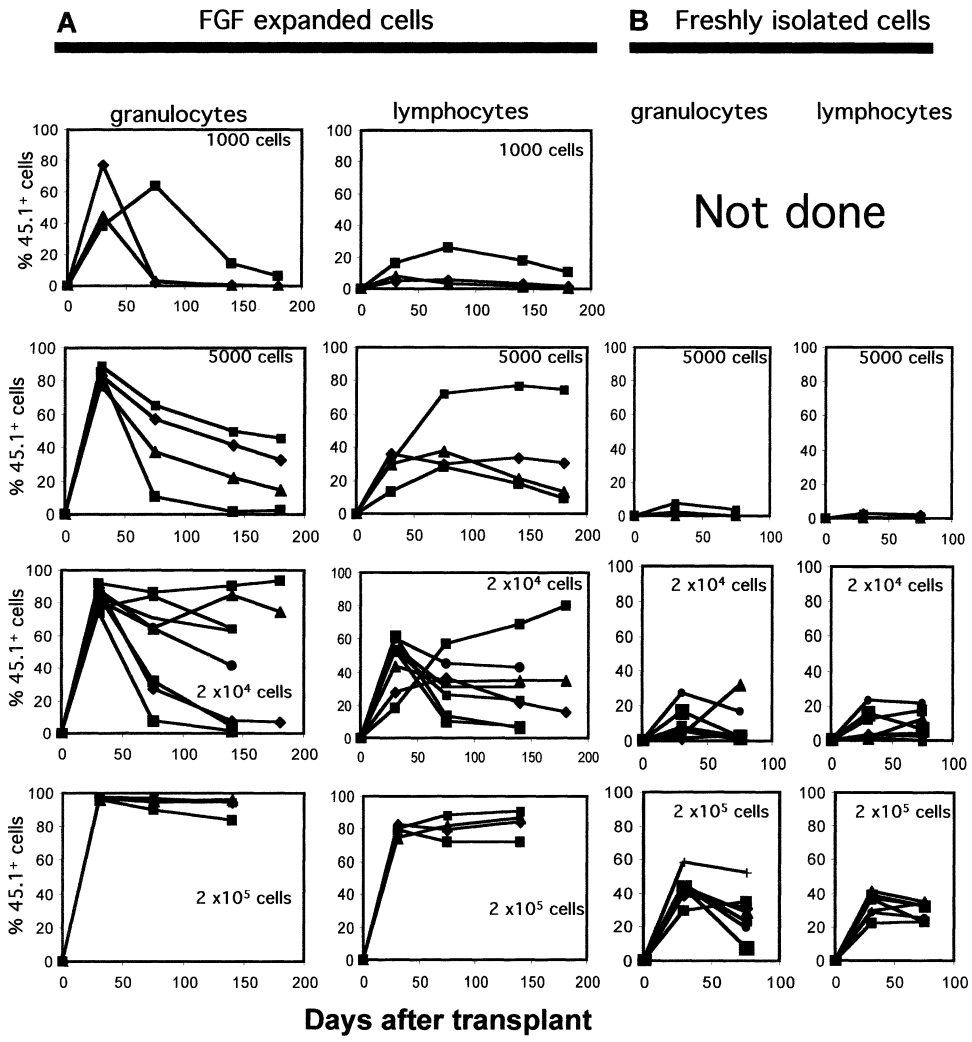

Figure 5. Long-Term Repopulating Potential of FGF-1-Expanded Bone Marrow Cells

A total of 1×10^3, 5×10^3, 2×10^4, or 2×10^5 FGF-1-expanded CD45.1$^+$ congenic cells were mixed with 2×10^5 wild-type C57BL/6 competitor cells and transplanted into lethally irradiated recipients. The fraction of expansion culture-derived granulocytes (left panels) and lymphocytes (right panels) for each individual recipient is shown (A). Granulocytes and lymphocytes were fractionated on the basis of standard forward/sideward scatter plots. To compare repopulating potential of FGF-1-expanded stem cells with freshly isolated bone marrow cells, we transplanted lethally irradiated recipients with 5×10^3, 2×10^4, or 2×10^5 CD45.1$^+$ fresh congenic bone marrow cells, again, together with 2×10^5 wild-type B6 competitor cells (B).

the bone marrow cells, we determined the fraction of B220$^+$, Thy-1$^+$, Gr-1$^+$, and Mac-1$^+$ CD45.1$^+$ peripheral blood cells in transplanted recipients: B cells (B220$^+$), T cells (Thy-1$^+$), and myeloid cells (Gr-1$^+$ and Mac-1$^+$) were all present in significant proportions (data not shown).

