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Insulin-like growth factor 2 modulates murine hematopoietic stem cell maintenance through upregulation of p57

Dolly D. Thomas[a,b], Andreia Gianotti Sommer[a,b], Alejandro B. Balazs[c], Isabel Beerman[d], George J. Murphy[b], Derrick Rossi[d], and Gustavo Mostoslavsky[a,b]

[a]*Section of Gastroenterology, Department of Medicine, Boston University School of Medicine, Boston, MA;* [b]*Center for Regenerative Medicine (CReM), Boston University and Boston Medical Center, Boston, MA;* [c]*Ragon Institute of MGH, MIT and Harvard, Cambridge, MA;* [d]*Department of Stem Cell and Regenerative Biology, Harvard Medical School, Boston, MA*

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Hematopoietic stem cells (HSC) rely on a highly regulated molecular network to balance self-renewal and lineage specification to sustain life-long hematopoiesis. Despite a plethora of studies aimed at identifying molecules governing HSC fate, our current knowledge of the genes responsible is limited. We have found insulin-like growth factor 2 (IGF2) to be expressed predominantly within long-term HSCs. This study examines IGF2 expression patterns and the effects of the gene in HSCs. Through the overexpression and knockdown of IGF2 within purified HSCs, we report that IGF2 expression increases HSC-derived multilineage colonies in vitro and enhances hematopoietic contribution in vivo on competitive bone marrow transplantation. The effects of IGF2 are mediated by direct upregulation of the CDKi p57, exclusively within long-term HSCs, via activation of the PI3K-Akt pathway. Increased expression of p57 resulted in a concomitant increase in HSCs in the G0/G1 stage of the cell cycle. Analysis of genomic DNA methylation revealed that HSCs exhibited a hypomethylated state within the promoter region of the CDKN1C (p57) gene, providing a potential mechanism for the exclusive effects of IGF2 within HSCs. Our studies indicate a novel role for IGF2 in regulating HSC cell cycle and illustrate potential novel therapeutic targets for hematologic diseases.

Lifelong blood cell production is contingent on the maintenance and orchestrated functions of the hematopoietic stem cell (HSC) pool. Because of the limited size of the HSC population, preservation of adult HSCs is critical and is reliant on a balance between cell cycle progression and quiescence. The majority of HSCs are retained in the G0 phase of the cell cycle and progress through the cell cycle slowly to prevent HSC exhaustion and offer protection against stress-induced damage [1–7]. Quiescence can therefore be regarded as a protective means to ensure HSC functionality and longevity.

To maintain hematologic homeostasis, HSCs are then required to readily exit quiescence and differentiate in a balanced manner. The HSC cell cycle is tightly regulated by several key cell cycle regulators and is largely modulated in response to the needs of the blood system [4]. The stage-specific expression and formation of cyclin–cyclin-dependent kinase (CDK) complexes allows for precise progression throughout the cycle. Cyclin-dependent kinase inhibitors (CDKi) counteract this function and limit the transition between individual phases. The balance of cyclin–CDK complexes and CDKi regulates stem cell fate, whereby CDKi are found in excess within quiescent HSCs.

Identifying the molecules and pathways involved in regulating these cell cycle processes is key to developing novel and improved approaches related to HSC manipulation for bone marrow transplantation and pivotal for expanding our understanding of hematologic disorders and malignancies. Thus far, a number of growth factor pathways have been implicated in modulating HSCs. These include Wnt [8], thrombopoietin (TPO) [9], angiopoietin-1 [10], and transforming growth factor β (TGFβ) [11,12].

Offprint requests to: Gustavo Mostoslavsky, Center for Regenerative Medicine (CReM), Boston University School of Medicine, 670 Albany Street, Suite 209, Boston, MA 02118; E-mail: gmostosl@bu.edu

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Several of these directly affect the expression of cell cycle regulators, particularly the cyclin-dependent kinase inhibitor p57. To identify additional regulators of HSC function, we analyzed publicly available microarray data sets for long-term and short-term HSCs and a third mixed population containing progenitors and mature blood cells. On the basis of our analysis, we identified insulin-like growth factor 2 (IGF2) to be upregulated in HSCs, and therefore, it became the focus of our studies.

Insulin-like growth factor 2 is a key modulator of many developmental processes and is widely expressed in a number of tissues throughout development and into adulthood [13–16]. Its activities are regulated by the expression of a number of receptors and IGF-binding proteins (IGFBPs). IGF2, produced by fetal liver stromal cells and cells of the aorta–gonad–mesonephros (AGM), enhances the number and function of long-term HSCs [17–19]. Human total bone marrow cells exhibit bi-allelic expression of IGF2 beyond development, whereas more mature cells of the peripheral blood exhibit mono-allelic expression [20]. These studies suggest that IGF2 may function as an HSC growth factor, though its exact role remains unclear. Here we report that IGF2 within adult HSCs preserves long-term HSCs through upregulation of the CDKi p57 via activation of the PI3K-Akt pathway. This allows for maintenance of HSC reserves in G0/G1. Importantly, we also report hypomethylation of the p57 gene promoter within HSCs. Our results highlight a novel and specific role for IGF2 in HSC function.

Methods

Mice

C57BL/6 (B6-Ly5.2) and B6.SJL-Ptprca Pep3b/BoyJ (CD45.1) mice were purchased from Jackson Laboratory (Bar Harbor, ME). All animals were maintained in the Laboratory Animal Science Center at the Boston University School of Medicine. Animal care and protocols were carried out in accordance with all approved IACUC protocols and procedures.

Purification of mouse HSCs by Hoechst staining

Mouse HSCs were purified from total bone marrow by Hoechst "side population" (SP) staining. Harvested femurs and tibias were homogenized using a mortar and pestle to release bone marrow into suspension. Cells were filtered through a 70-μm cell strainer and resuspended at a concentration of 4.5×10^6 cells/mL in Hanks' buffered saline solution (supplemented with 1% Hepes, 2% fetal bovine serum, and 1% penicillin–streptomycin) containing 8.8 μg/mL Hoechst 33342. Cells were incubated for 90 min at 37°C.

For depletion of red blood cells, stained cells were transferred onto a low-density gradient of Ficoll-Paque Plus and centrifuged at 2,000 rpm for 20 min at 20°C. Resulting cells were washed and resuspended in a solution containing 1 ng/mL propidium iodide and filtered through a 40-μm cell strainer before sorting using a Beckman-Coulter MoFlo or BDFACSAria cell sorter. Hoechst was excited using a 350-nm emission UV laser, and its signal was collected with a 405/30 filter (Hoechst blue) and a 670/40 filter (Hoechst Red). HSCs for culture were sorted directly to 96-well round-bottom plates.

Cloning of IGF2 lentiviral vectors

Insulin-like growth factor 2 was amplified from a pmigf2-3 vector (ATCC) by polymerase chain reaction (PCR). Resulting IGF2 cDNA was cloned into lentiviral vectors containing a constitutive elongation factor 1α (EF1α promoter), internal ribosome entry site (IRES) sequence, and ZsGreen reporter [21]. A short hairpin RNA (shRNA) was designed to knockdown IGF2 by annealing two polyacrylamide gel electrophoresis (PAGE)-purified 5′-phosphorylated primers. To anneal primers, equal volumes of 100 μmol/L were mixed and placed in boiling water. Primers were left to anneal for several hours. Double-stranded DNA inserts were cloned to a dual-promoter lentiviral construct, containing an EF1α promoter to drive expression of an enhanced green fluorescent protein (eGFP) reporter and an mU6 promoter to drive shRNA expression.

