IGF binding protein 2 supports the survival and cycling of hematopoietic stem cells

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The role of IGF binding protein 2 (IGFBP2) in cell growth is intriguing and largely undefined. Previously we identified IGFBP2 as an extrinsic factor that supports ex vivo expansion of hematopoietic stem cells (HSCs). Here we showed that IGFBP2-null mice have fewer HSCs than wild-type mice. While IGFBP2 has little cell-autonomous effect on HSC function, we found decreased in vivo repopulation of HSCs in primary and secondary transplanted IGFBP2-null recipients. Importantly, bone marrow stromal cells that are deficient for IGFBP2 have significantly decreased ability to support the expansion of repopulating HSCs. To investigate the mechanism by which IGFBP2 supports HSC activity, we demonstrated that HSCs in IGFBP2-null mice had decreased survival and cycling, down-regulated expression of antiapoptotic factor Bcl-2, and up-regulated expression of cell cycle inhibitors p21, p16, p19, p57, and PTEN. Moreover, we found that the C-terminus, but not the RGD domain, of extrinsic IGFBP2 was essential for support of HSC activity. Defective signaling of the IGF type I receptor did not rescue the decreased repopulation of HSCs in IGFBP2-null recipients, suggesting that the environmental effect of IGFBP2 on HSCs is independent of IGF-IR mediated signaling. Therefore, as an environmental factor, IGFBP2 supports the survival and cycling of HSCs. (*Blood*. 2011;118(12): 3236-3243)

Introduction

The number of hematopoietic stem cells (HSCs) is determined by the balance among different cell fates—self-renewal, differentiation, apoptosis, and migration—which are regulated by the intrinsic factors and environmental cues in vivo or in vitro.[1,2] We have identified several growth factors and secreted proteins that support the repopulation of HSCs and have developed an efficient serum-free system to support ex vivo expansion of mouse and human HSCs.[3-5] Insulin-like growth factor binding protein 2 (IGFBP2) is one of these secreted proteins; we isolated IGFBP2 from a cancer line that supports ex vivo expansion of HSCs.[6,7]

IGFBP2 is a member of the IGFBP family that is found in all vertebrates; it modulates the biologic effects of IGFs by controlling the distribution, function, and activity of IGF-1 and IGF-2.[8] IGFBP2 is expressed in the fetus and in several adult tissues and biologic fluids. It is also overexpressed in many tumors and in some cases its expression level correlates with grade of malignancy.[9-11] The level of IGFBP2 appears to be low in well-differentiated tumors but high in poorly differentiated tumors.[12]

The known functions of IGFBP2 are very interesting. IGFBP2 displays IGF-dependent inhibitory effects on normal somatic cell growth. However, several studies demonstrated that IGFBP2 has intrinsic bioactivities that are independent of IGF-1 or IGF-2. IGFBP2 stimulates proliferation, survival, differentiation, and motility of various types of cells.[9,13-20] Multiple mechanisms for these IGF-independent actions of IGFBP2 have been proposed. One line of studies supported the concept that intracellular IGFBP2 binds integrin and supports cell survival.[13] A second line of studies suggested that IGFBP2 acts as secreted proteins and binds to cell surface receptors. For example, when bound to the cell surface integrin, extrinsic IGFBP2 influences cell mobility and proliferation.[9-11,21] IGFBP2 also binds to Frizzled 8 and LDL receptor-related protein 6 and is proposed to antagonize Wnt signaling in heart cells.[22] Moreover, another line of research showed that extrinsic IGFBP2 can be taken up by cells on oxidative stress; it enters the cytosol after 12-24 hours.[11,23]

