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Insulin-like growth factor 2 (IGF2) modulates murine hematopoietic stem cell maintenance through upregulation of p57

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Abstract

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 predominantly expressed within long-term HSC. This study examines IGF2 expression patterns and the effects of the gene in HSC. Through the overexpression and knockdown of IGF2 within purified HSC, we demonstrate that IGF2 expression increases HSC-derived multilineage colonies *in vitro* and enhances hematopoietic contribution *in vivo* upon competitive bone marrow transplantation. The effects of IGF2 are mediated by direct upregulation of the CDKi p57, exclusively within long-term HSC, via activation of the PI3K-Akt pathway. Increased expression of p57 resulted in a concomitant increase of HSC in the G0/G1 stage of the cell cycle. Analysis of genomic DNA methylation revealed that HSC exhibited a hypomethylated state within the promoter region of the CDKN1C (p57) gene, providing a potential mechanism for the exclusive effects of IGF2 within HSC. Our studies demonstrate a novel role for IGF2 in regulating HSC cell cycle and illustrate potential novel therapeutic targets for hematological diseases.

Keywords

Hematopoietic Stem Cells; Insulin-like growth factor 2; P57 (Cyclin-dependent kinase inhibitor 1C); Cell Cycle

INTRODUCTION

Lifelong blood cell production is contingent upon the maintenance and orchestrated functions of the hematopoietic stem cell pool. Due to the limited size of the HSC population, preservation of adult HSC is critical and is reliant upon a balance between cell cycle progression and quiescence. The majority of HSC 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, HSC 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 through 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 regulate stem cell fate whereby CDKi are found in excess within quiescent HSC.

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 hematological disorders and malignancies. Thus far, a number of growth factor pathways have been implicated in modulating HSC. These include Wnt ⁸, TPO ⁹, Angiopoietin-1 ¹⁰, and TGF-beta ^{11,12}. 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 datasets for long-term and short-term HSC and a third mixed population containing progenitors and mature blood cells. Based on our analysis, we identified Insulin-like growth factor-2 (IGF2) to be upregulated in HSC and therefore became the focus of our studies.

IGF2 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 HSC ¹⁷⁻¹⁹. Beyond development, human total bone marrow cells exhibit biallelic expression of IGF2, while more mature cells of the peripheral blood exhibit monoallelic expression ²⁰. These studies suggest that IGF2 may function as an HSC growth factor, however its exact role remains unclear. Here we show that IGF2 within adult HSC preserves long-term HSC 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 show hypomethylation of the p57 gene promoter within HSC. Our results highlight a novel and specific role for IGF2 in HSC function.

MATERIALS AND METHODS

Mice

C57BL/6 (B6-Ly5.2) and B6.SJL-Ptprca Pep3b/BoyJ (CD45.1) mice were purchased from Jackson Laboratory (Bar Harbor, Maine). 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 hematopoietic stem cells (HSC) by Hoechst staining

Mouse HSC 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 70µm cell strainer and resuspended at a concentration of 4.5×10^6 cells/ml in Hanks Buffered Saline Solution (supplemented with 1% HEPES, 2% FBS, and 1% Penicillin-Streptomycin) containing 8.8 µg/ml of Hoechst 33342. Cells were incubated for 90 minutes 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 2000 rpm for 20 minutes at 20°C. Resulting cells were washed and resuspended in a solution containing 1 ng/ml of Propidium Iodide and filtered through a 40µm cell strainer before sorting using a Beckman-Coulter MoFlo or BDFACS Aria 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). HSC for culture were sorted directly to 96 well round bottom plates.

Cloning of IGF2 lentiviral vectors

IGF2 was amplified from a pmigf2-3 vector (ATCC) by PCR. Resulting IGF2 cDNA was cloned into lentiviral vectors containing a constitutive EF1α promoter, IRES sequence, and ZsGreen reporter²¹. A short hairpin RNA was designed to knockdown IGF2 by annealing two PAGE purified 5' phosphorylated primers. To anneal primers, equal volumes of 100 µM 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 eGFP reporter, and mU6 promoter to drive shRNA expression.