Lentiviral transduction of HSCs

All lentiviruses were prepared as previously described [21,22]. For overnight infection HSCs were sorted directly to StemSpan medium supplemented with 10 ng/mL stem cell factor (SCF), 100 ng/mL TPO, and 5 μg/mL polybrene. Lentiviral supernatants were added directly to HSC cultures and incubated overnight at 37°C. For spinfection, purified HSCs were centrifuged with lentiviral preparations at 800 RCF for 2 hours at 32°C prior to overnight incubation.

Flow cytometry analysis and cell sorting

To enrich for total HSCs by LSK (Lineage$^-$ Sca1$^+$cKit$^+$) staining, bone marrow cells were harvested and depleted of red blood cells. Resulting cells were resuspended in Flow Cytometry buffer (Ebiosciences) and stained with APC-conjugated Lineage antibody cocktail, fluorescein isothiocyanate (FITC)-conjugated Sca1, and phycoerythrin (PE)-conjugated cKit (CD117) antibodies (BD Pharmingen) for 20 min on ice. Stained cells were washed and resuspended in a solution containing 1 ng/mL propidium Iodide (Invitrogen) for dead cell exclusion and filtered. LSK were analyzed using a BD LSRII flow cytometer or sorted using a BD FACSARIA cell sorter.

To analyze chimerism in transplanted mice, peripheral blood was obtained by retro- orbital bleedings. All blood was collected into a 100 mmol/L solution of EDTA. For red cell lysis, samples were incubated in red blood cell lysing buffer (Sigma) for 30 min on ice and spun down. White blood cell pellets were resuspended in FACS staining buffer (Ebiosciences) and stained with PE-conjugated CD45.1 and allophycocyanin (APC)-conjugated CD45.2 (Ebiosciences) for 20 min on ice. Stained cells were washed and resuspended in a solution containing 1 ng/mL propidium Iodide (Invitrogen) to exclude dead cells, and analyzed on a BD LSRII flow cytometer.

For precise analysis and purification of long- and short-term HSC populations and hematopoietic progenitor populations, we used the SLAM family markers [23], with the following antibodies: PerCP-eFluor 710-conjugated CD150 (eBioscience), APC-Cy7-conjugated CD48 (eBioscience), APC-conjugated Lineage antibody cocktail, FITC-conjugated Sca1, and PE-conjugated cKit (CD117) antibodies (BD Pharmingen). Amine Aqua (Invitrogen)

was used to exclude dead cells. Red cell-depleted bone marrow cells were stained for 20 min on ice. Hematopoietic progenitors were defined as LSK CD48$^+$; short-term HSCs were gated as LSK CD150$^-$CD48$^-$; long-term HSCs were gated as LSK CD150$^+$CD48$^-$. For RNA, all populations were sorted directly to microcentrifuge tubes containing lysis buffer (Buffer RLT, Qiagen), using a BDFACSARIA cell sorter.

Colony-forming unit assay

Transduced HSCs were transferred directly to 3 mL of Methocult M3434 methylcellulose medium (StemCell Technologies). Medium containing cells was split to (2) 35-mm plates per the manufacturer's instructions. Cells were cultured for 7 days to promote colony formation. Resulting colonies were characterized and scored blindly by two independent investigators.

Quantitative real-time PCR

RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. First-strand cDNA was generated using either a QuantiTect Whole Transcriptome Kit (Qiagen) or Superscript III First Strand Kit (Invitrogen), both according to the manufacturers' protocols. Quantitative real-time PCR was performed on an Applied Biosystems Step One Plus Real-Time machine using TaqMan Universal PCR Master Mix (Applied Biosystems). Relative expression of the selected genes was normalized to that of β-actin (Mm01205647_g1) for each sample. The following TaqMan primers were used for gene expression studies: IGF2 (Mm00439564_m1), p57 (Mm01272135_g1), p27 (Mm00438167_g1), p21 (Mm00432448_m1), Cyclin D1 (Mm00432359_m1), Cyclin D2 (Mm00438070_m1), Cyclin D3 (Mm01612362_m1), Cyclin E1 (Mm00432367_m1), Cyclin E2 (Mm00438077_m1), CDK2 (Mm00443947_m1), CDK4 (Mm00726334_s1), CDK6 (Mm01311342_m1).

Cell cycle analysis

For DAPI staining, bone marrow cells were resuspended in 1 mL of phosphate-buffered saline (PBS), containing 1 ng/mL DAPI plus APC-conjugated Lineage antibody cocktail (BD Pharmingen), and stained for 25 min at room temperature. For Hoechst/Pyronin Y staining, cells were resuspended in 1 mL of PBS, containing 20 ng/mL Hoechst and 1 ng/mL of Pyronin Y, and stained at 37°C for 45 min. Cells were analyzed using a BD LSRII flow cytometer. Cells in G0 were characterized as Hoechst$^{-/low}$Pyronin Y$^{-/low}$, in G1 cells as Hoechst$^{-/low}$Pyronin Y$^+$, and in G2/S/M cells as Hoechst$^+$Pyronin Y$^+$.

Competitive repopulation transplants

Bone marrow transplantations were performed as described [21]. Briefly, 8-week-old C57BL/6 CD45.2 mice were lethally irradiated with two doses of 6.5 Gy 3 hours apart. Donor HSCs (purified by Hoechst staining) from B6.SJL-Ptprca Pep3b/BoyJ CD45.1 mice were transduced overnight with either IGF2 or mock lentiviral supernatants. Host mice were transplanted with 500 transduced HSCs via retro-orbital injection in competition with 5×10^5 total bone marrow cells isolated from C57BL/6 CD45.2 mice. For secondary transplants, total bone marrow cells (1×10^6) were obtained from recipient mice at 35 weeks post-transplantation and transplanted into secondary recipients. Peripheral blood was collected at 4-week intervals to evaluate levels of donor and recipient hematopoietic reconstitution. Donor CRU (competitive repopulating unit) values were calculated using the following equation: (competitor RU) × ([% donor contribution]/[100 − %donor contribution]). Competitor RUs are defined as 1 for every 10^5 competitor bone marrow cells transplanted.

Methylation analysis

Analysis of CpG methylation of the CDKN1c genomic region was performed as described [24].