The roles of IGFBP2 in the hematopoietic system are largely undefined. IGFBP2 supports ex vivo expansion of both mouse and human HSCs and is essential for the HSC-supportive activity of activated endothelium.[6,7,24] IGFBP2-null mice have lower spleen weights and total splenic lymphocyte numbers and decreased number and function of mouse osteoblasts in a gender-specific manner.[25,26] Knockdown of IGFBP2 in zebrafish downregulates the expression of transcription factor Scl and decreases the blood cell number and blood circulation.[27] The IGFBP2 level is negatively associated with the progress of acute leukemia[28,29] and the expression of IGFBP2 is a factor for the prediction of relapse of these blood cancer.[28,30-32] To gain mechanistic insights into the action of IGFBP2, we tried to address several questions: (1) Does IGFBP2 regulate HSC activity in vivo? (2) What cell fate(s) of HSCs does IGFBP2 regulate? (3) Which part of IGFBP2 is essential to its HSC supportive activity? In this study, we found that IGFBP2 had little cell-autonomous effect but environmental IGFBP2 positively supported HSC activity in the mouse bone marrow (BM). In IGFBP2 null mice, HSCs showed decreased survival and cycling, down-regulated expression of antiapoptotic factor Bcl-2, and up-regulated expression of cell cycle inhibitors. We further demonstrated that the C-terminus, but not

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the RGD domain, of secreted IGFBP2 is essential for support of HSC activity, and the environmental effect of IGFBP2 on HSCs is independent of IGF-IR mediated signaling.

Methods

Mice

C57BL/6 CD45.2 and CD45.1 mice were purchased from the National Cancer Institute and the University of Texas Southwestern Medical Center animal breeding core facility. The IGFBP2$^{+/-}$ mice originally obtained from Lexicon Genetics Inc were backcrossed to C57BL/6 CD45.2 mice 10 times to obtain IGFBP2-null and wild-type (WT) control littermates. IGF-IR$^{+/-}$ mice as previously described[33] were in a pure C57BL/6 background. Mice were maintained at the University of Texas Southwestern Medical Center animal facility. All animal experiments were performed with the approval of UT Southwestern Committee on Animal Care. To genotype mice, DNA was extracted from tail tips and a DNAeasy kit was used according to the manufacturer's instructions (Sigma-Aldrich). The IGFBP2 and/or LacZ-neomycin (neo) insert was amplified in a 3-primer PCR using primers 5′GGGTTCTCCTGGCTGGTGACTC3′ and 5′GAGTCTCCCTGGATCTGATTAAGG3′ for IGFBP2 and 5′GGGTTCTCCTGGCTGGTGACTC3′ and 5′ATAAACCCTCTTGCAGTTGCATC3′ for the lacZ-neomycin insert. The cycling conditions were 94°C for 2 minutes, followed by 35 cycles of 94°C for 45 seconds, 60°C for 45 seconds, and 72°C for 60 seconds, followed by a final extension of 72°C for 5 minutes. To perform RT-PCR to detect IGFBP2 expression in supplemental Figure 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article), primers 5′GGAGGGCGAAGCATGCGGCGTCTAC3′ and 5′GCCCATCTGCCGGTGCTGTTCATTGACCTT3′ were used. To perform real-time RT-PCR to detect IGFBP2 expression in other figures, a primer set purchased from QIAGEN (cat no. QT00269542) was used. Western blots were performed to detect the IGFBP2 protein using the goat anti-IGFBP2 antibody (SC-6002; Santa Cruz Biotechnology).

Mouse HSC culture

Indicated numbers of BM Lin$^-$Sca-1$^+$Kit$^+$Flk-2$^-$CD34$^-$ cells were isolated from 8- to 12-week-old C57BL/6 mice and were plated into wells of a U-bottom 96-well plate (3799; Corning). StemSpan serum-free medium (StemCell Technologies) was used as the basal medium. The basal medium supplemented with 10 μg/mL heparin (Sigma-Aldrich), 10 ng/mL mouse SCF (R&D Systems), 20 ng/mL mouse TPO (R&D Systems), and 10 ng/mL human FGF-1 (Invitrogen) was used as STF medium. FBS was included in the STF medium in the coculture experiment as described.[34] Cells were cultured at 37°C in 5% CO_2 and the normal level of O_2. For the purpose of competitive transplantation, cells from 12 culture wells were pooled and mixed with competitor/supportive cells before the indicated numbers of cells were transplanted into each mouse as we have done previously.[3-7]