To directly compare the repopulating potential of expanded cells with freshly isolated bone marrow cells, we also transplanted a cohort of animals with 5000, 2×10^4, or 2×10^5 nonexpanded congenic cells, together with 2×10^5 wild-type competitor cells (Figure 5B, right panels). Whereas transplantation of 5000 FGF-1-expanded cells resulted in high engraftment levels, 5000 freshly isolated cells (as expected) did not result in multilineage reconstitution. Only a cell dose of 2×10^5 bone marrow cells induced chimerism in all recipients, but levels of engraftment were substantially lower than those of an equal number of expanded cells.

A summary of all transplant data is shown in Figure 6. A cell dose of 1000 expanded cells resulted in 33% chimeric animals (defined as >2% donor lymphocyte and granulocyte contribution 10 weeks after transplant), whereas at least 20,000 freshly isolated cells were required to establish a similar frequency (Figure 6A). Limiting dilution analysis estimated that the frequency of long-term multilineage repopulating stem cells in FGF-1-expanded bone marrow cells after 4 weeks was ~1/2,500 cells, whereas this number was ~1/60,000 in freshly isolated bone marrow. The mean percentage of donor-derived leukocytes in chimeric mice as a function of the transplanted cell dose is shown in Figure 6B.

The extent of amplification of repopulating potential

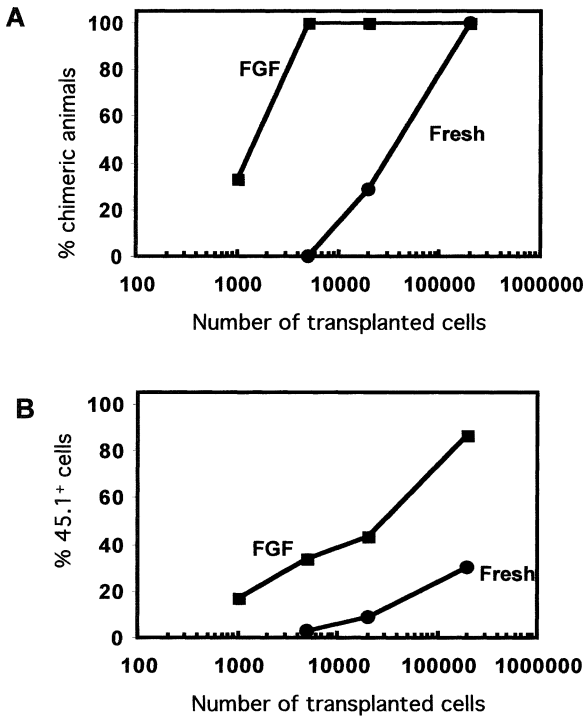

Figure 6. Comparison of Repopulating Potential of FGF-1-Expanded, or Fresh Marrow, Cells

The percentage of recipients transplanted with either FGF-1-expanded or fresh marrow cells showing donor-derived leukocytes as a function of transplanted cell dose is depicted (A). Animals were considered to be chimeric if >2% of both granulocytes and lymphocytes were of donor origin 2 months after transplant. The mean percentage of donor-derived leukocytes circulating in the peripheral blood of chimeric mice 2 months after transplant is shown in (B).

assayed in competitive transplantation of in vitro-expanded cells suggested that a graft containing only FGF-1-expanded cells (i.e., without additional freshly isolated competitor cells) should provide full radioprotection. Hence, we transplanted three groups of lethally irradiated recipients with either 2×10^5 unfractionated bone marrow cells, 10,000 FGF-1-expanded cells, or 20,000 FGF-1-expanded cells, and we determined survival and assayed white and red blood cell count recovery at various time points after transplant (Figure 7A). The results from this study again convincingly demonstrated the efficacy of FGF-1 to generate long-term repopulating stem cells. Whereas recipients typically need a transplant of at least 2×10^5 freshly isolated bone marrow cells to provide full radioprotection after a lethal irradiation of 10 Gy (data not shown), even at the lowest cell dose of 10,000 FGF-1-expanded cells, all animals were rescued. In addition, the recovery of peripheral white and red blood cells in recipients transplanted with FGF-1-expanded cells showed comparable kinetics to recipients that received a standard bone marrow transplant (Figure 7A). Also, in these recipients, which were transplanted with expanded cells only, we determined the proportion of granulocytes and lymphocytes that was donor (i.e., expansion culture)-derived. Not unexpectedly, virtually all granulocytes and lymphocytes were of donor origin, although lymphoid repopulation kinetics showed somewhat delayed engraftment kinetics (Figure 7B). In two out of six animals transplanted with 10,000 cells (again, estimated to contain approximately 4 long-term repopulating stem cells; Figure 6A) a decrease of donor-derived granulocytes was observed, but this decrease stabilized over time and was not observed for the lymphoid compartment.