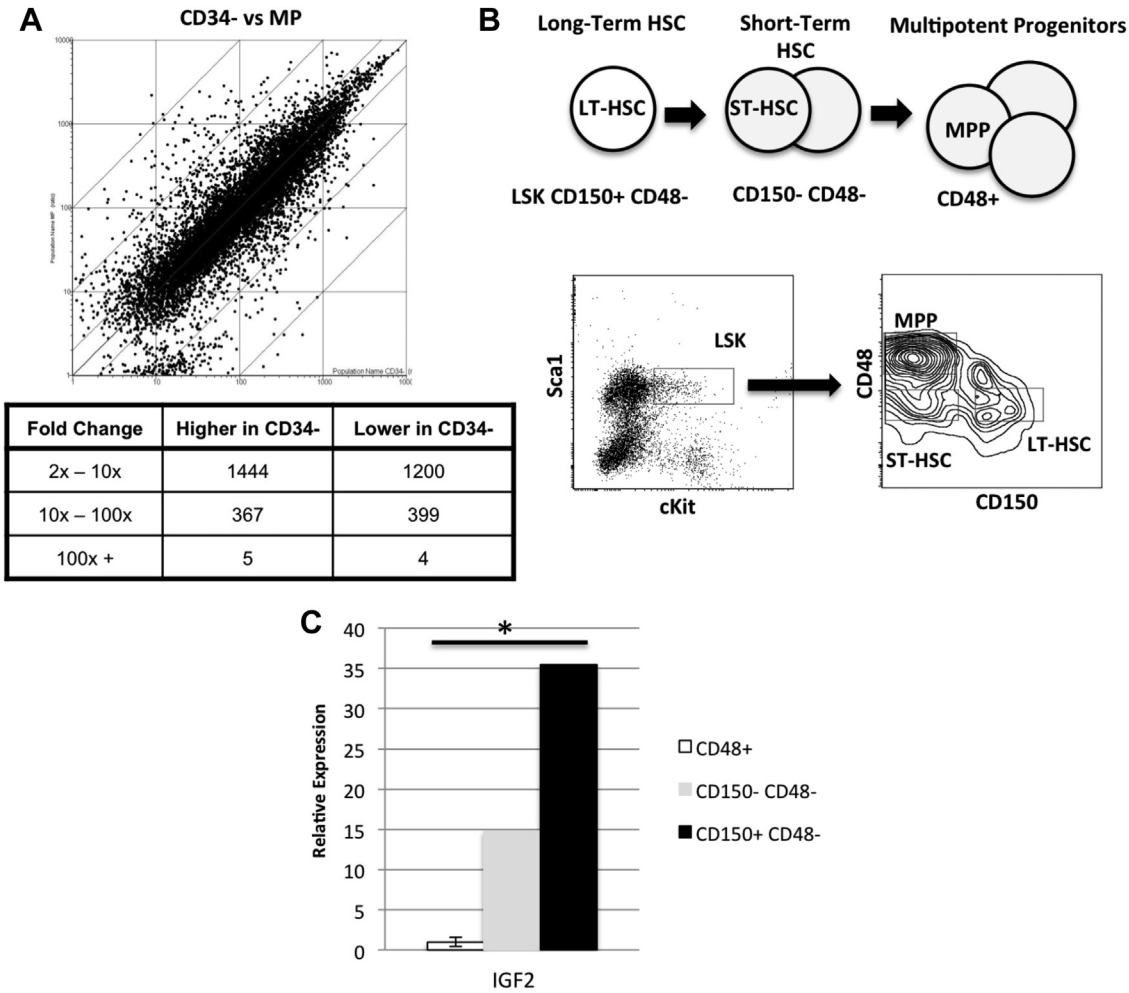
Results

IGF2 is preferentially expressed in long-term HSCs relative to ST-HSCs and progenitor populations

We hypothesized that genes responsible for HSC self-renewal would be preferentially expressed in long-term (LT)-HSCs relative to other early hematopoietic progenitors. To identify candidates, we re-analyzed microarray data from Hoechst$^-$ LSK CD34$^-$ LT-HSCs, Hoechst$^-$ LSK CD34$^+$ short-term (ST)-HSCs, and Hoechst$^+$CD34$^+$ progenitors [25,26]. Comparison of HSC and non-HSC transcriptomes revealed hundreds of HSC-enriched genes, including established HSC markers such as endothelial protein C receptor (EPCR) [27], endomucin [28], and CD105 [29] (Fig. 1A). Five genes exhibited a 100+ fold enrichment in HSCs versus non-HSCs, including IGF2, which was also fivefold enriched in LT-HSCs compared with ST-HSC. We independently validated preferential expression of IGF2 in LT-HSCs by real-time PCR in LT-HSCs, ST-HSCs, and non-HSC populations, purified using SLAM family receptors CD150 and CD48 (Fig. 1B, C) [23].

IGF2 enhances the multipotent capacity of long-term HSCs in vitro and in vivo

To test the effects of IGF2 expression within purified HSCs, we constructed a lentiviral vector to constitutively overexpress IGF2 cDNA. Overexpression vectors contained a ZsGreen reporter to monitor efficiency of transduction and to track the presence of transduced cells in functional assays. The same construct expressing a red fluorescent reporter (instead of IGF2) was used as a mock control. Purified HSCs (Hoechst$^-$) were transduced with an IGF2 or control lentivirus and plated to methylcellulose cultures to evaluate colony-forming potential. Transduction efficiencies ranged from 35%–45%, and overexpression of IGF2 was confirmed by qPCR and enzyme-linked immunosorbent assay (ELISA) (Supplementary Figure E1 [online only, available at www.exphem.org] and Figure 5A). HSCs overexpressing IGF2 (IGF2-HSCs) yielded an increased percentage of multipotent CFU-GEMM colonies compared with uninfected and mock infected controls (Fig. 2A). Conversely, knockdown of IGF2 resulted in a decrease in multilineage colonies, although this did not reach statistical significance, likely because of incomplete knockdown of IGF2 (Fig. 2B). Cultures were further analyzed by FACS for



Fold Change	Higher in CD34-	Lower in CD34-
2x – 10x	1444	1200
10x – 100x	367	399
100x +	5	4

Figure 1. Insulin-like growth factor 2 is differentially expressed in LT-HSCs compared with ST-HSCs and hematopoietic progenitors. (**A**) Graphical comparison of gene expression profiles for LT-HSCs (Hoechst$^-$ LSK CD34$^-$) and non-HSCs (Hoechst$^+$ MP). The table summarizes the number of genes up- or downregulated within long-term HSCs and the fold change in expression. (**B**) Sorting strategy for purification of LT-HSCs (LSK CD150$^+$CD48$^-$), ST-HSCs (LSK CD150$^-$CD48$^-$), and multipotent progenitors (MPPs: LSK CD48$^+$) based on the expression of SLAM family receptors, CD150 and CD48. (**C**) Quantitative PCR analysis of IGF2 expression within purified LT-HSCs and ST-HSCs compared with MPPs. *$p < 0.005$.

myeloid, lymphoid, and erythroid differentiation markers. There was no effect on lineage specification in response to IGF2 in vitro.

To assess the effects of IGF2 on HSC self-renewal and multipotency in vivo, we carried out competitive repopulation transplants (Fig. 3A). Short- and long-term reconstitution abilities of IGF2-HSCs (Hoechst-) and mock HSCs (Hoechst$^-$) were evaluated by analyzing donor and recipient peripheral blood contribution over 1 year. Consistent with our in vitro studies, IGF2-HSC transplanted mice had higher levels of donor-derived chimerism (Fig. 3B) and increased repopulating capacity (1.6-fold at 5 weeks, 3.8-fold at 8 weeks, 25-fold at 24 weeks) (Fig. 3C) compared with mock control cells, at short- and long-term time points (see Methods for CRU calculations). Contribution from IGF2- HSCs increased over time, indicating a sustained long-term effect of IGF2 in HSC function. Multilineage analysis revealed no effects on myeloid and lymphoid differentiation in response to IGF2 in vivo (Supplementary Figure E2, online only, available at www.exphem.org). Increased donor contribution can be attributed to a selective effect on HSC self-renewal rather than effects on the differentiation of downstream progeny or lineage skewing.