Lentiviral transduction of HSC

All lentiviruses were prepared as previously described^{21,22}. For overnight infection HSC were sorted directly to StemSpan media supplemented with 10 ng/ml 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 HSC 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 HSC 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, FITC- conjugated Sca1, and PE-conjugated cKit (CD117) antibodies (BD Pharmingen) for 20 minutes 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 100 mM solution of EDTA. For red cell lysis, samples were incubated in red blood cell lysing buffer (Sigma) for 30 minutes on ice and spun down. White blood cell pellets were resuspended in FACS staining buffer (Ebiosciences) and stained with PE- conjugated CD45.1 and APC-conjugated CD45.2 (Ebiosciences) for 20 minutes 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 utilized the SLAM family markers ²³, using 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 minutes on ice. Hematopoietic progenitors were defined as LSK CD48⁺; Short-term HSC were gated as LSK CD150⁻ CD48⁻; Long-term HSC 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 (CFU) assay

Transduced HSC were transferred directly to 3 mL of Methocult M3434 methylcellulose media (StemCell Technologies). Media containing cells was split to (2) 35 mm plates as per 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 (qPCR)

RNA was extracted using the RNeasy Mini Kit (Qiagen) according to 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 manufacturer's protocols. qPCR 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 Beta 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 PBS containing 1 ng/ml of DAPI plus APC- conjugated Lineage antibody cocktail (BD Pharmingen) and stained for 25 minutes 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 minutes. 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⁺, while G2/S/M cells were 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 three hours apart. Donor HSC (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 HSC via retroorbital injection in competition with 5×10⁵ total bone marrow cells isolated from C57BL/6 CD45.2 mice. For secondary transplants, total bone marrow cells (1×10⁶) 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 values were calculated using the following equation: (Competitor RU) × ((% donor contribution)/(100 - % donor contribution)). Competitor RU are defined as 1 for every 10⁵ 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 HSC relative to ST-HSC and progenitor populations

We hypothesized that genes responsible for HSC self-renewal would be preferentially expressed in LT-HSC relative to other early hematopoietic progenitors. To identify candidates, we re-analyzed microarray data from Hoechst⁻ LSK CD34⁻ LT-HSC, Hoechst⁻ LSK CD34⁺ ST-HSC 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 EPCR ²⁷, Endomucin ²⁸ and CD105 ²⁹ (Fig. 1A). Five genes exhibited a 100+ fold enrichment in HSC versus non-HSC, including IGF2, which was also 5-fold enriched in LT-HSC compared to ST-HSC. We independently validated preferential expression of IGF2 in LT-HSC by real-time PCR in LT-HSC, ST-HSC and non-HSC populations purified using SLAM family receptors CD150 and CD48 (Fig. 1B and 1C) ²³.

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

To test the effects of IGF2 expression within purified HSC, 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 HSC (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 ELISA (Supplemental Figure 1 and Figure 5A). HSC overexpressing IGF2 (IGF2-HSC) yielded an increased percentage of multipotent CFU-GEMM colonies compared to 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 due to incomplete knockdown of IGF2 (Fig. 2B). Cultures were further analyzed by FACS for myeloid, lymphoid and erythroid differentiation markers. There was no effect on lineage specification in response to IGF2 *in vitro* (data not shown).