Finally, we sacrificed an animal that had been transplanted with 10,000 FGF-1-expanded cells 4 months earlier and serially transplanted 5×10^5 or 5×10^6 bone marrow cells in a new cohort of ten lethally irradiated recipients. In the low-cell dose group, two animals died ~30 days after transplant. From one of these animals, we were able to collect a blood sample shortly before it died, and very low levels of donor cells were present in the peripheral blood, indicating graft failure (Figure 7C). However, in the other three recipients transplanted with this cell dose and in all recipients transplanted with the high cell dose, high levels of donor granulocytes and lymphocytes were present in the peripheral blood.

Discussion

Our data identify the FGF receptor as a novel and physiologically relevant stem cell marker: all long-term repopulating hematopoietic stem cells express high levels of FGFRs, and, conversely, absence of FGFR expression results in loss of stem cell potential. Our study defines the phenotype of stem cells with long-term repopulating ability as Lin^- $Sca\text{-}1^+$ $c\text{-}kit^+$ $CD34^-$ $FGFR^{++/+++}$. Most significantly, this is the first report, to our knowledge, to demonstrate that a large number of pluripotent, long-term repopulating hematopoietic stem cells can relatively simply be generated in vitro. The slow expansion kinetics suggest that FGF-1 acts exclusively on a small population of $FGFR^+$ primitive stem cells, whereas the majority of the cells, which do not express FGFRs, fail to survive in these culture conditions. However, we have so far been unable to expand purified hematopoietic stem cells (data not shown), so the exact nature of the FGF-1 target cell remains elusive. Consequently, although our data argue for a direct stimulating effect of FGF-1 on stem cells, we cannot exclude the possibility that FGF-1 acts either indirectly (an effect mediated through accessory cells) or in concert with other factors (released by accessory cells).

Upon transplant, which relieves the cells from enforced FGF-1 stimulation, expanded stem cells pursue a normal differentiation program. Expansion of hematopoietic stem cells has immediate clinical applications and has been unsuccessfully pursued by many research groups, using multiple "classical" hematopoietic growth factors. Although modest (3- to 4-fold) expansion of long-term repopulating stem cells in short-term cytokine-stimulated cultures has been documented (Conneally et al., 1997; Fraser et al., 1990), all protocols with genetically unperturbed stem cells so far have resulted in loss of stem cell activity (McNiece and Briddell, 2001; Srour et al., 1999). The only studies that have documented robust expansion of hematopoietic stem cells have used genetic protocols to selectively overexpress