To further confirm the effect of IGF2 on long-term HSC self-renewal and repopulation, secondary bone marrow transplantations were carried out (Fig. 3D). One million total bone marrow cells were isolated from IGF2-HSC transplanted primary mice and subsequently transplanted into lethally irradiated secondary recipients. IGF2 allows for the long-term repopulation of hematopoietic compartments within secondary recipients, and this contribution increased with time (2.62 ± 0.59% at 8 weeks compared with 10.55 ± 6.85% at 24 weeks),

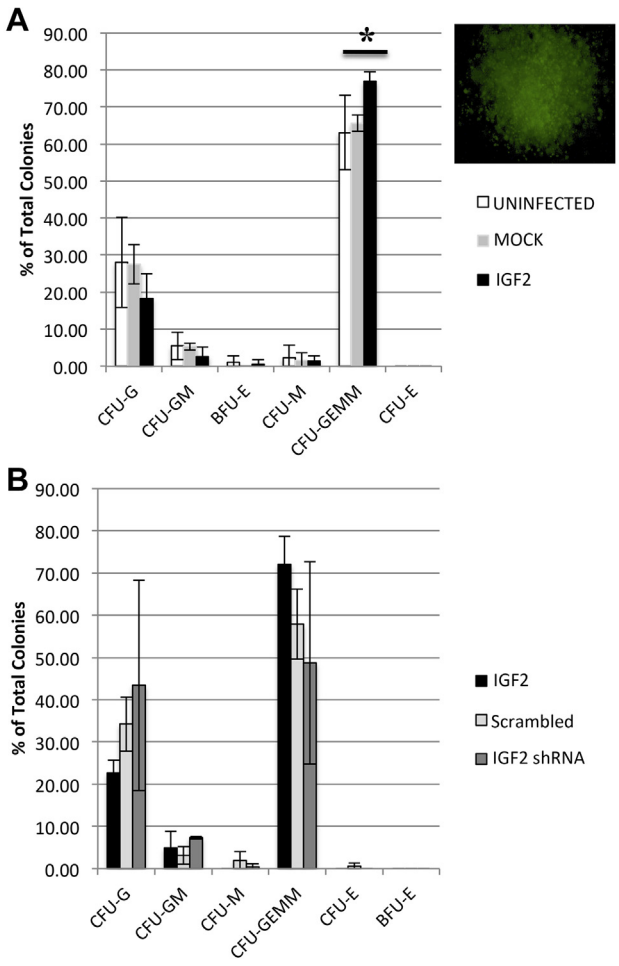


Figure 2. Overexpression of IGF2 within purified HSCs in vitro results in an increased percentage of multipotent GEMM colonies. (**A**) Purified HSCs were transduced with mock control (DsRed-IRES-ZsGreen) or IGF2 (IGF2-IRES-ZsGreen) lentiviruses. Transduced HSCs were plated to methylcellulose cultures for up to 7 days. Resulting colonies were characterized and quantified based on morphology. Representative image of a ZsGreen^{+} colony resulting from transduced HSCs. *$p < 0.01$. (**B**) The opposite results were observed when purified HSCs were transduced with lentiviruses expressing an IGF2 shRNA, compared with scrambled control or IGF2. Data are representative of three independent experiments ($n = 1$).

similar to what was observed in primary bone marrow transplants. Because of declining levels of contribution within our mock primary transplant group, ZsGreen^{+} cells were difficult to identify by FACS in the marrow of these mice. Therefore, we did not pursue secondary transplants for the mock control.

IGF2 increases p57 expression via activation of the PI3K-Akt pathway

To investigate a molecular mechanism for the observed effect of IGF2 within HSCs, we focused on the analysis of key cell cycle regulators, particularly those involved in G0/early G1. IGF2 has been reported to have direct effects on the expression of p57 within primary mouse embryonic fibroblasts [30]. To identify changes in the expression of CDK, cyclin, and CIP/KIP CDKi family members on IGF2 overexpression, LSK purified HSCs were transduced with IGF2 and incubated in minimal medium for 7 days. ZsGreen^{+} cells were purified to specifically analyze only IGF2 overexpressing cells. IGF2 overexpression increased mRNA levels of CIP/KIP CDKi family members and several early G1 cyclins, including Cyclin D3, with no effect on CDK expression (Fig. 4A). The most robust increase observed among CIP/KIP CDKi family members was that of p57 (greater than sixfold increase) (Fig. 4A).

To validate that the observed increase in p57 and Cyclin D3 was a specific effect of IGF2, we restored normal levels of IGF2 in IGF2-HSC by shRNA knockdown. For this purpose, purified HSCs (LSK) were initially transduced with an IGF2 overexpression vector containing a dTomato reporter and subsequently transduced with a second lentiviral vector encoding either an IGF2 shRNA or scrambled control, both containing a ZsGreen reporter. dTomato ZsGreen double-positive cells were purified and analyzed by real-time PCR for changes in CIP/KIP CDKi family members and Cyclin D3 expression. Knockdown of IGF2 resulted in a significant decrease in p57 expression (Fig. 4B), confirming an IGF2-specific effect on the expression of p57 within HSCs. Expression of p21 and Cyclin D3 was unchanged possibly because of inefficient knockdown of IGF2, or changes in the expression of these genes are a consequence of increased self-renewal. Expression of Cyclin D3 and p21 is upregulated within mobilized bone marrow HSCs undergoing self-renewing proliferation [31].

We also evaluated the effect of IGF2 on p57 in HSCs in vivo. IGF2-HSCs and mock control-HSCs were purified (based on Hoechst staining and ZsGreen expression) from the bone marrow of transplanted recipient mice, 6 months posttransplantation. Similar to what we observed in vitro, in vivo IGF2 overexpression resulted in an increase in p57 expression compared with mock-transduced HSCs (Fig. 5A, B). Interestingly, when we analyzed cells within the main population (MP; containing hematopoietic progenitors and mature blood cell types), overexpression of IGF2 did not result in a corresponding increase in p57, but rather in an increase in p27 (Fig. 5C; Supplementary Figure E3, online only, available at www.exphem.org). p27 has been described to be the most abundant CIP/KIP CDKi family member within progenitor populations and to have a greater impact on hematopoietic progenitors [31–33]. These results indicate that the observed IGF2-mediated increase in p57 expression is HSC specific.