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-HSC (Hoechst⁻) and Mock-HSC (Hoechst⁻) were evaluated by analyzing donor and recipient peripheral blood contribution over one 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 to Mock control cells, at short and long-term time points (See methods for CRU calculations). Contribution from IGF2-HSC 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* (Supplemental Figure 2). Increased donor contribution can be attributed to a selective effect on HSC self-renewal rather than effects on the differentiation of downstream progeny or caused by 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). 1×10^6 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 ($2.62\% \pm 0.59$ at 8 weeks compared to $10.55\% \pm 6.85$ at 24 weeks), 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 HSC, we focused on the analysis of key cell cycle regulators, particularly those involved in G0/early

G1. IGF2 has been shown to have direct effects on the expression of p57 within primary mouse embryonic fibroblasts³⁰. To identify changes in the expression of CDK, cyclin, and CIP/KIP CDKi family members upon IGF2 overexpression, LSK purified HSC were transduced with IGF2 and incubated in minimal media for seven 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 p57 (greater than 6-fold increase) (Fig. 4A).

In order 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 HSC (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 HSC. Expression of p21 and Cyclin D3 were unchanged possibly due to inefficient knockdown of IGF2 or changes in the expression of these genes is a consequence of increased self-renewal. Expression of Cyclin D3 and P21 is upregulated within mobilized bone marrow HSC undergoing self-renewing proliferation³¹.

We also evaluated the effect of IGF2 on p57 in HSC *in vivo*. IGF2-HSC and Mock control-HSC were purified (based on Hoechst staining and ZsGreen expression) from the bone marrow of transplanted recipient mice, six months post-transplantation. Similar to what we observed *in vitro*, *in vivo* IGF2 overexpression resulted in an increase in p57 expression compared to Mock- transduced HSC (Fig. 5A and 5B). 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 an increase in p27 (Fig. 5C and Supplemental Figure 3). P27 has been previously 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.

IGF 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 p57³⁶. Therefore we tested whether activation of PI3K-Akt was the mechanism for IGF2 mediated upregulation of p57 expression within HSC. To do this we treated IGF2-HSC 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 HSC.

IGF2 regulates the HSC cell cycle

p57 is the most abundant CDKi within LT-HSC and is an important regulator of quiescence³⁷. p57 deficient HSC exhibited impaired reconstitution capacity upon bone marrow transplantation and decreased quiescence³⁷. Growth factors such as TGF-beta have been shown to induce cell cycle arrest in human hematopoietic cells through upregulation of p57¹². We hypothesized that IGF2 mediated upregulation of p57 could have similar effects on HSC cell cycle. In order 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 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 to mock control cells.

Increased CDKN1C promoter accessibility in HSC compared to 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 HSC and other hematopoietic cell types. Promoter accessibility is often correlated with changes in methylation within CpG islands (reviewed in^{38,39}), 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 differentiation²⁴. 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, HSC 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 HSC. The methylation of key cell cycle genes, such as p57 may dictate different responses to IGF2 or other cytokines amongst different hematopoietic cell populations.

DISCUSSION

IGF2 is a key developmental growth factor with direct effects on mammalian growth and differentiation^{17–18, 40–41}. Based on our analysis of HSC transcriptomes, we found IGF2 to be preferentially expressed within LT-HSC. IGF2 was similarly identified to be abundantly expressed within LT-HSC, expressed within common lymphoid progenitors (CLP) and megakaryocyte erythroid progenitors (MEP) and silenced within somatic tissues postnatally⁴². Our own analysis of long-term and short-term HSC and LSK CD48+ progenitors show similar patterns in IGF2 expression. Importantly IGF2 is downregulated more than three-fold within proliferating HSC subsequent to 5-Fluoruracil (5-FU) treatment, suggesting an involvement in HSC maintenance⁴².

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 HSC, 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 HSC results in decreased retention in G0 and impaired self-renewal potential, leading to significant defects in donor contribution upon primary and secondary transplants compared to WT controls³⁷. This illustrates the requirement for p57 to ensure maintenance of a quiescent, self-renewing population of HSC.