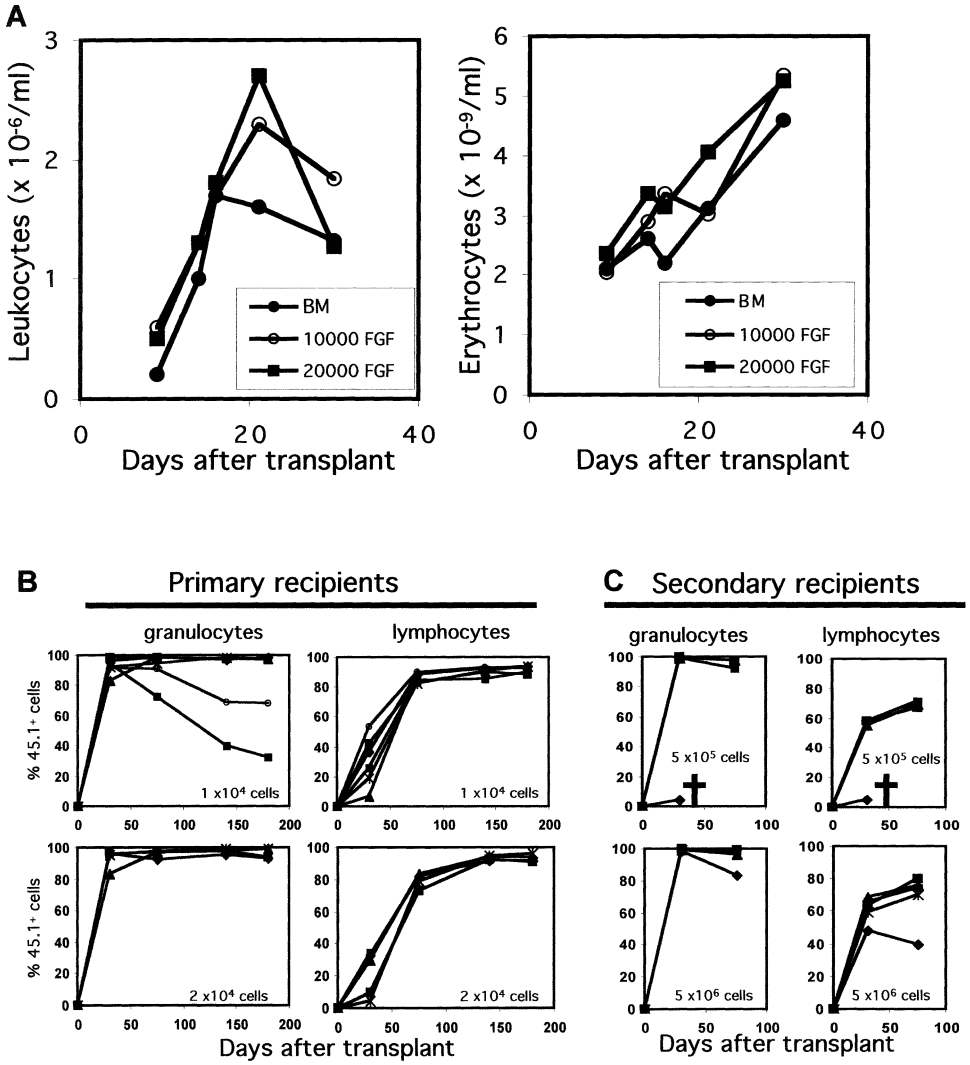

Figure 7. Radioprotective Ability of Expanded Cells in Primary and Secondary Recipients

Lethally irradiated recipients were transplanted with 1×10^4 or 2×10^4 CD45.1 FGF-1-expanded cells alone, and recovery of white and red blood cell counts at various time points after transplant was compared with recipients transplanted with 2×10^5 unfractionated bone marrow cells (A). Donor derived myeloid and lymphoid engraftment in recipients transplanted with 1×10^4 or 2×10^4 CD45.1 FGF-1-expanded cells ($n = 5$ per group) is shown in (B). Four months after transplant an animal that had been reconstituted with 1×10^4 FGF-1-expanded cells was sacrificed, and 5×10^5 or 5×10^6 bone marrow cells were serially transplanted in a second cohort of lethally irradiated recipients (C). Two out of five recipients transplanted with the lower cell dose died 30 days after transplant because of graft failure. All other animals survived and showed fully donor-derived hematopoiesis.

genes, such as Mdr-1 (Bunting et al., 1998), Notch1 (Varnum-Finney et al., 2000), or, most convincingly, HoxB4 in stem cells (Antonchuk et al., 2002; Sauvageau et al., 1995). Retroviral overexpression of HoxB4 in embryonic stem cells induces definitive hematopoietic potential after transplant in lethally irradiated recipients (Kyba et al., 2002). To test whether FGF-1 induced the expression of HoxB4, we have compared the expression level of this gene in FGF-expanded cells to that in freshly isolated marrow cells. Four weeks after culturing bone marrow cells in the presence of FGF-1, HoxB4 expression was readily detectable by RT-PCR. However, the extent of expression was not significantly different from that observed in fresh marrow (data not shown), suggesting that FGF-1-induced stem cell expansion did not result from enhanced expression of HoxB4.

We have not yet made any attempts to optimize our expansion protocol, and it is conceivable that even more robust amplification can be obtained when culturing conditions are altered or culture time is extended. In addition, whether the potent expansion activity is restricted to FGF-1 remains to be seen, but, considering the redundancy of the various FGF family members, this may be unlikely.