Insulin-like growth factor signaling results in activation of the PI3K-Akt pathway to drive expression of a number of genes [34,35]. Within mammary epithelial cells, IGF1-mediated activation of the PI3K-Akt pathway resulted in upregulation of p57 [36]. Therefore we tested whether

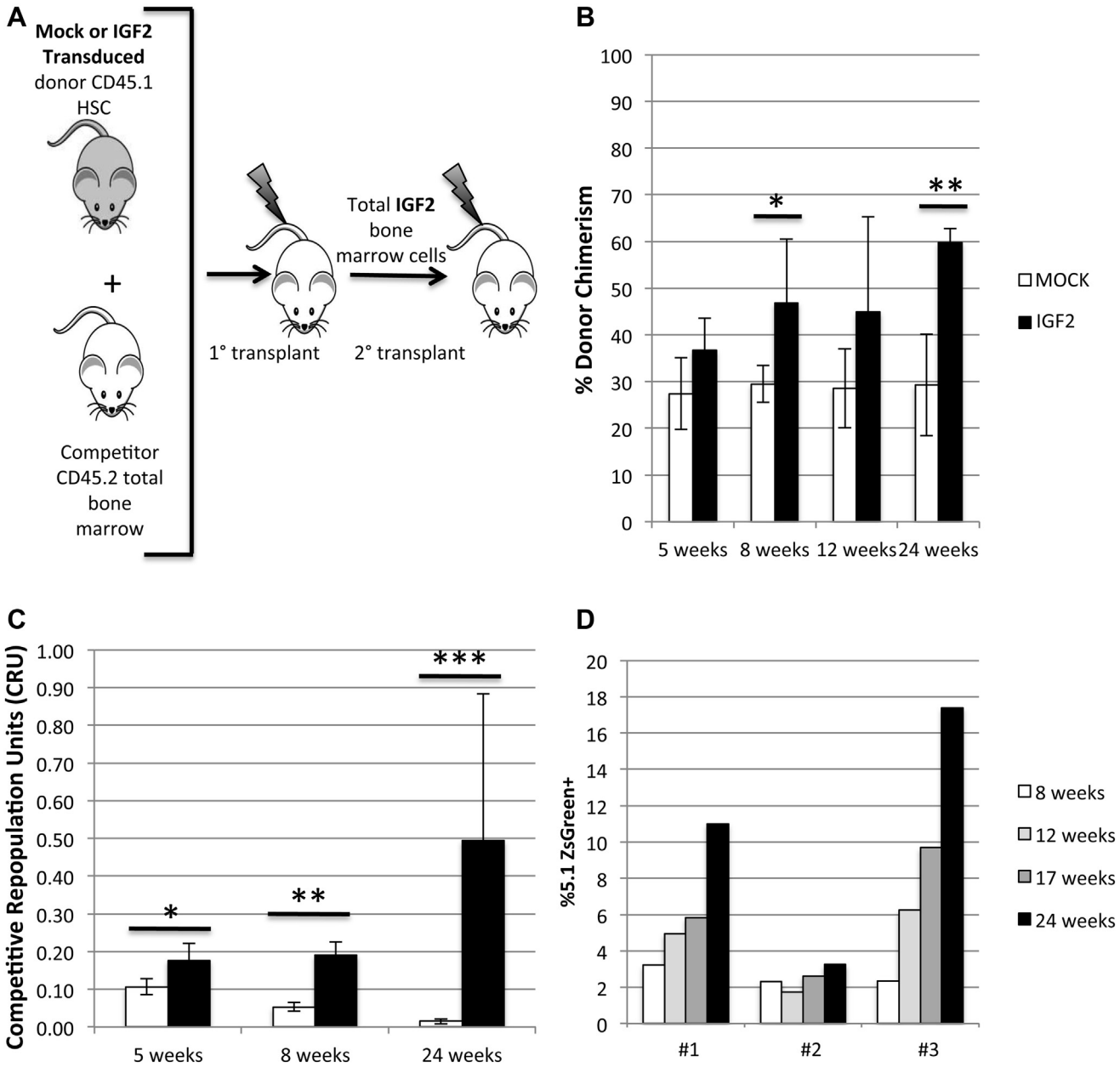


Figure 3. Overexpression of IGF2 within purified HSCs results in increased donor contribution in both primary and secondary bone marrow transplantations (BMTs). (**A**) Experimental scheme for primary and secondary BMTs. (**B**) Mock (*white columns*) and IGF2 (*black columns*) transduced HSCs were transplanted to lethally irradiated recipients. To evaluate levels of donor chimerism, peripheral blood was analyzed by FACS at different time points posttransplantation. $N = 3$–5 transplanted mice per group, data representative of 3 independent transplants. *$p < 0.1$. **$p < 0.03$. (**C**) To quantify HSC repopulating function, percentages of ZsGreen^{+} donor contribution were used to calculate competitive repopulation units (CRU). Graph depicts average CRU values for mock and IGF2 transplanted groups. *$p < 0.07$. **$p < 0.005$. ***$p < 0.01$. (**D**) Contribution from IGF2 transduced HSCs on secondary bone marrow transplantation. Data are for three individual recipient animals at different time points posttransplantation.

activation of PI3K-Akt was the mechanism for IGF-mediated upregulation of p57 expression within HSCs. To do this, we treated IGF2-HSCs with the PI3K inhibitor LY294002. Indeed, LY294002 completely ablated the IGF2-mediated increase in p57 expression (Fig. 6), suggesting that activation of the PI3K-Akt pathway is required for IGF2-mediated upregulation of p57 in HSCs.

IGF2 regulates the HSC cell cycle

p57 is the most abundant CDKi within LT-HSCs and is an important regulator of quiescence [37]. p57-deficient HSCs exhibited impaired reconstitution capacity on bone marrow transplantation and decreased quiescence [37]. Growth factors, such as TGFβ, have been reported to induce cell cycle arrest in human hematopoietic cells through upregulation of