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 upon bone marrow transplantation. This is due to enhanced preservation and maintenance of HSC function. Levels of HSC donor contribution upon secondary bone marrow transplantation further demonstrate maintenance of IGF2- HSC, and do not support the notion of excessive proliferation and stem cell exhaustion (Figure 3D). This analysis reveals 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 known to be important for HSC. Inhibition of the PI3K-Akt pathway within HSC completely attenuated the upregulation of p57 in response to IGF2 (Figure 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, TGF-beta and TPO regulate HSC quiescence and maintain self-renewal via activation of Akt^{9–12, 47}. Interestingly, both TGF-beta 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 amongst different blood cell populations. Throughout differentiation, the acquired gain or loss of epigenetic marks lead to the suppression of a number of stem cell associated genes and genes associated with alternate lineages or expression of lineage specific genes respectively²⁴. Based on our methylation analysis, the p57 promoter exists in a hypomethylated state within HSC while mature blood lineages exhibit increased methylation at this same promoter. We hypothesize that decreased methylation of CpG sites within HSC may allow for increased expression of p57 in response to IGF2 while 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 exhaustion⁴⁸. However deletion of the H19-DMR region affects additional pathways involved in maintenance of LT-HSC that may compound the effects described, particularly upon transplant.

Clinically our findings may have implications for the expansion of HSC for bone marrow transplant procedures. Growth factors such as SCF, Flt-3 ligand, IL-3, IL-6, G-CSF (granulocyte colony-stimulating factor), and TPO are the most common cytokines used for expansion of human CD34+ cells^{49–51}. Several of these have been shown 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 previously associated with the development of hematological cancers^{52–55}; therefore approaches where 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 hematological 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 HSC. This causes an increase in the population of HSC within G0/G1. We believe that due to 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 HSC renders the promoter more accessible to these transcription factors in response to IGF2. Our own preliminary data mining (not shown) 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 previously been shown 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 NSC⁵⁸. These data further support the role of IGF2 in regulating stem cell properties, and correlates with our findings of a role of IGF2 in adult HSC. Our data supports 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 HSC and proposed mechanism complement the complexity of the current HSC regulatory network.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Cheshier SH, Morrison SJ, Liao X, Weissman IL. In vivo proliferation and cell cycle kinetics of long-term self-renewing hematopoietic stem cells. *Proc Natl Acad Sci U S A*. 1999; 96:3120–3125. [PubMed: 10077647]