We show that lack of FGFR expression by stem cells is strongly associated with loss of stem cell potential, implying that FGFs play an important role in maintaining hematopoietic stem cell pool size. Considering the pleiotropic effects of many members of the FGF family, it is conceivable that these proteins also function to maintain homeostasis of other somatic stem cell compartments. For example, growth of neural stem cells in

vitro has been shown to be critically dependent on FGF-2 (Gage et al., 1995; Gritti et al., 1999), hepatic progenitor cells can be grown in FGF-4 (Schwartz et al., 2002), and FGF-10 maintains the stem cell compartment in developing mouse incisors (Harada et al., 2002). Thus, FGFRs may specify pan-stem cell properties, and the elucidation of downstream signaling events in stem cells stimulated by FGFs may reveal crucial information on molecular pathways associated with asymmetrical divisions and fate decisions.

Experimental Procedures

Cell Purification of Lin$^-$ FGFR$^+$ Cells

Bone marrow cells were isolated from C57BL/6 mice. After standard erythrocyte lysis, nucleated cells were incubated with normal rat serum for 15 min at 4°C. Subsequently, cells were stained with a panel of biotinylated lineage-specific antibodies (Murine Progenitor Enrichment Cocktail, containing anti-CD5, anti-B220, anti-Mac-1, anti-TER119, anti-Gr-1, and anti-7-4; Stem Cell Technologies), FITC-anti-Sca-1, and APC-anti-c-kit or FITC-anti-CD34 (Pharmingen) and 10 ng/10^6 cells Fc-FGF in the presence of 2.5 μg/ml heparin (Dikov et al., 1998) for 30 min at 4°C. Cells were washed twice and incubated for 30 min with streptavidin-PerCP (Pharmingen) and phycoerythrin-labeled goat F(ab′)$_2$ anti-human IgG1 (Southern Biotechnology Associates). The anti-human IgG antibody will recognize the Fc domain of the Fc-FGF fusion protein and, thus, detect specific Fc-FGF binding. After two washes cells were resuspended in PBS plus 1% BSA and purified with a MoFlo flow cytometer. The lineage-depleted bone marrow cell population was defined as the 5% of cells showing the least PerCP fluorescence intensity.

Immunofluoresence Staining

Lin$^-$ FGFR$^{++/+++}$ and Lin$^-$ FGFR$^-$ cells were purified, and cytospot slides were made. Cells were fixed with cold methanol for 10 min, washed with TBS, and stained overnight with a rabbit polyclonal IgG anti-human FGFR-1 antibody (clone SC-121; Santa Cruz Biotechnology). After repeated washing cells were stained with FITC-labeled swine anti-rabbit IgG (DAKO) and DAPI. Fluorescence was evaluated with a confocal laser microscope.

In Vivo Transplantation Assays

FACS-purified C57BL/6-CD45.2 Lin$^-$ FGFR$^{++/+++}$ and Lin$^-$ FGFR$^-$ cells were diluted appropriately and mixed with 2×10^5 unfractionated competitor bone marrow cells harvested from B6.SJL-CD45.1 congenic mice. Cells were transplanted intravenously in lethally (10 Gy) irradiated mice, each transplant group consisting of five recipients. Blood samples from recipient animals were analyzed by flow cytometry to detect the presence of CD45.1- or CD45.2-positive cells. The CD45 antigens are expressed on all peripheral leukocytes.

In a second series of experiments, B6.SJL-CD45.1 congenic bone marrow cells were cultured in serum-free medium containing FGF-1 (see below) for 4 weeks. After 4 weeks, adherent and nonadherent cells were harvested, counted, and transplanted (alone or together with 2×10^5 freshly isolated wild-type C57BL/6 bone marrow cells) in lethally (10 Gy) irradiated recipients. At selected time points white blood cells and erythrocytes were quantified in the peripheral blood of recipient animals, and the presence of CD45.1- or CD45.2-positive leukocytes was detected by incubating leukocytes with fluorescently labeled CD45.1- and CD45.2-specific antibodies (Pharmingen). In addition, the percentage of donor-derived T cells, B cells, and myeloid cells was determined by costaining cells with anti-Thy1, anti-B220, and anti-Gr-1 and anti-Mac-1 antibodies, respectively (Pharmingen).

For serial transplantation we sacrificed an animal that had been transplanted 4 months earlier with 10,000 FGF-1-expanded cells and that showed high levels (>95%) of donor cell engraftment. Cells were isolated from both femora, counted and appropriately resuspended, and 5×10^5 or 5×10^6 cells were intravenously transplanted in lethally irradiated C57BL/6 wild-type recipients.