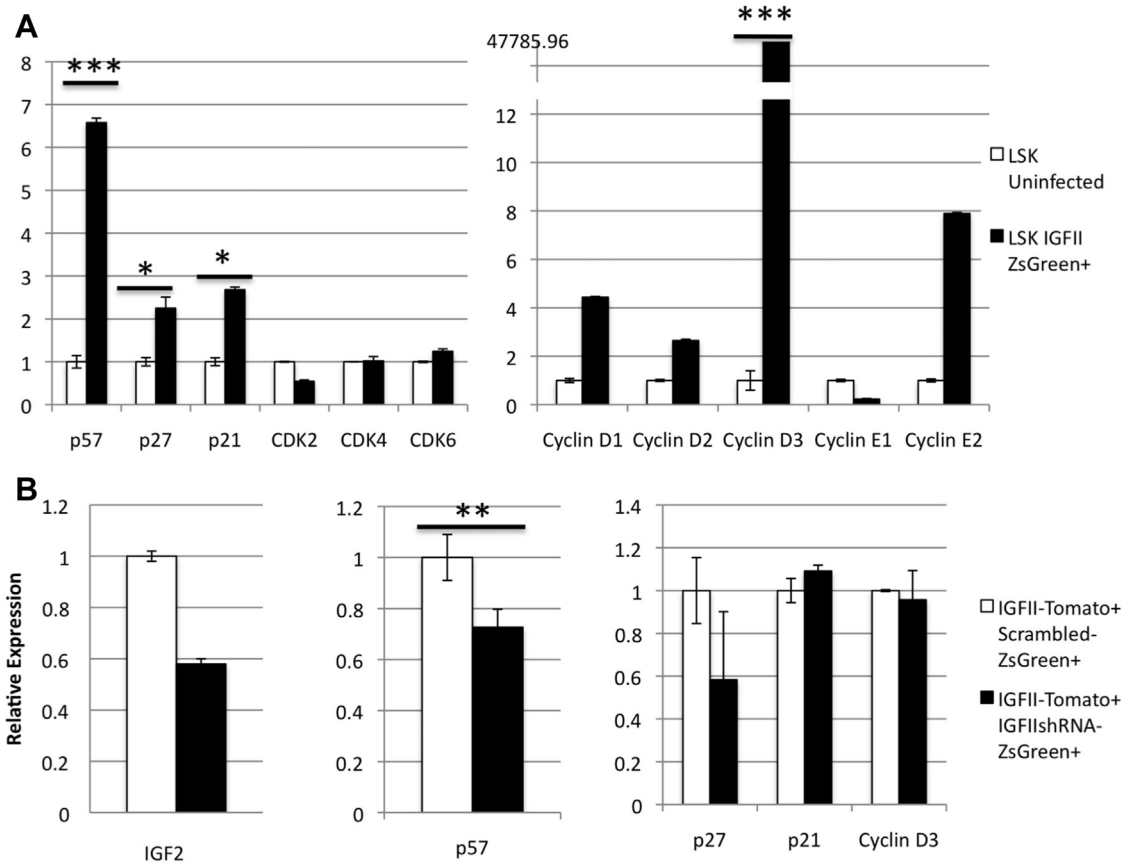


Figure 4. Overexpression of IGF2 in HSCs induces direct upregulation of p57. (**A**) Expression of cell cycle regulators as determined by qPCR analysis within control (*white columns*) and IGF2-HSCs (*black columns*). $*p < 0.05$. $***p < 0.005$. (**B**) Quantitative PCR analysis of IGF2-Tomato$^+$Scrambled ZsGreen$^+$ HSC (*white columns*) and IGF2-Tomato$^+$ IGF2shRNA-ZsGreen$^+$ HSCs (*black columns*) to assess changes in gene expression on knockdown of IGF2. $**p < 0.02$.

p57 [12]. We hypothesized that IGF2-mediated upregulation of p57 could have similar effects on the HSC cell cycle. To evaluate the effect of IGF2, bone marrow cells were purified from IGF2 or mock control transplant recipients and analyzed for cell cycle status. Lineage$^-$ ZsGreen$^+$ cells were analyzed by DAPI staining to identify the percentages of cells in G0/G1 and S/G2/M (Fig. 7A). Our analysis revealed that IGF2 overexpression resulted

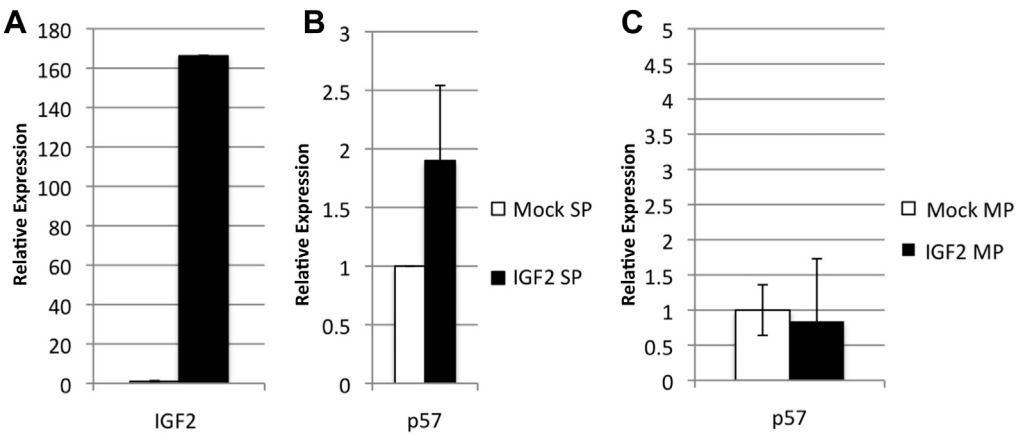


Figure 5. Insulin-like growth factor 2-mediated upregulation of p57 is HSC specific. (**A**) Expression of IGF2 in mock control (*white column*) and IGF2 overexpressing (*black column*) cells. (**B**) Expression of p57 in purified mock control (*white column*) and IGF2 overexpressing (*black column*) HSCs (Hoechst$^-$ SP). (**C**) Expression of p57 in purified control (*white column*) and IGF2 overexpressing (*black column*) hematopoietic progenitors (Hoechst$^+$ MP).

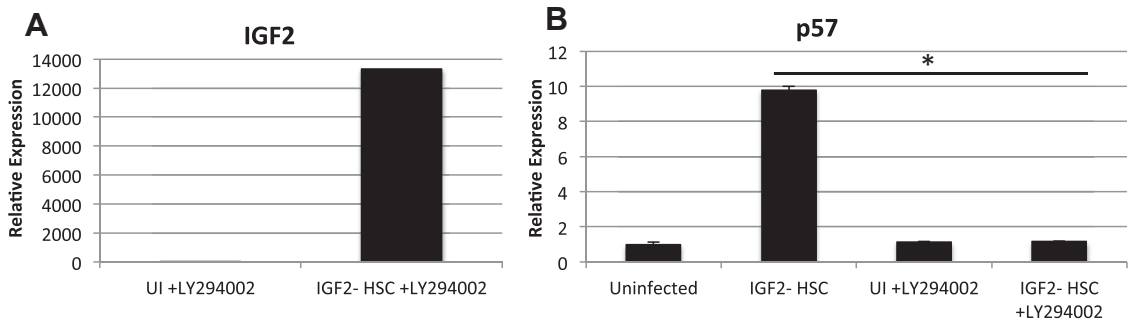


Figure 6. Activation of the PI3K-Akt pathway is required for IGF2-mediated upregulation of p57 within HSCs. (**A**) IGF2 expression within uninfected (UI) HSCs (LSK) and IGF2 expressing (IGF2-HSC) HSCs treated with LY294002 as determined by real-time PCR. (**B**) Expression of p57 within treated and untreated IGF2-HSCs. *$p < 0.02$.

in an increase in the proportion of cells in G0/G1, with a concomitant decrease in the percentage of cycling cells. To further delineate between cells in G0 and G1, cells were stained with Hoechst and Pyronin Y (Fig. 7B). IGF2 increased the percentage of cells in G0 compared with mock control cells.

Increased CDKN1C promoter accessibility in HSCs compared with other hematopoietic populations

The HSC-specific effects of IGF2 on p57 expression prompted us to consider potential differences in CDKN1C (p57) gene promoter accessibility between HSCs and other hematopoietic cell types. Promoter accessibility often correlates with changes in methylation within CpG islands (reviewed in Cedar and Bergman [38] and Laurent et al. [39]), with hypomethylation being associated with an open state (and, therefore, prompt for transcription) and hypermethylation associated with compacted chromatin and low transcription. Detailed DNA methylation maps for the entire repertoire of hematopoietic lineages highlight specific changes in DNA methylation associated with hematopoietic differentiation [24]. Analysis of the methylation status of CpG islands 5 kb upstream and 1 kb downstream of the transcription start site of the CDKN1C gene within different purified hematopoietic populations revealed that in contrast to other cells, HSCs exhibited a lower percentage of methylation within all CpG sites analyzed (Fig. 8). This strongly suggests that p57 transcription is specifically and increasingly accessible in HSCs. The methylation of key cell cycle genes, such as p57, may dictate different responses to IGF2 or other cytokines among different hematopoietic cell populations.