2. Nygren JM, Bryder D, Jacobsen SE. Prolonged cell cycle transit is a defining and developmentally conserved hematopoietic stem cell property. *J Immunol*. 2006; 177:201–208. [PubMed: 16785515]
3. Orford KW, Scadden DT. Deconstructing stem cell self-renewal: genetic insights into cell-cycle regulation. *Nat Rev Genet*. 2008; 9:115–128. [PubMed: 18202695]
4. Wilson A, Laurenti E, Oser G, et al. Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell*. 2008; 135:1118–1129. [PubMed: 19062086]
5. Wilson A, Oser GM, Jaworski M, et al. Dormant and self-renewing hematopoietic stem cells and their niches. *Ann N Y Acad Sci*. 2007; 1106:64–75. [PubMed: 17442778]
6. Foudi A, Hochedlinger K, Van Buren D, et al. Analysis of histone 2B-GFP retention reveals slowly cycling hematopoietic stem cells. *Nat Biotechnol*. 2009; 27:84–90. [PubMed: 19060879]
7. van der Wath RC, Wilson A, Laurenti E, Trumpp A, Lio P. Estimating dormant and active hematopoietic stem cell kinetics through extensive modeling of bromodeoxyuridine label-retaining cell dynamics. *PLoS One*. 2009; 4:e6972. [PubMed: 19771180]
8. Fleming HE, Janzen V, Lo Celso C, et al. Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. *Cell Stem Cell*. 2008; 2:274–283. [PubMed: 18371452]
9. Yoshihara H, Arai F, Hosokawa K, et al. Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. *Cell Stem Cell*. 2007; 1:685–697. [PubMed: 18371409]
10. Arai F, Hirao A, Ohmura M, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. *Cell*. 2004; 118:149–161. [PubMed: 15260986]
11. Larsson J, Blank U, Helgadottir H, et al. TGF-beta signaling-deficient hematopoietic stem cells have normal self-renewal and regenerative ability in vivo despite increased proliferative capacity in vitro. *Blood*. 2003; 102:3129–3135. [PubMed: 12842983]
12. Scandura JM, Bocconi P, Massague J, Nimer SD. Transforming growth factor beta- induced cell cycle arrest of human hematopoietic cells requires p57KIP2 up-regulation. *Proc Natl Acad Sci U S A*. 2004; 101:15231–15236. [PubMed: 15477587]
13. Schofield PN, Tate VE. Regulation of human IGF-II transcription in fetal and adult tissues. *Development*. 1987; 101:793–803. [PubMed: 3503697]
14. DeChiara TM, Efstratiadis A, Robertson EJ. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature*. 1990; 345:78–80. [PubMed: 2330056]
15. Wu Q, Kawahara M, Kono T. Synergistic role of Igf2 and Dlk1 in fetal liver development and hematopoiesis in bi-maternal mice. *J Reprod Dev*. 2008; 54:177–182. [PubMed: 18344616]
16. Kono T, Obata Y, Wu Q, et al. Birth of parthenogenetic mice that can develop to adulthood. *Nature*. 2004; 428:860–864. [PubMed: 15103378]
17. Zhang CC, Lodish HF. Insulin-like growth factor 2 expressed in a novel fetal liver cell population is a growth factor for hematopoietic stem cells. *Blood*. 2004; 103:2513–2521. [PubMed: 14592820]
18. Mascarenhas MI, Parker A, Dzierzak E, Ottersbach K. Identification of novel regulators of hematopoietic stem cell development through refinement of stem cell localization and expression profiling. *Blood*. 2009; 114:4645–4653. [PubMed: 19794138]