Stem Cell Expansion

A total of 5×10^6 unfractionated bone marrow cells were incubated in 5 ml StemSpan ex vivo expansion medium (Stem Cell Technologies, Vancouver, British Columbia, Canada) in the presence of 10 ng/ml recombinant human FGF-1 (Invitrogen) or 10 ng/ml recombinant rat SCF (kindly provided by Amgen, Thousand Oaks, CA) in the presence of 10 μg/ml heparin (Sigma, St. Louis, MO). Nonadherent cells were harvested weekly and counted, and an aliquot was used to initiate standard GM-CSF plus SCF-stimulated methylcellulose CFU-GM cell assays. Cells that were not used for progenitor cell assays were reintroduced in the expansion culture. Twice weekly, one-half of the medium in the flask was replaced with fresh media containing the same concentration of growth factors without depopulation. At selected time points cytospin preparations were made, and slides were stained with May-Gruenwald/Giemsa.

Cobblestone Area-Forming Cell Assays

CAFC assays were used to quantitate the number of hematopoietic progenitor cells or stem cells in the FACS-purified cell populations and in the FGF-expanded cell culture. To this end test cells were seeded in a limiting dilution fashion in 96-well plates containing a preestablished FBMD-1 stromal cell layer, exactly as described earlier (de Haan et al., 2000).

RT-PCR

Expression of distinct FGFR transcripts was assessed by RT-PCR with the following specific primers: FGFR-1, 5′-GTGGAAGACCTGG ACCACAT-3′ (forward) and 5′-TGAGAGAAGACAGAGTCCTCCC-3′ (reverse) (fragment size, 143 bp); FGFR-2, 5′-GAGGCTGTCTCAG AGCCTGT-3′ (forward) and 5′-CTTGCGGCTGTCCACTTATC-3′ (reverse) (fragment size, 164 bp); FGFR-3, 5′-TCAAAGCTTGGGAGAC AGTG-3′ (forward) and 5′-CTAGGCCCCAAGAAAGTCAC-3′ (reverse) (fragment size, 138 bp); FGFR-4, 5′-GTCGCAGATCCTCT CTCTGG-3′ (forward) and 5′-TCTGAGGATGAGTCCAAGGG-3′ (reverse) (fragment size, 152 bp). DNA sequencing was employed to confirm specificity of the PCR products. HoxB4 primers were 5′-CCACAAGTTGCCCAACACC-3′ (forward) and 5′ GCTTGGGGGGCA CTAGAG-3′ (reverse) (fragment size, 108 bp).

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References

Antonchuk, J., Sauvageau, G., and Humphries, R.K. (2002). HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. Cell *109*, 39–45.

Arman, E., Haffner-Krausz, R., Chen, Y., Heath, J.K., and Lonai, P. (1998). Targeted disruption of fibroblast growth factor (FGF) receptor 2 suggests a role for FGF signaling in pregastrulation mammalian development. Proc. Natl. Acad. Sci. USA *95*, 5082–5087.

Bartunek, P., Pajer, P., Karafiat, V., Blendinger, G., Dvorak, M., and Zenke, M. (2002). bFGF signaling and v-Myb cooperate in sustained growth of primitive erythroid progenitors. Oncogene *21*, 400–410.

Beer, H.D., Vindevoghel, L., Gait, M.J., Revest, J.M., Duan, D.R., Mason, I., Dickson, C., and Werner, S. (2000). Fibroblast growth factor (FGF) receptor 1-IIIb is a naturally occurring functional receptor for FGFs that is preferentially expressed in the skin and the brain. J. Biol. Chem. *275*, 16091–16097.

Berardi, A.C., Wang, A., Abraham, J., and Scadden, D.T. (1995). Basic fibroblast growth factor mediates its effects on committed

myeloid progenitors by direct action and has no effect on hematopoietic stem cells. Blood *86*, 2123–2129.

Bunting, K.D., Galipeau, J., Topham, D., Benaim, E., and Sorrentino, B.P. (1998). Transduction of murine bone marrow cells with an MDR1 vector enables ex vivo stem cell expansion, but these expanded grafts cause a myeloproliferative syndrome in transplanted mice. Blood *92*, 2269–2279.

Burger, P.E., Coetzee, S., McKeehan, W.L., Kan, M., Cook, P., Fan, Y., Suda, T., Hebbel, R.P., Novitzky, N., Muller, W.A., and Wilson, E.L. (2002). Fibroblast growth factor receptor-1 is expressed by endothelial progenitor cells. Blood *100*, 3527–3535.

Colvin, J.S., Bohne, B.A., Harding, G.W., McEwen, D.G., and Ornitz, D.M. (1996). Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. Nat. Genet. *12*, 390–397.

