Insulin-like growth factor 2 expressed in a novel fetal liver cell population is a growth factor for hematopoietic stem cells

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Hematopoietic stem cells (HSCs) undergo dramatic expansion during fetal liver development, but attempts to expand their numbers ex vivo have failed. We hypothesized that unidentified fetal liver cells produce growth factors that support HSC proliferation. Here we describe a novel population of CD34+ and Ter119− day-15 fetal liver cells that support HSC expansion in culture, as determined by limiting dilution mouse reconstitution analyses. DNA array experiments showed that, among other proteins, insulin-like growth factor 2 (IGF-2) is specifically expressed in fetal liver CD34+ cells but not in several cells that do not support HSCs. Treatment of fetal liver CD34+Ter119− cells with anti–IGF-2 abrogated their HSC supportive activity, suggesting that IGF-2 is the key molecule produced by these cells that stimulates HSC expansion. All mouse fetal liver and adult bone marrow HSCs express receptors for IGF-2. Indeed, when combined with other growth factors, IGF-2 supports a 2-fold expansion of day-15 fetal liver Lin−Sca-1−c-Kit+ long-term (LT)–HSC numbers. Thus, fetal liver CD34+Ter119− cells are a novel stromal population that is capable of supporting HSC expansion, and IGF-2, produced by these cells, is an important growth factor for fetal liver and, as we show, adult bone marrow HSCs.

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Introduction

In vivo stromal cells form a complex microenvironment for hematopoietic stem cells (HSCs) that controls their multiple fates, including apoptosis and migration as well as the cell divisions that lead to formation either of daughter HSCs or of lineage-committed progenitors that are capable of limited proliferation.1,2 Culture of HSCs in vitro, away from their natural microenvironment, leads to apoptosis or differentiation but not HSC self-renewal.

Genetic studies in mice, together with studies in which purified proteins were added to cultures of partially purified HSC populations, have led to the identification of secreted proteins that, in combination with others, maintain or expand the number of bone marrow HSCs. In mice, mutations either in stem cell factor (SCF) or its receptor, the protein tyrosine kinase Kit, lead to a profound reduction of HSC activities and numbers.3,4 In vitro, SCF can prevent apoptosis of HSCs and is a growth factor for primitive lymphoid and myeloid hematopoietic progenitor or stem cells.5,6 Both TPO−/− mice and mice with mutations in its receptor, mpl, have a marked reduction in HSC activity,7 and thrombopoietin (TPO) can promote survival and expansion of bone marrow HSCs in culture. Addition of interleukin-11 (IL-11) together with SCF or Flt-3 ligand (FL) maintains and expands bone marrow HSCs.8 Flt-3 ligand (FL) receptor-deficient mice have many deficiencies in hematopoiesis,9 but elimination of the IL-11 receptor does not affect HSC numbers.10 FL has been included in numerous culture systems for HSCs,6 and several papers suggested that the FL receptor is expressed in some fetal liver long-term (LT)–HSCs but not in bone marrow LT-HSCs.8,15-17 Addition of fibroblast growth factor-1 (FGF-1) resulted in robust expansion of repopulating HSCs when total bone marrow cells were cultured in serum-free medium.16 Because purified HSCs cannot be expanded using FGF-1, the exact nature of the FGF-1 target cell population remains to be determined.16 Recently, purified and lipid-modified Wnt3a protein, when added in combination with fetal bovine serum and SCF, enhanced ex vivo expansion of purified bone marrow c-Kit+Thy-1.1+Sca-1−Lin− hematopoietic progenitor cells.17-18 However, mouse repopulation was studied only at 4 or 6 weeks after transplantation. Thus, it is not known whether Wnt3a supports expansion of long-term repopulating stem cells.

Although fetal liver HSCs have a greater proliferative potential than adult bone marrow HSCs and undergo significant expansion in vivo,19-21 attempts to expand fetal liver HSCs ex vivo have failed.11 We hypothesized that unknown growth proteins are produced by as yet unidentified populations of fetal liver cells that stimulate the expansion of fetal liver HSCs. Here we studied the effects of differentiated fetal liver hematopoietic cells on HSCs cultured ex vivo and identified a novel cell population, day-15 fetal liver CD34+ cells that support expansion of HSC numbers in culture. We show that insulin-like growth factor 2 (IGF-2) is specifically produced by fetal liver CD34+ cells and is the key molecule produced by these cells that stimulates HSC expansion. Furthermore, when combined with other growth factors, IGF-2 is capable of markedly enhancing ex vivo expansion of long-term repopulating fetal liver and adult bone marrow HSCs. Thus, IGF-2 is a novel growth factor for HSCs.

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Materials and methods

Cell culture

Lin<sup>-</sup>Sca-1<sup>-</sup>Kit<sup>+</sup> or side population (SP) cells were cultured alone or cocultured with the same number of fetal liver CD3<sup>+</sup> cells as indicated, at a density of 50 Lin<sup>-</sup>Sca-1<sup>-</sup>Kit<sup>+</sup> or SP cells per 30 μL in Iscove modified Dulbecco medium (IMDM) with 10% fetal bovine serum (FBS) (StemCell Technologies, Vancouver, BC), 1% bovine serum albumin (BSA) (Sigma, St Louis, MO), and 50 μM β-mercaptoethanol (Invitrogen, Carlsbad, CA) in a well of a U-bottom 96-well plate. Murine IGF-2 (R&D Systems, Minneapolis, MN), 20 ng/mL SFC (R&D Systems), 30 ng/mL Fli-3 ligand (R&D Systems), and 10 ng/mL IL-6 (R&D Systems), or 50 ng/mL SCF and 100 ng/mL TPO (R&D Systems), as well as anti–IGF-2 neutralizing antibody (R&D Systems) were used as indicated.