19. Zhang CC, Kaba M, Iizuka S, Huynh H, Lodish HF. Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. *Blood*. 2008; 111:3415–3423. [PubMed: 18202223]
20. Morison IM, Eccles MR, Reeve AE. Imprinting of insulin-like growth factor 2 is modulated during hematopoiesis. *Blood*. 2000; 96:3023–3028. [PubMed: 11049980]
21. Mostoslavsky G, Fabian AJ, Rooney S, Alt FW, Mulligan R. Complete correction of murine Artemis immunodeficiency by lentiviral vector-mediated gene transfer. *Proc Natl Acad Sci U S A*. 2006 Oct 31; 103(44):16406–16411. [PubMed: 17062750]
22. Thomas D, Mostoslavsky G. Efficient transduction of hematopoietic stem cells and its potential for gene correction of hematopoietic diseases. *Methods Mol Biol*. 2014; 1114:441–450. [PubMed: 24557921]
23. Kiel MJ, Yilmaz OH, Iwashita T, Terhorst C, Morrison SJ. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell*. 2005; 121:1109–1121. [PubMed: 15989959]
24. Bock C, Beerman I, Lien WH, et al. DNA methylation dynamics during in vivo differentiation of blood and skin stem cells. *Mol Cell*. 2012; 47:633–647. [PubMed: 22841485]
25. Ramalho-Santos M, Yoon S, Matsuzaki Y, Mulligan RC, Melton DA. “Stemness”: transcriptional profiling of embryonic and adult stem cells. *Science*. 2002; 298:597–600. [PubMed: 12228720]
26. Goodell MA, Brose K, Paradis G, Conner AS, Mulligan RC. Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. *J Exp Med*. 1996; 183:1797–1806. [PubMed: 8666936]
27. Balazs AB, Fabian AJ, Esmon CT, Mulligan RC. Endothelial protein C receptor (CD201) explicitly identifies hematopoietic stem cells in murine bone marrow. *Blood*. 2006; 107:2317–2321. [PubMed: 16304059]
28. Matsubara A, Iwama A, Yamazaki S, et al. Endomucin, a CD34-like sialomucin, marks hematopoietic stem cells throughout development. *J Exp Med*. 2005; 202:1483–1492. [PubMed: 16314436]
29. Chen CZ, Li M, de Graaf D, Monti S, Gottgens B, Sanchez MJ, Lander ES, Golub TR, Green AR, Lodish HF. Identification of endoglin as a functional marker that defines long-term repopulating hematopoietic stem cells. *Proc Natl Acad Sci U S A*. 2002 Nov 26; 99(24):15468–15473. [PubMed: 12438646]
30. Grandjean V, Smith J, Schofield PN, Ferguson-Smith AC. Increased IGF-II protein affects p57kip2 expression in vivo and in vitro: implications for Beckwith-Wiedemann syndrome. *Proc Natl Acad Sci U S A*. 2000; 97:5279–5284. [PubMed: 10779549]
31. Passegue E, Wagers AJ, Giuriato S, Anderson WC, Weissman IL. Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. *J Exp Med*. 2005; 202:1599–1611. [PubMed: 16330818]
32. Pietras EM, Warr MR, Passegue E. Cell cycle regulation in hematopoietic stem cells. *J Cell Biol*. 2011; 195:709–720. [PubMed: 22123859]
33. Cheng T, Rodrigues N, Dombkowski D, Stier S, Scadden DT. Stem cell repopulation efficiency but not pool size is governed by p27(kip1). *Nat Med*. 2000; 6:1235–1240. [PubMed: 11062534]
34. Hamamura K, Zhang P, Yokota H. IGF2-driven PI3 kinase and TGFbeta signaling pathways in chondrogenesis. *Cell Biol Int*. 2008; 32:1238–1246. [PubMed: 18675921]
35. Chen L, Jiang W, Huang J, et al. Insulin-like growth factor 2 (IGF-2) potentiates BMP-9-induced osteogenic differentiation and bone formation. *J Bone Miner Res*. 2010; 25:2447–2459. [PubMed: 20499340]
36. Worster DT, Schmelzle T, Solimini NL, et al. Akt and ERK control the proliferative response of mammary epithelial cells to the growth factors IGF-1 and EGF through the cell cycle inhibitor p57Kip2. *Sci Signal*. 2012; ra19. [PubMed: 22394561]
37. Matsumoto A, Takeishi S, Kanie T, et al. p57 is required for quiescence and maintenance of adult hematopoietic stem cells. *Cell Stem Cell*. 2011; 9:262–271. [PubMed: 21885021]
38. Cedar H, Bergman Y. Programming of DNA methylation patterns. *Annu Rev Biochem*. 2012; 81:97–117. [PubMed: 22404632]