Conneally, E., Cashman, J., Petzer, A., and Eaves, C. (1997). Expansion in vitro of transplantable human cord blood stem cells demonstrated using a quantitative assay of their lympho-myeloid repopulating activity in nonobese diabetic-scid/scid mice. Proc. Natl. Acad. Sci. USA *94*, 9836–9841.

de Haan, G., Szilvassy, S.J., Meyerrose, T.E., Dontje, B., Grimes, B., and Van Zant, G. (2000). Distinct functional properties of highly purified hematopoietic stem cells from mouse strains differing in stem cell numbers. Blood *96*, 1374–1379.

De Moerlooze, L., Spencer-Dene, B., Revest, J., Hajihosseini, M., Rosewell, I., and Dickson, C. (2000). An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis. Development *127*, 483–492.

Deng, C.X., Wynshaw-Boris, A., Shen, M.M., Daugherty, C., Ornitz, D.M., and Leder, P. (1994). Murine FGFR-1 is required for early postimplantation growth and axial organization. Genes Dev. *8*, 3045–3057.

Dikov, M.M., Reich, M.B., Dworkin, L., Thomas, J.W., and Miller, G.G. (1998). A functional fibroblast growth factor-1 immunoglobulin fusion protein. J. Biol. Chem. *273*, 15811–15817.

Faloon, P., Arentson, E., Kazarov, A., Deng, C.X., Porcher, C., Orkin, S., and Choi, K. (2000). Basic fibroblast growth factor positively regulates hematopoietic development. Development *127*, 1931–1941.

Fraser, C.C., Eaves, C.J., Szilvassy, S.J., and Humphries, R.K. (1990). Expansion in vitro of retrovirally marked totipotent hematopoietic stem cells. Blood *76*, 1071–1076.

Gabbianelli, M., Sargiacomo, M., Pelosi, E., Testa, U., Isacchi, G., and Peschle, C. (1990). "Pure" human hematopoietic progenitors: permissive action of basic fibroblast growth factor. Science *249*, 1561–1564.

Gage, F.H., Coates, P.W., Palmer, T.D., Kuhn, H.G., Fisher, L.J., Suhonen, J.O., Peterson, D.A., Suhr, S.T., and Ray, J. (1995). Survival and differentiation of adult neuronal progenitor cells transplanted to the adult brain. Proc. Natl. Acad. Sci. USA *92*, 11879–11883.

Goldfarb, M. (2001). Signaling by fibroblast growth factors: the inside story. Sci. STKE *2001*, PE37.

Gritti, A., Frolichsthal-Schoeller, P., Galli, R., Parati, E.A., Cova, L., Pagano, S.F., Bjornson, C.R., and Vescovi, A.L. (1999). Epidermal and fibroblast growth factors behave as mitogenic regulators for a single multipotent stem cell-like population from the subventricular region of the adult mouse forebrain. J. Neurosci. *19*, 3287–3297.

Guasch, G., Ollendorff, V., Borg, J.P., Birnbaum, D., and Pebusque, M.J. (2001). 8p12 stem cell myeloproliferative disorder: the FOP-fibroblast growth factor receptor 1 fusion protein of the t(6;8) translocation induces cell survival mediated by mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt/mTOR pathways. Mol. Cell. Biol. *21*, 8129–8142.

Harada, H., Toyono, T., Toyoshima, K., Yamasaki, M., Itoh, N., Kato, S., Sekine, K., and Ohuchi, H. (2002). FGF10 maintains stem cell compartment in developing mouse incisors. Development *129*, 1533–1541.

Kyba, M., Perlingeiro, R.C., and Daley, G.Q. (2002). HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. Cell *109*, 29–37.

McNiece, I., and Briddell, R. (2001). Ex vivo expansion of hematopoietic progenitor cells and mature cells. Exp. Hematol. *29*, 3–11.

Mugneret, F., Chaffanet, M., Maynadie, M., Guasch, G., Favre, B., Casasnovas, O., Birnbaum, D., and Pebusque, M.J. (2000). The 8p12 myeloproliferative disorder. t(8;19)(p12;q13.3): a novel translocation involving the FGFR1 gene. Br. J. Haematol. *111*, 647–649.

Ogawa, M. (2002). Changing phenotypes of hematopoietic stem cells. Exp. Hematol. *30*, 3–6.

Okada, S., Nakauchi, H., Nagayoshi, K., Nishikawa, S., Miura, Y., and Suda, T. (1992). In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. Blood *80*, 3044–3050.