FACS and analysis

Donor fetal liver or bone marrow cells were isolated from C57BL/6 CD45.2 mice. To sort Lin<sup>-</sup>Sca-1<sup>-</sup>Kit<sup>+</sup> cells, nucleated cells were incubated with rat serum for 15 minutes at 4°C at a density of 5 × 10<sup>5</sup>/mL. Cells were then stained with a biotinylated Lin<sup>+</sup> antibody cocktail (for fetal liver cells, anti-CD3, anti-CD5, anti-B220, anti–Gr-1, anti-Ter119, and anti–7–4; for bone marrow cells, anti–Mac-1 was also included; StemCell Technologies and BD Pharmingen, San Diego, CA) for 15 minutes at 4°C. Cells were washed and incubated for 20 minutes with streptavidin-allophycocyanin (streptavidin-APC), anti-c-Kit (BD Pharmingen), and anti–Sca-1–fluorescein isothiocyanate (anti-Sca-1–FITC). After 2 washes the cells were isolated by fluorescence-activated cell sorting (FACS) in a MoFlo cell sorter. Fetal liver CD3<sup>+</sup> cells were sorted after staining with the anti–CD3–PE antibody. Adult mouse bone marrow SP cells were isolated essentially as described.22

To sort IGF-2–hFc<sup>+</sup> and IGF-2–hFc<sup>−</sup> cells, 10<sup>7</sup> cells were resuspended in 1 mL conditioned medium (with about 1 μg/mL IGF-2–hFc determined by Western blotting) at 4°C for 30 minutes, followed by staining with anti-human immunoglobulin G1 (IgG1) Fc-PE (Jackson ImmunoResearch, West Grove, PA). The conditioned medium from mock-transfected cells was used as control. When necessary, the containing of biotinylated Lin<sup>+</sup> antibody cocktail followed by streptavidin-APC and anti-Sca-1–FITC was performed before sorting Lin<sup>-</sup>Sca-1<sup>-</sup>IGF-2–hFc<sup>+</sup> and Lin<sup>-</sup>Sca-1<sup>-</sup>IGF-2–hFc<sup>−</sup> cells.

For reconstitution analysis, peripheral blood cells were collected by retro-orbital bleeding, followed by lysis of red blood cells and staining with anti-CD45.2–FITC, anti-CD45.1–PE, anti–Thy1.2–PE, anti-B220–PE, anti–Mac-1–PE, anti–Gr-1–PE, or anti–Ter119–PE monoclonal antibodies (BD Pharmingen). When FACS analyses was used for characterizing CD3<sup>+</sup> cells, 15 day fetal liver cells or adult spleen cells were stained with isotype control, anti–CD3–FITC, anti–CD4–PE, anti–CD8–PE, anti–Thy1.2–PE, and anti–Ter119–PE monoclonal antibodies (BD Pharmingen). FACS analyses were performed on a FACSCalibur instrument.

Competitive reconstitution analysis

Both activity assays and limiting dilution assays were performed using the competitive reconstitution protocol. Indicated numbers of CD45.2 donor cells were mixed with 2 × 10<sup>4</sup> (1 × 10<sup>5</sup> used in Figures 4A and 6B) freshly isolated CD45.1 competitor bone marrow cells, which were then injected intravenously via the retro-orbital route into a group of CD45.1 mice previously irradiated with a total dose of 10 Gy. To measure reconstitution of mice undergoing transplantation, peripheral blood was collected by retro-orbital bleeding at the indicated times after transplantation, and the presence of CD45.1<sup>+</sup> and CD45.2<sup>+</sup> cells in lymphoid and myeloid compartments was measured.

Calculation of stem cell frequency

In limiting dilution assays, the frequency of repopulating cells (competitive repopulating units (CRUs)) was estimated on the basis of Poisson statistics as described; the ranges of CRUs are given as 95% confidence intervals. For the experiment in Figure 6D, because the numbers of injected cells were insufficient for limiting dilution assays, the frequencies of HSCs were calculated as follows. If n = unknown frequency of HSCs in the population and m = injected cell number, the probability of a mouse not repopulated by the donor cells is P = (1 – n)<sup>m</sup>. For a group of mice in Figure 6D, the P value, which is the ratio of the number of nonchimeric mice to the total number of mice, and m, which is the number of injected cells, are both known and n can be calculated. When all the recipient mice were repopulated, we could not calculate the actual n value; instead we calculated a mock n value by assuming that 1 of the recipient mice was not repopulated. The actual ln value was represented as less than 1 per mock n value.

DNA array experiments and analyses

Fifteen micrograms of total RNA and cRNA were prepared for hybridization to Affymetrix U74Av2 mouse chips according to manufacturer’s instructions. The analysis was performed as detailed in our recent paper.

Measuring the expression of IGF-2 and its receptors

Reverse transcriptase–polymerase chain reaction (RT-PCR) was performed on cDNAs isolated from FACS-sorted murine day 15 fetal liver Lin<sup>-</sup>Sca-1<sup>-</sup>Kit<sup>+</sup> cells, CD3<sup>+</sup> cells, CD3<sup>+</sup>Ter119<sup>+</sup> cells, CD3<sup>+</sup>Ter119<sup>-</sup> cells, IGF-2–hFc<sup>+</sup> cells, and IGF-2–hFc<sup>−</sup> cells to check expression of IGF-2, IGF type 1 receptor (IGF1R), IGF type 2 receptor (IGF2R), insulin receptor (IR), and glycerolaldehydes-3-phosphate dehydrogenase (GAPDH). Forward primer for IGF-2: GTCGATGGTGTCCTTCTCA; reverse primer for IGF-2: AAGCAGCCTCTCCACGAGT. Forward primer for IGF1R: CAAGCTGTGTTCTCCGGAA; reverse primer for IGF1R: TGTAGGATCCGGTGATCC. 5′ primer for IGF2R: GCACCAAGATGAAGACCTGA; reverse primer for IGF2R: ACATCGGGTACTGTGTTGCTG. For IR: AAGTTGGCACAACCATCTG; reverse primer for IR: GTGAAGGTTTGGCAGAA. For GAPDH: AACTTTGCGATTGTGAAGG; reverse primer for GAPDH: ACACATTGGGCGTAGGAACA. The level of murine IGF-2 in the medium of cultured cells was measured by enzyme-linked immunosorbant assay (ELISA) as described in the DuoSet ELISA Development Kit for mouse IGF-2 (R&D Systems).