Flow cytometry

Donor BM cells were isolated from 8- to 12-week-old C57BL/6 CD45.2 (or CD45.1 as indicated) mice. Lin$^-$Sca-1$^+$Kit$^+$Flk2$^-$CD34$^-$ cells were isolated by staining with a biotinylated lineage cocktail (anti-CD3, anti-CD5, anti-B220, anti–Mac-1, anti–Gr-1, anti-Ter119; StemCell Technologies) followed by streptavidin-PE/Cy5.5, anti–Sca-1–FITC, anti–Kit-APC, anti–Flk-2–PE, and anti-CD34–PE. Various progenitors, including CMP, GMP, MEP, and CLP were analyzed as described.[35] For repopulation analysis of mouse HSCs, peripheral blood cells of recipient mice were collected by retro-orbital bleeding. Red blood cells were lysed, and samples were stained with anti-CD45.2–FITC, anti-CD45.1–PE, anti-Thy1.2–PE (for T-lymphoid lineage), anti-B220–PE (for B-lymphoid lineage), anti–Mac-1–PE or anti–Gr-1–PE (cells costaining with anti–Mac-1 and anti–Gr-1 were deemed to be of the myeloid lineage) monoclonal antibodies (BD Pharmingen). The "percent repopulation" shown in all figures was based on the staining results of anti-CD45.2–FITC and anti-CD45.1–PE. In all cases FACS analysis of the T, B, and myeloid lineages was also performed to confirm multi-lineage reconstitution as previously described.[3-7,36]

Cell cycle and apoptosis analysis

The cell cycle analysis with Hoechst and pyronin Y staining was performed as described.[34] Briefly, the Lin$^-$Sca-1$^+$Kit$^+$Flk2$^-$CD34$^-$ cells were collected in Hanks buffered salt solution medium containing 10% FBS, 1 g/L glucose, and 20mM HEPES (pH 7.2). Cells were washed, Hoechst 33 342 (Invitrogen) was added to 20 μg/mL, and cells were incubated at 37°C for 45 minutes after which pyronin Y (1 μg/mL, Sigma-Aldrich) was added. Cells were incubated for another 15 minutes at 37°C, washed, and resuspended in cold PBS. Samples were immediately analyzed by flow cytometry (FACSAria, BD Biosciences).

To examine the BrdU incorporation, mice were given 3 intraperitoneal injections of BrdU (Sigma-Aldrich; 3 mg every 24 hours) in PBS and maintained on 0.2 mg/mL BrdU in the drinking water for 72 hours. After 72 hours, the BM was harvested and stained with antibodies against lineage markers, c-Kit, and Sca-1. Cells were fixed, permeablized, and denatured, and anti-BrdU–PE (BD Pharmingen) was used to examine the BrdU incorporation as described.[34] To examine the apoptosis, Lin$^-$Sca-1$^+$Kit$^+$ cells were stained with PE-conjugated anti–annexin V and 7-AAD according to manufacturer's manual (BD Pharmingen).

Competitive reconstitution analysis

The indicated numbers of mouse CD45.2 or CD45.1 donor cells were mixed with 1×10^5 freshly isolated CD45.1 or CD45.2 competitor BM cells and the mixture was injected intravenously via the retro-orbital route into each of a group of 6- to 9-week-old CD45.1 or CD45.2 mice previously irradiated with a total dose of 10 Gy. One million BM cells collected from primary recipients were used for the secondary transplantation as described.[34]