Ollendorff, V., Guasch, G., Isnardon, D., Galindo, R., Birnbaum, D., and Pebusque, M.J. (1999). Characterization of FIM-FGFR1, the fusion product of the myeloproliferative disorder-associated t(8;13) translocation. J. Biol. Chem. *274*, 26922–26930.

Ornitz, D.M., and Itoh, N. (2001). Fibroblast growth factors. Genome Biol *2*, REVIEWS3005.

Osawa, M., Hanada, K., Hamada, H., and Nakauchi, H. (1996). Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science *273*, 242–245.

Ploemacher, R.E., van der Sluijs, J.P., van Beurden, C.A., Baert, M.R., and Chan, P.L. (1991). Use of limiting-dilution type long-term marrow cultures in frequency analysis of marrow-repopulating and spleen colony-forming hematopoietic stem cells in the mouse. Blood *78*, 2527–2533.

Popovici, C., Zhang, B., Gregoire, M.J., Jonveaux, P., Lafage-Pochitaloff, M., Birnbaum, D., and Pebusque, M.J. (1999). The t(6;8)(q27;p11) translocation in a stem cell myeloproliferative disorder fuses a novel gene, FOP, to fibroblast growth factor receptor 1. Blood *93*, 1381–1389.

Quito, F.L., Beh, J., Bashayan, O., Basilico, C., and Basch, R.S. (1996). Effects of fibroblast growth factor-4 (k-FGF) on long-term cultures of human bone marrow cells. Blood *87*, 1282–1291.

Ratajczak, M.Z., Ratajczak, J., Skorska, M., Marlicz, W., Calabretta, B., Pletcher, C.H., Jr., Moore, J., and Gewirtz, A.M. (1996). Effect of basic (FGF-2) and acidic (FGF-1) fibroblast growth factors on early haemopoietic cell development. Br. J. Haematol. *93*, 772–782.

Sato, T., Laver, J.H., and Ogawa, M. (1999). Reversible expression of CD34 by murine hematopoietic stem cells. Blood *94*, 2548–2554.

Sauvageau, G., Thorsteinsdottir, U., Eaves, C.J., Lawrence, H.J., Largman, C., Lansdorp, P.M., and Humphries, R.K. (1995). Overexpression of HOXB4 in hematopoietic cells causes the selective expansion of more primitive populations in vitro and in vivo. Genes Dev. *9*, 1753–1765.

Schwartz, R.E., Reyes, M., Koodie, L., Jiang, Y., Blackstad, M., Lund, T., Lenvik, T., Johnson, S., Hu, W.S., and Verfaillie, C.M. (2002). Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. J. Clin. Invest. *109*, 1291–1302.

Srour, E.F., Abonour, R., Cornetta, K., and Traycoff, C.M. (1999). Ex vivo expansion of hematopoietic stem and progenitor cells: are we there yet? J. Hematother. *8*, 93–102.

Varnum-Finney, B., Xu, L., Brashem-Stein, C., Nourigat, C., Flowers, D., Bakkour, S., Pear, W.S., and Bernstein, I.D. (2000). Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. Nat. Med. *6*, 1278–1281.

Vasiliauskas, D., and Stern, C.D. (2001). Patterning the embryonic axis: FGF signaling and how vertebrate embryos measure time. Cell *106*, 133–136.

Wang, Y., Spatz, M.K., Kannan, K., Hayk, H., Avivi, A., Gorivodsky, M., Pines, M., Yayon, A., Lonai, P., and Givol, D. (1999). A mouse model for achondroplasia produced by targeting fibroblast growth factor receptor 3. Proc. Natl. Acad. Sci. USA *96*, 4455–4460.

Weinstein, M., Xu, X., Ohyama, K., and Deng, C.X. (1998). FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung. Development *125*, 3615–3623.

Wilson, E.L., Rifkin, D.B., Kelly, F., Hannocks, M.J., and Gabrilove,

J.L. (1991). Basic fibroblast growth factor stimulates myelopoiesis in long-term human bone marrow cultures. Blood *77*, 954–960.

Xiao, S., McCarthy, J.G., Aster, J.C., and Fletcher, J.A. (2000). ZNF198-FGFR1 transforming activity depends on a novel proline-rich ZNF198 oligomerization domain. Blood *96*, 699–704.

Yuen, D., Mittal, L., Deng, C.X., and Choi, K. (1998). Generation of a primitive erythroid cell line and promotion of its growth by basic fibroblast growth factor. Blood *91*, 3202–3209.