Expression of IGF-2–hFc

The DNAs encoding Met1-Glu91 of human prepro–IGF-2 and Pro100-Lys330 of human IgG1 Fc sequences were linked by a DNA sequence encoding IEGRMD linker peptide. The whole 1.1-kilobase (kb) fragment of human prepro–IGF-2 and Met1-Glu91 of human prepro–IGF-2 and Pro100-Lys330 of human IgG1 Fc was inserted into the KpnI and XhoI sites of pcDNA3.1, downstream of cytomegalovirus (CMV) promoter. The plasmid was transfected into 293T cells using lipofectamine 2000 (Invitrogen), and conditioned medium at 48 hours after transfection was collected. The conditioned medium with about 1 μg/mL IGF-2–hFc determined by Western blotting was used in the subsequent staining of fetal liver or bone marrow cells.

Results

Coculture of fetal liver HSCs with fetal liver CD3<sup>+</sup> cells increases the number of long-term repopulating stem cells

Because fetal liver HSCs undergo significant expansion during fetal development, we hypothesized that certain lineage-positive (Lin<sup>+</sup>) cells might produce protein(s) that support HSCs. A medium containing fetal calf serum, SCF, FL, and IL-6 cannot in itself support the maintenance of purified fetal liver HSC’s, presumably because it is missing some essential growth factor(s). In preliminary studies we confirmed this by showing that culture of day-15 fetal liver Lin<sup>+</sup> cells in this medium for 3 or 6 days resulted in loss of HSC activity. However, adding day-15 fetal liver Lin<sup>+</sup> cells in this culture system for 3 days supported a net 3-fold expansion in the number of in vivo repopulating HSCs (data not shown).

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suggesting that some Lin+ cells are expressing HSC supportive growth factor(s).

To identify the Lin+ cell population(s) that support HSC ex vivo expansion, we fractionated day-15 fetal liver cells by flow cytometry into 4 populations: CD3+, B220+, Gr-1+, and Ter119+, which were then individually cocultured with Lin- cells. Only fetal liver CD3+ cells supported increases in HSC numbers and activity during a 3- or 6-day culture; the activity of HSCs was maintained from day 3 to day 6 and dropped after 6 days of culture (data not shown and Figure 1). Controls showed, as expected, no hematopoietic reconstitution ability in either freshly isolated or 3-day-cultured day 15 fetal liver CD3+ cells (104 cells tested) 1 to 8 months after transplantation.

The limiting dilution experiment in Figure 1A shows that day-15 fetal liver CD3+ cells directly stimulate ex vivo expansion of purified LT-HSCs during a 3-day culture. The CRU frequency for freshly isolated fetal liver Lin-Sca-1+Kit+ (LSK) cells is 1 per 50 (95% confidence interval for mean, 1/36 to 1/57; n = 41). That is, as calculated from Poisson statistics, injection on average of 50 freshly isolated LSK cells is sufficient to repopulate 63% (1 – 1/e) of mice undergoing transplantation. After culture in medium containing serum, SCF, FL, and IL-6, with or without CD3+ cells, the number of cells was too few to be counted reliably. However, after a 3-day culture without CD3+ cells, all HSC activity was lost, as judged by the fact (“□” data point) that all 10 mice undergoing transplantation with the progeny of 100 input LSK cells failed to reconstitute (less than 1% donor contribution to nucleated blood cells; Figure 1).

After a 3-day culture of LSK cells with an equivalent number of day-15 fetal liver CD3+ cells, the number of functional LT-HSCs had increased about 2.5-fold (P < .05, Student t test), as evidenced by the fact that 63% of the mice undergoing transplantation with the progeny of, on average, only 22 initial LSK cells became reconstituted. In other words, the CRU frequency was 1 per 22 input equivalent LSK cells (95% confidence interval for mean, 1/13 to 1/26; n = 35). Figure 1B shows that fetal liver LSK cells cocultured with CD3+ cells repopulated, 4 months after transplantation, all blood lineages we tested (Figure 1B), attesting to net expansion of LT-HSCs.

**IGF-2 is a key factor expressed in day-15 fetal liver CD3+ cells to support HSCs**

At day 15, CD3+ cells represent 2% to 3% of total fetal liver cells. Although they are nonadherent to plastic and remain in suspension during culture, they are very different from CD3+ cells found in adult spleen or bone marrow (Figure 2A and data not shown). CD3+ cells isolated from adult spleen are mature T cells; they are Thy1.2+ (Figure 2Axi) and either CD4+ or CD8+ (Figure 2Axi-x). Most of them are Ter119+ (Figure 2Axii). Fetal liver CD3+ cells, in contrast, are Thy1.2 CD4+CD8− (Figure 2Axi-xv), and half are Ter119+ (Figure 2Avi). In addition, the level of CD3 expression on fetal liver CD3+ cells is less than that on adult splenic CD3+ cells (Figure 2A, compare plots ii and viii). Fetal liver HSCs cocultured with CD3+ cells isolated from adult spleen or bone marrow lose most if not all repopulation ability (data not shown).