Quantitative RT-PCR

Total RNA was isolated from FACS-collected BM Lin$^-$Sca-1$^+$Kit$^+$Flk2$^-$CD34$^-$ cells, differentiated lineage cells, or nonhematopoietic cells. First-strand cDNA was synthesized using SuperScript II RT (Invitrogen). Samples were analyzed in triplicate 25-μL reactions (300nM each primer, 12.5 μL of Master mix) as adapted from the standard protocol provided in SYBR Green PCR Master Mix and RT-PCR Protocols provided by Applied Biosystems. Primers were purchased from QIAGEN or Sigma-Aldrich. The default PCR protocol was used on an Applied Biosystems Prism 7000 Sequence Detection System. The mRNA level in each population was normalized to the level of β-actin RNA transcripts present in the same sample as described.[6]

Colony assays

IGFBP2-null or WT BM cells were resuspended in IMDM with 2% FBS and then seeded into methylcellulose medium M3334 (StemCell Technologies) for CFU-E, M3434 (StemCell Technologies) for CFU-GEMM, CFU-GM, and BFU-E, M3630 (StemCell Technologies) for CFU-Pre-B assays, according to the manufacturer's protocols and as described previously.[34]

Results

IGFBP2-null mice have fewer stem cells

To determine the effect of IGFBP2 on HSCs in vivo, we first examined the BM hematopoietic compartment of IGFBP2-null mice in C57BL/6 background. The lack of IGFBP2 was confirmed in BM and serum of these mice by RT-PCR and Western blotting, respectively (supplemental Figure 1A-B). As reported before,[25] the IGFBP2-null mice did not show an overt phenotype. The total BM cells in IGFBP2-null mice were similar to those in WT mice (supplemental Figure 1C). Importantly, however, IGFBP2-null

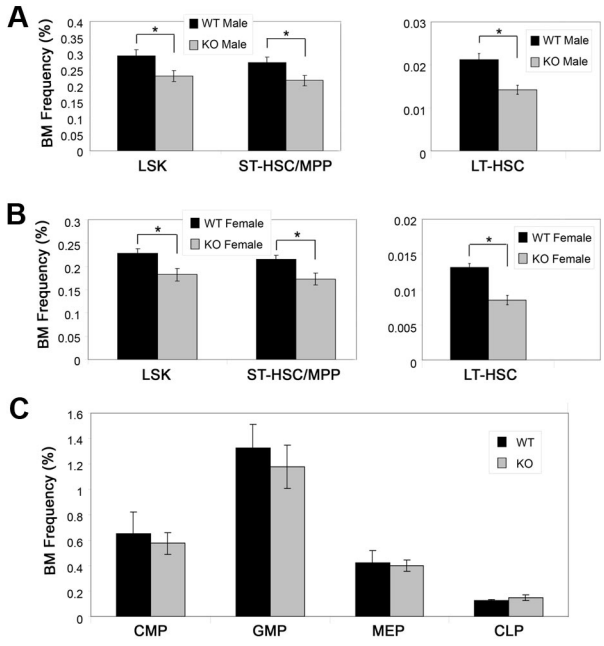

Figure 1. IGFBP2-null mice have fewer HSCs than wild-type mice. The frequencies of BM Lin$^-$Sca-1$^+$Kit$^+$ cells, ST-HSCs/MPPs as Lin$^-$Sca-1$^+$Kit$^+$Flk2$^+$CD34$^+$ cells, and LT-HSCs as Lin$^-$Sca-1$^+$Kit$^+$Flk2$^-$CD34$^-$ cells in IGFBP2-null and WT male littermates (A; n = 8) and female littermates (B; n = 7-8) were compared. (C) The frequencies of BM progenitors CMP, GMP, MEP, and CLP in IGFBP2-null and WT mice (n = 4-5) were measured.

mice had significantly lower frequency of BM Lin$^-$Sca-1$^+$Kit$^+$ (LSK) cells, short-term (ST)–HSCs/multipotent progenitors (MPPs), and long-term (LT)–HSCs than WT mice of either gender (Figure 1A-B, and supplemental Figure 1D-E). Nevertheless, various BM hematopoietic progenitors, including CMP, GMP, MEP, and CLP, were not significantly different at steady state (Figure 1C, and supplemental Figure 1F). In addition to analysis of the phenotypic hematopoietic progenitors, we also used a functional measure, the colony forming unit (CFU) assay, to quantitate some hematopoietic progenitors. We found that the null mice had more BM CFU-E and CFU-GM than did the WT mice; however, BM BFU-E, CFU-Pre-B, and CFU-GEMM did not differ significantly (supplemental Figure 1G-I).