39. Laurent L, Wong E, Li G, et al. Dynamic changes in the human methylome during differentiation. *Genome Res.* 2010; 20:320–331. [PubMed: 20133333]
40. Ferguson-Smith AC, Cattanach BM, Barton SC, Beechey CV, Surani MA. Embryological and molecular investigations of parental imprinting on mouse chromosome 7. *Nature.* 1991; 351:667–670. [PubMed: 2052093]
41. DeChiara TM, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell.* 1991; 64:849–859. [PubMed: 1997210]
42. Berg JS, Lin KK, Sonnet C, Boles N, Weksburg D, Nguyen H, Holt L, Rickwood D, Daly R, Goodell M. Imprinted genes that regulate early mammalian growth are coexpressed in somatic stem cells. *Plos One.* 6(10):e26410.
43. Zou P, Yoshihara H, Hosokawa K, et al. p57(Kip2) and p27(Kip1) cooperate to maintain hematopoietic stem cell quiescence through interactions with Hsc70. *Cell Stem Cell.* 2011; 9:247–261. [PubMed: 21885020]
44. Rossi L, Lin KK, Boles NC, et al. Less is more: unveiling the functional core of hematopoietic stem cells through knockout mice. *Cell Stem Cell.* 2012; 11:302–317. [PubMed: 22958929]
45. Juntilla MM, Patil VD, Calamito M, Joshi RP, Birnbaum MJ, Koretzky GA. AKT1 and AKT2 maintain hematopoietic stem cell function by regulating reactive oxygen species. *Blood.* 2010; 115:4030–4038. [PubMed: 20354168]
46. Kobayashi H, Butler JM, O'Donnell R, et al. Angiocrine factors from Akt-activated endothelial cells balance self-renewal and differentiation of haematopoietic stem cells. *Nat Cell Biol.* 2010; 12:1046–1056. [PubMed: 20972423]
47. Yamazaki S, Iwama A, Takayanagi S, et al. Cytokine signals modulated via lipid rafts mimic niche signals and induce hibernation in hematopoietic stem cells. *EMBO J.* 2006; 25:3515–3523. [PubMed: 16858398]
48. Venkatraman A, He X, Thorvaldsen J, et al. Maternal-imprinting at H19-Igf2 locus maintains adult hematopoietic stem cell quiescence. *Nature.* 2013; 500(7462):345–349. [PubMed: 23863936]
49. Goncalves TL, Benvenuto DM, Bonfanti G. Specific factors influence the success of autologous and allogeneic hematopoietic stem cell transplantation. *Oxid Med Cell Longev.* 2009; 2:82–87. [PubMed: 20357929]
50. Dahlberg A, Delaney C, Bernstein ID. Ex vivo expansion of human hematopoietic stem and progenitor cells. *Blood.* 2011; 117:6083–6090. [PubMed: 21436068]
51. Walasek MA, van Os R, de Haan G. Hematopoietic stem cell expansion: challenges and opportunities. *Ann N Y Acad Sci.* 2012; 1266:138–150. [PubMed: 22901265]
52. Wu HK, Weksberg R, Minden MD, Squire JA. Loss of imprinting of human insulin-like growth factor II gene, IGF2, in acute myeloid leukemia. *Biochem Biophys Res Commun.* 1997; 231:466–472. [PubMed: 9070302]
53. Randhawa GS, Cui H, Barletta JA, et al. Loss of imprinting in disease progression in chronic myelogenous leukemia. *Blood.* 1998; 91:3144–3147. [PubMed: 9558368]
54. Vorwerk P, Wex H, Hohmann B, Mohnike K, Schmidt U, Mittler U. Expression of components of the IGF signalling system in childhood acute lymphoblastic leukaemia. *Mol Pathol.* 2002; 55:40–45. [PubMed: 11836446]
55. Bock O, Tessema M, Serinsoz E, von Wasielewski R, Busche G, Kreipe H. Aberrant expression of insulin-like growth factor-2 (IGF-2) in Philadelphia chromosome negative chronic myeloproliferative disorders. *Leuk Res.* 2004; 28:1145–1151. [PubMed: 15380337]
56. Ziegler AN, Schneider JS, Qin M, et al. IGF-II promotes stemness of neural restricted precursors. *Stem Cells.* 2012; 30:1265–1276. [PubMed: 22593020]
57. Lehtinen MK, Zappaterra MW, Chen X, et al. The cerebrospinal fluid provides a proliferative niche for neural progenitor cells. *Neuron.* 2011; 69:893–905. [PubMed: 21382550]
58. Bracko O, Singer T, Aigner S, et al. Gene expression profiling of neural stem cells and their neuronal progeny reveals IGF2 as a regulator of adult hippocampal neurogenesis. *J Neurosci.* 2012; 32:3376–3387. [PubMed: 22399759]