In an attempt to determine what factor(s) are made by day-15 fetal liver CD3+ cells that support HSC expansion, we compared the transcriptional profile of day-15 fetal liver CD3+ cells with those of 2 negative cell populations: adult splenic CD3+ cells and day-15 fetal liver Gr-1+ cells. Table 1 shows that several potentially important secreted and membrane proteins are differentially expressed on fetal liver CD3+ cells compared to adult splenic CD3+ cells. Of these, several proteins (IGF-2, IGF-I, IGF binding protein 6, IGF binding protein 1, and IGF binding protein 3) are known to support HSC expansion in vitro. However, only IGF-2 is expressed at high levels in the fetal liver (Figure 2A, compare plots ii and vi). Furthermore, adult splenic CD3+ cells do not express IGF-2 (Figure 2A, compare plots ii and vii).

**Figure 1. Coculture of fetal liver HSCs with fetal liver CD3+ cells increases in vivo repopulating stem cells.** (A) In vivo limiting dilution analysis of the repopulating ability of day-15 fetal liver Lin–Sca-1−Kit+ cells before and after in vitro culture with day-15 fetal liver CD3+ cells. Freshly isolated day-15 fetal liver Lin−Sca-1−Kit+ cells were mixed with the same number of freshly isolated day-15 fetal liver CD3+ cells. Half of the mixed cells were used for direct transplantation (+ and solid line). The other half were cultured for 3 days in a volume of 30 μL followed by transplantation of the entire culture (□ and dashed line). In addition, 100 freshly isolated day-15 fetal liver Lin−Sca-1−Kit+ cells were cultured alone without CD3+ cells for 3 days followed by transplantation (▲). The medium contained fetal calf serum supplemented with SCF, FL, and IL-6. Irradiated CD45.1 congenic mice were injected with 2 × 104 CD45.1 bone marrow cells and the indicated numbers of freshly isolated CD45.2 Lin−Sca-1−Kit+ fetal liver cells or their progeny after culture. Plotted is the percentage of recipient mice containing less than 1% CD45.2 lymphoid (B220+) and myeloid (Gr-1+/Mac-1+) subpopulations in nucleated peripheral blood cells 4 months after transplantation versus the number of initial Lin−Sca-1−Kit+ cells; note that the abscissa is presented as the number of LSK cells initially added to the culture. The curve was anchored by the 0 cells/100 negative mice point. Injection of the culture of 50 or 100 initial Lin−Sca-1−Kit+ cells with CD3+ cells resulted in repopulation of 100% of the mice. A data point of 0% negative mice cannot be plotted on a logarithmic axis; so to be conservative we assume and plot that only 90% of the mice injected with the culture derived from these 50 input LSK cells are positive, or 10% negative. Dotted lines show the determination of the CRU frequency values by the method of maximum likelihood (at 37% negative mice). (B) Details of a typical competitive repopulation by 100 CD45.2 fetal liver Lin−Sca-1−Kit+ cells cultured alone or with fetal liver CD3+ cells (as shown in panel A) 4 months after transplantation into lethally irradiated CD45.1 recipient mice. Data shown are representative FACS plots of peripheral blood mononuclear cells from 1 mouse in each group. To assess the repopulation frequencies of the donor cells, cell isolates were double stained with antibodies against CD45.2 (donor) and CD45.1 (recipient), Thy1.2 (T cells), B220 (B cells), Mac-1 (macrophages), and Gr-1 (granulocytes). As illustrated in the top panels, Lin−Sca-1−Kit+ cells cultured in the absence of CD3+ cells yielded no reconstitution of any of the lineages analyzed.
RT-PCR was performed on cDNAs isolated from FACS-sorted murine day-15 fetal liver Lin−/H11002 FITC and anti-Ter119–(v, xi), and anti-CD3/H11001 supplemented with SCF, FL, and IL-6 as follows: 7 × 10⁵ LSK cells. FACS-sorted fetal liver CD3/H11001 the isotype control (I, vii), anti-CD3–CD4, CD8, Thy1.2, and Ter119 in murine fetal liver and adult spleen cells. Cells freshly isolated from day-15 fetal liver (i-vi) or from adult spleen (vii-xii) were stained with dehydrogenase (GAPDH) was measured as an indicator of the amount of RNA. (C) Fetal liver CD3/H11001 below zero represent no detectable repopulation. *Significantly different from lane 1 and 2 values; P < .05. **Significantly different from lane 3 value; P < .005.

Figure 2. IGF-2 produced by the fetal liver CD3−Ter119− cells is the key factor that supports cultured HSCs. (A) FACS analysis of surface expression of CD3, CD4, CD8, Thy1.2, and Ter119 in murine fetal liver and adult spleen cells. Cells freshly isolated from day-15 fetal liver (i-v) or from adult spleen (vii-xii) were stained with the isotype control (I, vii), anti-CD3–FITC (ii, viii), anti-CD3–FITC and anti-CD4–PE (iii, ix), anti-CD3–FITC and anti-CD8–PE (iv, x), anti-CD3–FITC and anti-Thy1.2–PE (v, xi), and anti-CD3–FITC and anti-Ter119–PE (vi, xii), respectively. (B) RT-PCR analyses of expression of IGF-2 and IGF-2 receptors in fetal liver cells. On the left, RT-PCR was performed on cDNAs isolated from FACS-sorted murine day-15 fetal liver CD3−Ter119− and CD3−Ter119− cells to determine expression of IGF-2. Expression of glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was measured as an indicator of the amount of RNA. (C) Fetal liver CD3−Ter119− cells secrete more IGF-2 than do CD3−Ter119− cells when cocultured with LSK cells. FACS-sorted fetal liver CD3−Ter119− cells, CD3−Ter119− cells, and LSK cells were cultured for 2 days in 100 µl serum containing medium supplemented with SCF, FL, and IL-6 as follows: 7 × 10⁵ CD3−Ter119− cells (i), 7 × 10⁵ CD3−Ter119− cells (ii), 2 × 10⁵ LSK cells (iii), 7 × 10⁵ CD3−Ter119− cells and 2 × 10⁵ LSK cells (iv), or 7 × 10⁵ CD3−Ter119− cells and 2 × 10⁵ LSK cells (v). ELISA was then performed to determine the level of IGF-2 in the medium. Error bars represent SEM. (D) Fetal liver CD3−Ter119− cells supported cultured HSCs by producing IGF-2. Fifty freshly isolated day 15 CD45.2 fetal liver Lin−/H11002 Kit−/H11001 cells and 2 × 10⁵ competitor CD45.1 bone marrow cells, the total culture, derived from 50 initial Lin−/H11002 Kit−/H11001 cells, was coinjected into CD45.1 recipients (n = 4 to 5). Peripheral blood cells from mice undergoing transplantation were analyzed for the presence of CD45.2+ cells in lymphoid and myeloid compartments at 4 months after transplantation. Each ▲ represents the percentage of repopulation from a single recipient mouse. Bars represent the average percentage of repopulation for each group; data points below zero represent no detectable repopulation. *Significantly different from lane 1 and 2 values; P < .05. **Significantly different from lane 3 value; P < .005.