IGFBP2 has little cell-autonomous effect on HSCs

Because IGFBP2-null mice have a decreased frequency of BM HSCs, we sought to identify the cell population(s) in the mouse BM that express IGFBP2, and to ask whether IGFBP2 has a cell-autonomous effect on HSCs. We collected different hematopoietic populations from mouse BM by flow cytometry and determined the levels of expression of IGFBP2 using real-time RT-PCR. We detected little *IGFBP2* expression in Lin$^-$Sca-1$^+$Kit$^+$Flk2$^-$CD34$^-$ cells. The level of *IGFBP2* mRNA was 2-fold greater in the BM CD45$^+$ hematopoietic populations, and nonhematopoietic CD45$^-$ stromal cells had ~ 8-fold higher levels compared with HSCs (Figure 2A). We then sorted various BM stromal cell populations and measured *IGFBP2* expression. In general, *IGFBP2* mRNA was expressed more abundantly in CD105$^+$, CD44$^+$, SSEA4$^+$, CD29$^+$, and Sca-1$^+$ BM CD45$^-$ stromal cells than in HSCs (supplemental Figure 2). These cells may be enriched for mesenchymal stromal cells.[37] By contrast, CD45$^-$CD31$^-$ cells expressed greater amounts than CD45$^-$CD31$^+$ endothelial cells. These results suggest that IGFBP2 may be predominantly expressed by mesenchymal stromal cells but not endothelial cells in the mouse BM.

To test whether IGFBP2 has cell-autonomous function on HSCs, we used the CD45 congenic mouse model to perform competitive BM transplantation. We injected 500 freshly isolated CD45.2 Lin$^-$Sca-1$^+$Kit$^+$Flk2$^-$CD34$^-$ donor cells from WT and IGFBP2-null mice into lethally irradiated CD45.1 recipients, along with 1×10^5 CD45.1 total BM cells as competitors. The donors were analyzed for ST (6 weeks) and LT (17 weeks) repopulation after transplantation (Figure 2B). No significant difference was observed for the ST and LT donor repopulating activities between the WT and IGFBP2-null HSCs. Both WT and null donor cells repopulated myeloid and lymphoid lineages (Figure 2C). Taken together, our results suggest that IGFBP2 has minimal cell-autonomous effect on HSCs.

IGFBP2 from the host environment supports HSC activity

Because IGFBP2 was expressed abundantly by the nonhematopoietic BM stromal cells (supplemental Figure 2), we sought to test whether IGFBP2 from the BM environment had any effect on HSC function. Here, we transplanted 5×10^5 total CD45.1 BM cells into lethally irradiated CD45.2 WT or IGFBP2-null mice without competitors. We measured percentages of donor-derived phenotypic HSCs (CD45.1$^+$ Lin$^-$Sca-1$^+$Kit$^+$Flk2$^-$CD34$^-$ cells) from