HIGHLIGHTS

- IGF2 is preferentially expressed within long-term HSC.
- Within HSC, IGF2 activates the PI3K-Akt pathway to upregulate expression of p57.
- HSC exhibit decreased methylation at CpG sites within the p57 gene promoter.
- The described mechanism illustrates a novel regulatory function for IGF2 in HSC.

Figure 1. IGF2 is differentially expressed in LT-HSC compared to ST-HSC and hematopoietic progenitors. **(A)** Graphical comparison of gene expression profiles for long-term HSC (Hoechst⁻ LSK CD34⁻) and non-HSC (Hoechst⁺ MP). Table summarizes the number of genes up- or downregulated within long-term HSC and fold change in expression. **(B)** Sorting strategy for purification of long-term HSC (LT-HSC: LSK CD150⁺ CD48⁻), short-term HSC (ST-HSC: LSK CD150⁻ CD48⁻), and multipotent progenitors (MPP: LSK CD48⁺) based on the expression of SLAM family receptors, CD150 and CD48. **(C)** Quantitative PCR analysis of IGF2 expression within purified LT-HSC and ST-HSC compared to MPP *p<.005.

Figure 2.

Overexpression of IGF2 within purified HSC *in vitro* results in an increased percentage of multipotent GEMM colonies. **(A)** Purified HSC were transduced with Mock control (DsRed-IRES-ZsGreen) or IGF2 (IGF2-IRES-ZsGreen) lentiviruses. Transduced HSC were plated to methylcellulose cultures for up to 7 days. Resulting colonies were characterized and quantified based on morphology. Representative image of a ZsGreen positive colony resulting from transduced HSC. * $p < .01$ **(B)** The opposite results were observed when purified HSC were transduced with lentiviruses expressing an IGF2 shRNA, compared to Scrambled control or IGF2. Data is representative of three independent experiments ($n=1$).

Figure 3.

Overexpression of IGF2 within purified HSC results in increased donor contribution in both primary and secondary bone marrow transplantations (BMT). **(A)** Experimental scheme for primary and secondary BMT. **(B)** Mock (white columns) and IGF2 (black columns) transduced HSC were transplanted to lethally irradiated recipients. To evaluate levels of donor chimerism peripheral blood was analyzed by FACS at different time points post-transplantation. (N= 3–5 transplanted mice per group, data representative of 3 independent transplants), * $p < 0.1$ ** $p < .03$. **(C)** To quantify HSC repopulating function, percentages of ZsGreen⁺ donor contribution were used to calculate competitive repopulation units (CRU). Graph shows average CRU values for Mock and IGF2 transplanted groups. * $p < .07$; ** $p < .005$; *** $p < .01$. **(D)** Contribution from IGF2 transduced HSC upon secondary bone marrow transplantation. Data shows three individual recipient animals at different time points post-transplantation.

Figure 4.

Overexpression of IGF2 in HSC induces direct upregulation of p57. **(A)** Expression of cell cycle regulators as determined by quantitative PCR analysis within control (white columns) and IGF2-HSC (black columns). * $p < .05$, *** $p < .005$. **(B)** Quantitative PCR analysis of IGF2-Tomato⁺ Scrambled ZsGreen⁺ HSC (white columns) and IGF2-Tomato⁺ IGF2shRNA-ZsGreen⁺ HSC (black columns) to assess changes in gene expression upon knockdown of IGF2. ** $p < .02$.

Figure 5.

IGF2 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) HSC (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 HSC. (A) IGF2 expression within uninfected (UI) HSC (LSK) and IGF2 expressing (IGF2-HSC) HSC treated with LY294002 as determined by real time PCR. (B) Expression of p57 within treated and untreated IGF2-HSC * $p < 0.02$.

Figure 7.

Overexpression of IGF2 in HSC 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. * $p < .05$ ** $p < 0.1$. **(B)** 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 panel shows the percentage of bone marrow cells in G0 isolated from BMT recipients transplanted with either Mock transduced HSC, IGF2-HSC or secondary BMT recipients * $p < .05$; ** $p < 0.1$.

Figure 8.

Assessment of CpG methylation within the CDKN1C (p57) gene reveals decreased levels of methylation within HSC. Graph displays percentage of methylation within several hematopoietic cell types in an area covering approximately 5Kb of the genome around the CDKN1C gene. Levels of methylation are lowest in HSC at all CpG sites analyzed.