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Listed genes have normalized value more than 2.0 in fetal liver CD3+ cells and are at least 3-fold greater than adult CD3+ and 2-fold greater than fetal liver Gr-1+ normalized values. Total RNA and cRNA was prepared for hybridization to Affymetrix U74Av2 mouse chips according to manufacturer’s instructions, and the analysis was performed as in our recent paper.23
expressed, including IGF-2, Dlk1-like, CD59, and PRNP. Dlk1-like was previously shown to support maintenance of fetal liver HSCs in long-term culture. Because IGF-2 was highly expressed in fetal liver CD3+ and not by adult CD3+ or fetal liver Gr-1+ cells, we hypothesized that this protein, not previously implicated in HSC biology, might mediate expansion of HSCs.

Figure 2B shows that IGF-2 as well as the 3 known IGF-2 receptors—IGF1R, IGF type 2 receptor (IGF2R), and insulin receptor (IR)—are expressed in fetal liver CD3+ cells. In contrast, fetal liver LSK cells express all 3 IGF-2 receptors but do not contain detectable amounts of IGF-2 mRNA.

About half of the day-15 fetal liver CD3+ cells are Ter119+ (Figure 2A, plot 6), which is a marker for terminally differentiating erythroblasts and mature erythrocytes. Figure 2D shows that the CD3+Ter119+ cells showed a much better ability than their CD3+Ter119− counterparts to support the in vivo repopulating activity of LSK cells cocultured for 3 days (Figure 2D, compares lanes 3 and 2, P < .05). Thus, the HSC supportive activity of day-15 fetal liver CD3+ cells lies within the Ter119− subtraction.

Addition of neutralizing anti–IGF-2 antibody to the coculture of LSK and CD3+Ter119− cells completely blocked expansion of HSC activity by the cocultured CD3+Ter119− cells (Figure 2D, compare lanes 4 and 3, P < .005), suggesting that IGF-2 is a key factor produced by fetal liver CD3+Ter119+ cells to support HSCs in culture.

Interestingly, within the fetal liver CD3+ cell population, both CD3+Ter119+ and CD3+Ter119− cells express IGF-2 mRNA (Figure 2B, right) whereas the latter population supports HSC expansion. Thus, we measured the level of IGF-2 secreted by cultured fetal liver CD3+Ter119+ and CD3+Ter119− cells using ELISA (Figure 2C). When cultured alone, both populations secreted moderate levels (25 to 30 ng/mL) of IGF-2 (Figure 2C, lanes 1 and 2). When cocultured with fetal liver LSK cells, which in themselves produce little IGF-2 (Figure 2C, lane 3), CD3+Ter119− cells express significantly more IGF-2 (250 ng/mL) than their Ter119− counterparts (40 ng/mL IGF-2) (Figure 2C, compare lanes 5 and 4). Taken together, these results strongly support the notion that IGF-2, produced by fetal liver CD3+Ter119− cells, is a key factor that supports the growth of HSCs ex vivo and that exposure to HSCs increases IGF-2 production by these supportive cells.

All fetal liver HSCs express receptors for IGF-2

While fetal liver LSK cells express receptors for IGF-2 (Figure 2B), only a minority of these cells actually are HSCs. Existing antibodies against IGF1R and IGF2R are suitable for applications such as Western blotting but not for flow cytometry. Therefore, we generated a fusion protein of prepro–IGF-2 and a human IgG1 Fc fragment (IGF-2–hFc) that could be used in flow cytometry to detect and isolate cells expressing receptors for IGF-2. To this end, a plasmid expressing the IGF-2–hFc was constructed (Figure 3A, top) and transfected into 293T cells. The IGF-2–hFc fusion protein was secreted and could be detected by antibodies against either human IGF-2 or human IgG1 Fc (Figure 3A, bottom panels). The 2 bands observed on the Western blots suggest that some of the IGF-2–hFc has undergone partial protease degradation.