Discussion

Insulin-like growth factor 2 is a key developmental growth factor with direct effects on mammalian growth and differentiation [17,18,40,41]. On the basis of our analysis of HSC

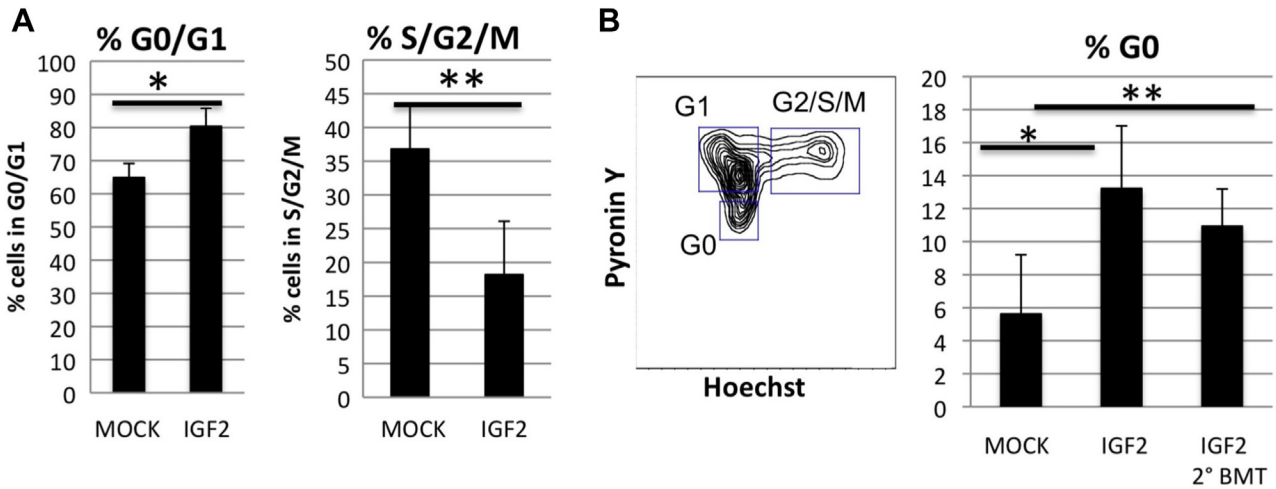


Figure 7. Overexpression of IGF2 in HSCs increases levels of quiescence. (**A**) Lineage$^-$ ZsGreen$^+$ bone marrow cells were analyzed for cell cycle status by DAPI staining. Graphs compare the percentages of resting and cycling cells obtained from mock (DsRed-IRES-ZsGreen) or IGF2 (IGF2-IRES-ZsGreen) primary transplant recipients. *$p < 0.05$. **$p < 0.1$. (**B**) Left: Representative Hoechst/Pyronin Y FACS profile used to distinguish the percentage of cells in G0 from those in G1 or actively cycling (G2/S/M). Right: Percentage of bone marrow cells in G0 isolated from BMT recipients transplanted with either mock transduced HSCs or IGF2-HSCs or secondary BMT recipients. *$p < 0.05$. **$p < 0.1$.

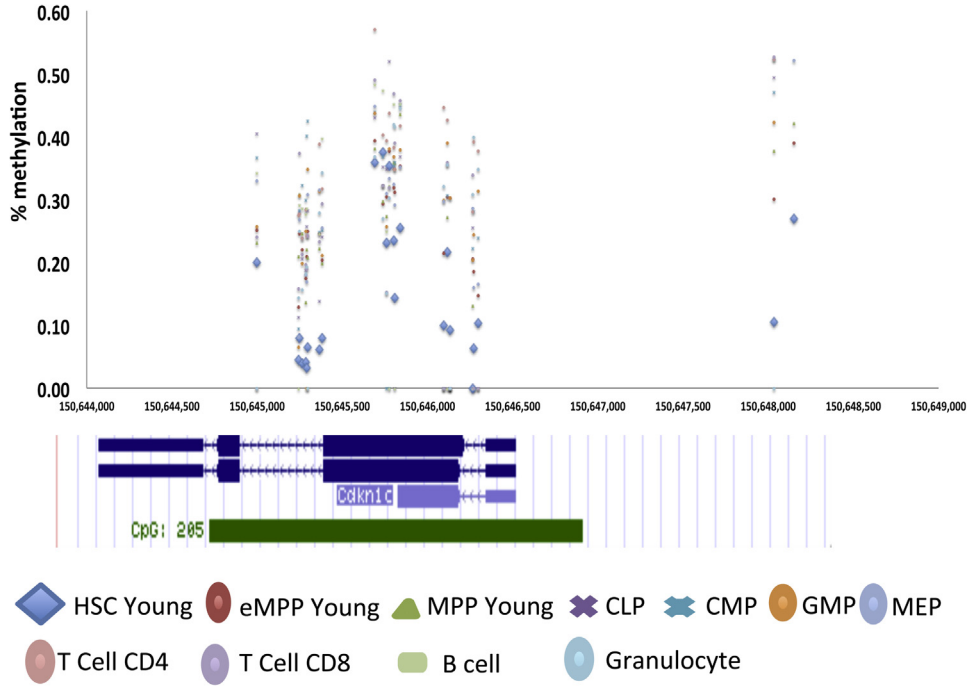


Figure 8. Assessment of CpG methylation within the CDKN1C (p57) gene reveals decreased levels of methylation within HSCs. Graph depicts percentage of methylation within several hematopoietic cell types in an area covering approximately 5 kb of the genome around the CDKN1C gene. Levels of methylation are lowest in HSCs at all CpG sites analyzed. (Color version of figure available online.)

transcriptomes, we found IGF2 to be preferentially expressed within LT-HSCs. IGF2 was similarly identified to be abundantly expressed within LT-HSCs, expressed within common lymphoid progenitors (CLPs) and megakaryocyte erythroid progenitors (MEPs), and silenced within somatic tissues postnatally [42]. Our own analysis of long-term and short-term HSC and LSK CD48+ progenitors reveals similar patterns in IGF2 expression. Importantly IGF2 is downregulated more than threefold within proliferating HSCs subsequent to 5-fluoruracil (5-FU) treatment, suggesting an involvement in HSC maintenance [42].

Taken together, our data present a model in which IGF2 upregulates expression of the cyclin-dependent kinase inhibitor, p57, through activation of the PI3K-Akt pathway. Increased p57 expression allows for maintenance of HSCs, as seen by an increase of cells in G0/G1. p57 is an essential regulator of HSC maintenance through the balanced regulation of quiescence and self-renewal [37,43]. Loss of p57 within HSCs results in decreased retention in G0 and impaired self-renewal potential, leading to significant defects in donor contribution on primary and secondary transplants compared with wild-type controls [37]. This illustrates the requirement for p57 to ensure maintenance of a quiescent, self-renewing population of HSCs.

Wherein loss of p57 results in deleterious effects on HSC maintenance and function, our studies revealed that IGF2-induced upregulation of p57 translates into more robust HSC contribution on bone marrow transplantation. This is due to enhanced preservation and maintenance of HSC function. Levels of HSC donor contribution on secondary bone marrow transplantation further demonstrate maintenance of IGF2-HSCs and do not support the notion of excessive proliferation and stem cell exhaustion (Fig. 3D). This analysis reveals that IGF2-mediated upregulation of p57 functions to maintain the HSC pool and its overall function.