As judged by Western blotting, we estimate that medium from transfected 293T cells contains about 1 μg/mL IGF-2–hFc. Figure 3B shows that, after incubation in this conditioned medium, about 36% of total fetal liver cells stain positive for IGF-2–hFc (Figure 3Biv), as do 76% (67.0% + 9.3%) of Lin− fetal liver cells and 83% (9.3 of 11.2) of Lin−Sca-1+ fetal liver cells (Figure 3Bv). As
HSC activity was retained (Figure 5Aii), but only half of the animals were repopulated by the progeny of 50 LSK cells. However, when 500 ng/mL IGF-2 was added to either medium, significant increases of HSC activities were observed, and all mice undergoing transplantation exhibited chimerism (Figure 5A, compare lanes iii versus i, \( P < 0.05 \); lanes iv versus ii, \( P < 0.005 \)). Impressively, the combination of SCF, TPO, and IGF-2 substantially increased HSC activity, and each of 4 recipient mice was strongly reconstituted by the cultured progeny of only 50 original LSK cells (Figure 5Aiv). This suggests that IGF-2 is capable of strongly stimulating expansion HSCs in culture. Surprisingly, IGF-1, which only binds to IGFIR but not the IGF2R, does not support HSC maintenance or expansion in our culture system (data not shown).

Figure 5B definitely shows that IGF-2 increases the number of fetal liver stem cells. Freshly isolated fetal liver LSK cells were directly transplanted or were cultured for 3 days in serum-containing medium supplemented with SCF, TPO, and IGF-2. The CRU frequency for freshly isolated LSK cells was 1 per 50 (95% confidence interval for mean, 1/40 to 1/157; \( n = 31 \)). After culture in medium containing serum, 500 ng/mL IGF-2, SCF, and TPO, the number of cells was again too few to be counted reliably. However, the number of functional LT-HSCs had increased about 2-fold (50 vs 25; \( P < .05 \), Student t test), as evidenced by the fact that 63% of the mice undergoing transplantation with the progeny of, on average, only 25 initial LSK cells became reconstituted. In other words, the CRU frequency was 1 per 25 input equivalent LSK cells (95% confidence interval for mean, 1/9 to 1/50; \( n = 26 \)). To our

expected, there is no IGF-2 staining of total fetal liver cells incubated in control-conditioned medium (Figure 3Biii). Both Western blot and RT-PCR analyses showed that fetal liver cells unable to bind the IGF-2–hFc fusion protein express little or undetectable IGF1R or IGF2R while those that bind the IGF-2–hFc fusion protein express both (Figure 3C-D). The insulin receptor, which binds IGF-2 weakly, is expressed by both cell populations (Figure 3D). Therefore, IGF-2–hFc specifically binds to IGFIR and IGF2R expressed on the surface of fetal liver cells.

Figure 4 demonstrates that all fetal liver HSCs express receptors for IGF-2. In Figure 4A, total fetal liver cells were sorted according to their ability to bind the IGF-2–hFc fusion protein. Those that did not bind the fusion protein were unable to reconstitute these mice, either at 3 weeks (measuring primarily short-term [ST]–HSCs) or 6 months after transplantation, primarily detecting LT-HSCs. In contrast, total fetal liver cells arbitrarily sorted according to their weak (+) or strong (+++) ability to bind the IGF-2–hFc fusion protein supported both long-term and short-term engraftment (Figure 4A). Similarly, 100 sorted fetal liver Lin–Sca-1+ cells unable to bind the IGF-2–hFc fusion protein were unable to reconstitute irradiated mice at 6 months after transplantation while 50 Lin–Sca-1–IGF-2–hFc+ cells supported significant long-term reconstitution (Figure 4B). Further experiments (data not shown) showed that even 1000 fetal liver Lin–Sca-1–IGF-2–hFc+ cells contained no detectable population. Thus, all fetal liver HSC activity resides in the Lin–Sca-1–IGF-2–hFc+ population, confirming the results of Figure 4A that all long-term repopulating HSCs bind IGF-2.

IGF-2 stimulates ex vivo expansion of purified fetal liver HSCs

When 50 fetal liver LSK cells were cultured in the presence of serum supplemented with SCF, FL, and IL-6 for 3 days, all HSC activity was lost, similar to the results in Figure 1 (Figure 5Ai). After culture in serum supplemented with SCF and TPO, some
knowledge, this is the first report showing the expansion of purified fetal liver HSCs ex vivo.

**IGF-2 stimulates ex vivo expansion of purified adult bone marrow HSCs**

IGF-2 is thought not to be ubiquitously expressed in adult mice, but Figure 6A shows that about 42% of total adult bone marrow cells do bind significant amounts of the IGF-2–hFc fusion protein (Figure 6A, plot 4), as do 41% (39.1% + 2.1%) of Lin<sup>−</sup> bone marrow cells and 47% (2.1 of 4.5) of Lin<sup>−</sup> Sca-1<sup>−</sup> bone marrow cells (Figure 6Av). Total bone marrow cells that did not bind the IGF-2–hFc fusion protein reconstituted mice at 3 weeks but not at 6 months after transplantation. In contrast, total bone marrow cells arbitrarily sorted according to their weak (+) or strong (+++) ability to bind the IGF-2–hFc fusion protein supported both strong short-term and long-term engraftment (Figure 6B). Similarly, 100 sorted bone marrow Lin<sup>−</sup> Sca-1<sup>−</sup> cells unable to bind the IGF-2–hFc fusion protein were unable to reconstitute irradiated mice 6 months after transplantation. In contrast, 50 Lin<sup>−</sup> Sca-1<sup>−</sup> IGF-2–hFc<sup>+</sup> cells supported significant long-term reconstitution (Figure 6C). Thus, like their fetal counterparts, all long-term repopulating bone marrow HSCs bind IGF-2.

Compared with freshly isolated bone marrow SP cells (Figure 6Di), SP cells cultured for 3 days in serum-containing medium with SCF, FL, and IL-6 but without IGF-2 maintained some stem cell activity (Figure 6Dii). Importantly, addition of 1 μg/mL IGF-2 increased the repopulating activity and the stem cell frequency (Figure 6D, compare lanes iii versus i and ii, P < .05). Based on the finding that 5 of the 9 mice injected with 50 freshly isolated SP cells showed significant chimerism (Figure 6Di), we estimate that the HSC frequency in fresh bone marrow is 1 per 62 SP cells. After culture with IGF-2 the frequency increases to more than 1 per 26 input equivalent cultured SP cells, representing at least a 2-fold increase in HSC numbers.

**Discussion**

Here we describe a new population of nonadherent fetal liver Ter119<sup>−</sup>CD3<sup>+</sup> cells that are capable of supporting a 2.5-fold expansion of LT-HSCs in culture and that are different from the other known population of CD3<sup>+</sup> cells, adult T cells. We further showed that fetal liver CD3<sup>+</sup> cells specifically express high levels of IGF-2 and that IGF-2 is the key factor in these cells that supports HSCs in culture. Importantly, all fetal liver and adult bone marrow HSCs express IGF-2 receptors, and culture of day 15 fetal liver LSK cells with IGF-2 increased HSC numbers and activities. IGF-2 also supported ex vivo expansion of adult bone marrow HSCs.

Figure 6. Culture with IGF-2 increases in vivo repopulating activity of adult bone marrow HSCs. (A) About 50% of adult bone marrow Lin<sup>−</sup> Sca-1<sup>−</sup> cells specifically bind IGF-2–hFc. Adult bone marrow cells were incubated at 4°C for 30 minutes with conditioned medium from control transfected 293T cells (ii) or cells transfected with the IGF-2–hFc expression vector (iv,v); the latter medium contained about 1 μg/mL IGF-2–hFc. Cells were then stained with antihuman IgG1-PE, followed by the biotinylated Lin<sup>−</sup> antibody cocktail, streptavidin-APC, and anti-Sca-1<sup>−</sup>-FITC. Total bone marrow cells and Lin<sup>−</sup> cells were then stained, gated (i,ii), and analyzed as in Figure 3B to quantify the binding to IGF-2–hFc and the level of Sca-1. (B) All repopulating HSCs in the total population of bone marrow cells bind IGF-2–hFc. Of the 42.0% of total CD45.2 bone marrow cells that bind the IGF-2–hFc fusion protein (panel Av), the top 30% and the lower 70% were arbitrarily sorted into the + and + fractions, respectively. Similarly, of the 58.0% that cannot bind the IGF-2–hFc fusion protein (as shown in panel A), the top 70% and the lower 30% were arbitrarily sorted into the − and − fractions, respectively. From each group of total bone marrow cells, 10<sup>5</sup> cells were transplanted together with 10<sup>5</sup> CD45.1 competitor cells into lethally irradiated CD45.1 mice (n = 4 to 5). Peripheral blood cells were analyzed for the presence of CD45.2<sup>+</sup> cells in lymphoid and myeloid compartments at 3 weeks and 6 months after transplantation. (C) All repopulating HSCs in the Lin<sup>−</sup> Sca-1<sup>−</sup> subpopulation of bone marrow cells bind IGF-2–hFc. Fifty CD45.2<sup>+</sup> bone marrow Lin<sup>−</sup> Sca-1<sup>−</sup> IGF-2–hFc<sup>+</sup> and 100 Lin<sup>−</sup> Sca-1<sup>−</sup> IGF-2–hFc<sup>−</sup> cells, sorted as depicted in panel Av, were transplanted together with 2 × 10<sup>5</sup> CD45.1 competitor cells into lethally irradiated CD45.1 mice (n = 4 to 5). Peripheral blood cells were analyzed for the presence of CD45.2<sup>+</sup> cells in lymphoid and myeloid compartments at 6 months after transplantation. Error bars represent SEM. (D) Culture of adult bone marrow SP cells with IGF-2 enhances in vivo repopulating stem cell activity. Fifty freshly isolated adult bone marrow CD45.2<sup>+</sup> bone marrow SP cells were transplanted directly (with 2 × 10<sup>5</sup> CD45.1 competitor bone marrow cells per mouse, n = 9) into CD45.1 congenic mice (i). From the same isolation 50 SP cells were cultured 3 days in medium alone (ii) or in medium with 1 μg/mL IGF-2 (iii). Serum-containing medium supplemented with SCF, FL, and IL-6 was used. Together with 2 × 10<sup>5</sup> competitor CD45.1 bone marrow cells, the entire culture derived from these initial 50 SP cells was coinjected into CD45.1 recipients (n = 8 to 9). Peripheral blood cells from mice undergoing transplantation were analyzed for the presence of CD45.2<sup>+</sup> cells in lymphoid and myeloid compartments at 3.5 months after transplantation. *Significantly different from panel i and ii values; P < .05.
Thus, fetal liver CD3+ cells are a novel population that is capable of supporting HSC expansion, and IGF-2, produced by these cells, is a growth factor for fetal liver and adult bone marrow HSCs.

**Fetal liver CD3+ cells represent a new type of hematopoietic stromal cells**

In vivo, stromal cells form a complex microenvironment for HSCs that controls their multiple fates, including apoptosis, migration, and the cell divisions that lead to formation either of daughter HSCs or of lineage-committed progenitors that are capable of limited proliferation. Different types of stromal and other cell types can have either positive or negative effects on fates of HSCs. Certain differentiated hematopoietic cells negatively regulate the ability of transplanted HSCs to repopulate the hematopoietic system. Consistent with these findings, in a preliminary study we observed that fetal liver B220+, Gr-1+, and Ter119+ cells did not support maintenance of fetal liver HSCs in culture while CD3+Ter119− cells did. Thus, differentiated hematopoietic cells are similar to stromal cells in that they provide both positive and negative signals to cocultured HSCs.