Our work places IGF2 at the apex of an established pathway, important for HSC. Inhibition of the PI3K-Akt pathway within HSCs completely attenuated the upregulation of p57 in response to IGF2 (Fig. 6). The Akt pathway is essential for maintenance of HSC quiescence and has been confirmed to work cooperatively with a host of other signaling factors to directly affect the HSC cell cycle [44–46]. Several other HSC regulatory factors converge on the Akt pathway. Angiopoietin-1, TGFβ, and TPO regulate HSC quiescence and maintain self-renewal via activation of Akt [9–12,47]. Interestingly, both TGFβ and TPO regulate HSC quiescence by mediating expression of p57 [9–12]. We have now identified IGF2 as an additional regulatory factor that functions similarly to modulate HSC function.

Our studies point to HSC-specific upregulation of p57 in response to IGF2. We attribute this to differences in methylation at the p57 promoter among different blood cell populations. Throughout differentiation, the acquired gain or loss of epigenetic marks leads to the suppression of a number of stem cell-associated genes and genes associated with

alternate lineages or expression of lineage-specific genes, respectively [24]. Based on our methylation analysis, the p57 promoter exists in a hypomethylated state within HSCs, while mature blood lineages exhibit increased methylation at this same promoter. We hypothesize that decreased methylation of CpG sites within HSCs may allow for increased expression of p57 in response to IGF2, whereas hypermethylation at these same CpG sites within more mature hematopoietic lineages prevents upregulation of p57. This may be an important mechanism for preserving the proliferative capacity of progenitors. Further experiments are required to validate the link between methylation of the p57 promoter and the ability of IGF2 and other growth factors to induce its expression.

Although several studies corroborate our findings on the involvement of IGF2 in HSC maintenance [17–19], interestingly, our findings do not coincide with results presented by Venkatraman et al. They describe that conditional deletion of the maternal H19-DMR imprinting control region results in activation of the IGF2 signaling pathway (as determined by the phosphorylation and translocation of FoxO3), leading to HSC activation and proliferation and eventual exhaustion [48]. However, deletion of the H19-DMR region affects additional pathways involved in maintenance of LT-HSCs.

Clinically our findings may have implications for the expansion of HSCs for bone marrow transplant procedures. Growth factors, such as SCF, Flt-3 ligand, interleukin (IL)-3, IL-6, granulocyte colony-stimulating factor (G-CSF), and TPO, are the most common cytokines used for expansion of human $CD34^{+}$ cells [49–51]. Several of these have been found to have direct effects on HSC self-renewal and quiescence. We postulate that IGF2 could be similarly used to manipulate HSC fate for bone marrow transplantation. Aberrant IGF2 expression has been associated with the development of hematologic cancers [52–55]; therefore, approaches in which the expression of IGF2 is tightly regulated or confined to the HSC compartment might be necessary. Our own in vivo analysis did not reveal any hematologic abnormalities within transplanted host mice for the duration of our studies (up to 12 months).

To then summarize this work, IGF2 induces the expression of p57 via activation of the PI3K-Akt pathway within HSCs. This causes an increase in the population of HSCs within G0/G1. We believe that because of the hypermethylated state of the p57 promoter within more mature hematopoietic lineages, IGF2-induced, Akt-associated transcription factors are unable to readily access the p57 gene promoter, therefore interfering with induction of p57 within these cells. Hypomethylation of the gene promoter within HSCs renders the promoter more accessible to these transcription factors in response to IGF2. Our own preliminary data mining suggests the presence of several binding sites for the transcription factor AP-1 within the p57 gene promoter. We hypothesize that activation of AP-1 via Akt may lead to the induction of p57. Further studies on the potential role of AP-1 will help to expand this model.

Lastly, IGF2 has been reported to be upregulated and to have a defined regulatory function within neural stem cell (NSC) populations [56,57]. Specifically, IGF2 expanded neural stem/progenitor cells and promoted self-renewal through signaling by way of the IR-A or IGF1R. In addition, activation of the PI3K-Akt pathway was required for IGF2 regulation of hippocampal NSC [58]. These data further support the role of IGF2 in regulating stem cell properties and correlate with our findings of a role for IGF2 in adult HSCs. Our data support an HSC-specific function for IGF2 by way of the CDKi p57, via activation of the PI3K-Akt pathway. The identification of IGF2 as a regulator of HSCs and proposed mechanism complement the complexity of the current HSC regulatory network.

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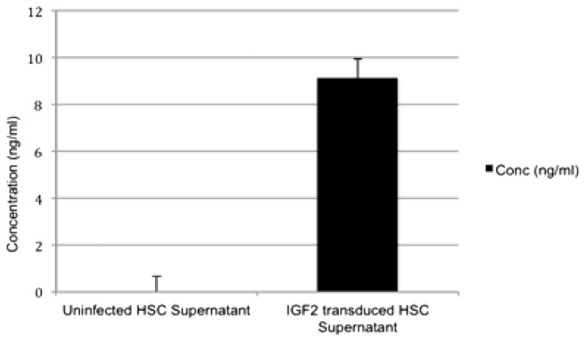
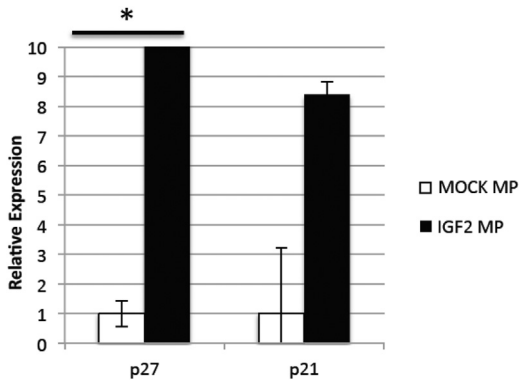
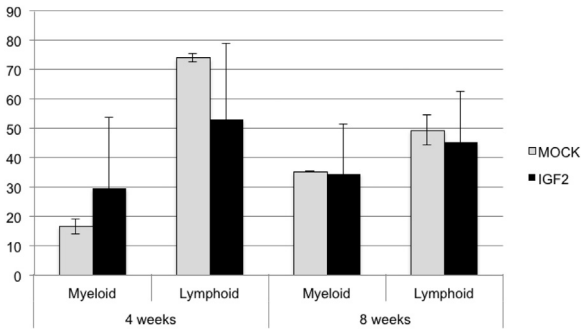


Supplementary Figure E1. Insulin-like growth factor 2-transduced HSCs produce increased levels of IGF2 protein. Medium was collected from untransduced and IGF2-transduced HSC cultures and tested for the presence of soluble IGF2.



Supplementary Figure E3. Insulin-like growth factor 2 overexpression increases expression of p27 and p21 within cells of the main population (MP). Expression of p27 and p21 within hematopoietic progenitor cells purified from Mock (*white columns*) or IGF2 (*black columns*) transplanted mice. *$p = 0.01$.



Supplementary Figure E2. Insulin-like growth factor 2 has no effect on lineage specification in vivo. Graph depicts the percentage of peripheral blood cells positive for myeloid and lymphoid differentiation markers at 4 and 8 weeks posttransplantation. *Gray columns* are representative of peripheral blood (PB) cells from mock transplant mice. *Black columns* are representative of levels within our IGF2 transplant mice.