Fetal liver CD3+Ter119− cells are mainly erythroid precursors (C.C.Z., M. Socolovsky, H.F.L., unpublished observations, April 2003), while the nature of the CD3+Ter119− population is unknown. Although both CD3+ fractions express IGF-2, only CD3+Ter119− cells are capable of supporting HSC activity in culture. Because both CD3+Ter119− and CD3+Ter119+ populations express IGF-2, it is likely that CD3+Ter119− cells also produce other factor(s) that facilitate IGF-2's function in supporting HSC activity. Alternatively, CD3+Ter119− but not CD3+Ter119− cells might physically interact with HSCs; this hypothesis is supported by our observation that the level of IGF-2 secreted by CD3+Ter119− cells increases when cultured in the presence of HSCs. In addition, fetal liver CD3+Ter119− cells might express negatively acting factors that counteract the positive effects of IGF-2.

Fetal liver CD3+Ter119− cells are different from a CD3+ “facilitating” cell population in adult bone marrow that enhances allografts. Fetal liver CD3+Ter119− cells do not enhance engraftment when freshly cotransplanted with donor cells (data not shown). They lack expression of the classical CD8 and Thy-1 T-cell markers while facilitating cells are CD8+Thy-1+. Furthermore, the function of the facilitating cells was mediated by the CD3 T-cell receptor (TCR) β-FcP33 complex while HSC supportive activity of fetal liver CD3+ cells is mediated by IGF-2.

**IGF-2 is the principal growth factor produced by fetal liver CD3+ cells that supports expansion of HSCs**

Normally maternally imprinted, IGF-2 has been suggested to play important roles in embryonic but not adult growth and development. Targeted disruption of IGF-2 leads to significant fetal growth retardation, partially as a result of decreased placenta growth. Although no role in HSC biology was previously identified, IGF-2 has been used to establish pluripotential cell lines and maintain undifferentiated stem cells from the inner cell mass in culture. IGF-2 stimulates growth and/or differentiation and inhibits apoptosis of a number of cell types including hematopoietic progenitors. While stimulating the growth of human bone marrow CD34+ cells, IGF-2 also increases the formation of myeloid and erythroid progenitors such as granulocyte macrophage colony-forming units (CFU-GMs), erythroid burst-forming units (BFU-Es), and erythroid colony-forming units (CFU-Es) and regulates T-cell development and stimulates T-cell proliferation. However, this is the first report showing that IGF-2 is a growth factor for HSCs.

Different IGF-2 receptors have either a positive or a negative effect on cell proliferation. Two receptor protein tyrosine kinases, IGF1R and IR, mediate the mitogenic activities of IGF-2, while IGF2R mediates degradation of IGF-2. Lack of IGF1R results in fetal growth retardation, and IGF1R-deficient mice invariably die within minutes of birth. In contrast, mice genetically deficient in the negatively acting IGF2R show increased serum and tissue levels of IGF-2 that result in cell overgrowth, developmental abnormalities, and prenatal death.

Multiple IGF-2 receptors could explain the fact that we need to add 500 ng/mL (67 nM) IGF-2 to support HSC expansion during culture of LSK cells; preliminary experiments (not shown) indicated that neither 25 ng/mL nor 50 ng/mL IGF-2 support cultured HSCs when combined with SCF, FL, and IL-6. Given the low nanomolar values of the dissociation constants for binding of IGF-2 to both the IGF1R and IGF2R, it is possible that, at low concentrations, the balance of binding of IGF-2 to its 3 types of receptors triggers the HSC differentiation; hypothetically, only higher concentrations of hormone could effectively prevent apoptosis and promote self-renewal. This is consistent with the “threshold” model positing that a low concentration of a growth factor stimulates stem cell differentiation but that high levels of the factor lead to a net expansion of stem cells.

The situation may be different when fetal liver CD3+Ter119− cells are cocultured with HSCs. Because cells tend to closely interact in the U-bottomed wells we used, it is possible that CD3+Ter119− cells physically contact HSCs and thus that the local concentration of IGF-2 around an HSC is much higher than in the rest of the medium. On the other hand, IGF2R+ cells might degrade IGF-2, and this would mandate a high initial concentration of hormone to support expansion of HSCs over a 3-day culture period.

Both SCF and TPO are required to maintain normal HSC levels, mainly by preventing their apoptosis. IGFs prevent apoptosis and trigger proliferation of different types of cells, principally through both the mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI-3K) pathways. Here we showed that the combination of IGF-2 with SCF and TPO supports a more than 2-fold increase in HSC numbers. Most likely, when combined with SCF and TPO, IGF-2 is both antiapoptotic and mitogenic for HSCs. We are trying to determine whether IGF-2 affects apoptosis of HSCs or whether it acts by accelerating the rate at which HSCs enter the cell cycle. IGF-2 may also increase the proportion of the daughter cells that become HSCs.

In adult rodents, IGF-2 is reportedly not expressed and its functions are suggested to be replaced by IGF-1. However, we found that some non-HSC and stromal bone marrow cells express high levels of IGF-2 (C.C.Z., B. Luo, H.F.L., unpublished observations, July 2003). Therefore, IGF-2 likely is functionally important in the adult bone marrow to maintain homeostasis of adult stem cells, consistent with our observations that all adult bone marrow HSCs express receptors for IGF-2 and that IGF-2 supports expansion of bone marrow HSCs in culture.

In summary, we identified IGF-2 as a novel stem cell growth factor and for the first time achieved a 2- to 3-fold net expansion of fetal liver HSCs in the presence of IGF-2. Although rodent IGF-2 is thought to play important roles only in fetal development, we showed that IGF-2 also stimulated the ex vivo expansion of adult bone marrow HSCs. Because IGF-2 has pleiotropic effects, the expression of receptors for IGF-2 may define pan–stem cell properties, and IGF-2 may have the potential to act on a broader spectrum of stem cells, both ex vivo and in vivo.
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References


Insulin-like growth factor 2 expressed in a novel fetal liver cell population is a growth factor for hematopoietic stem cells

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