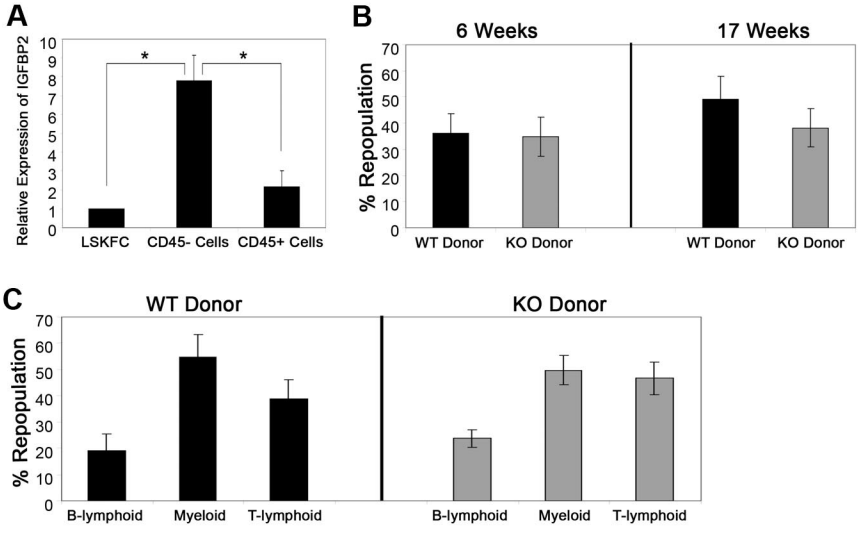

Figure 2. IGFBP2 has little cell-autonomous effect on HSCs. (A) Lin$^-$Sca-1$^+$Kit$^+$Flk2$^-$CD34$^-$, hematopoietic CD45$^+$, and nonhematopoietic CD45$^-$ stromal cells were freshly isolated from BM, and IGFBP2 gene expression was determined by real-time RT-PCR (n = 4-5). (B) Five hundred freshly isolated CD45.2 Lin$^-$Sca-1$^+$Kit$^+$Flk2$^-$CD34$^-$ cells from WT or IGFBP2-null mice were cotransplanted with 1×10^5 CD45.1 total BM cells into CD45.1 recipients, and the ST and LT donor repopulating activities were evaluated at indicated time after transplantation (n = 8). (C) Different donor lineages from long-term repopulation were determined. Representative data from 1 of 2 independent experiments that gave similar results are shown.

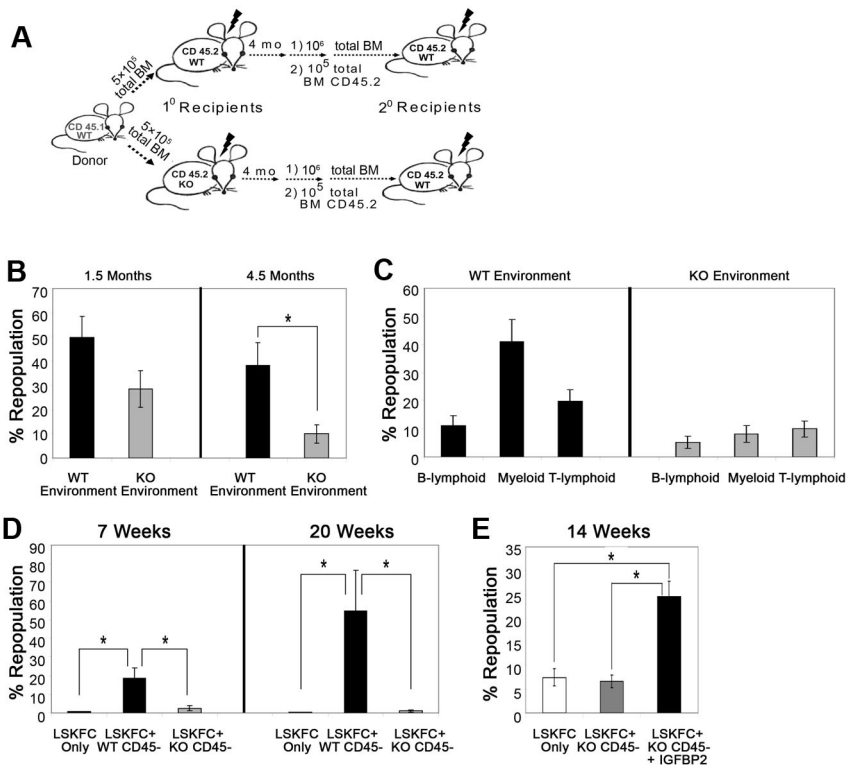

Figure 3. IGFBP2 supports the repopulation of HSCs in the BM environment. (A) A schematic representation of BM transplantation. (B) CD45.1 total BM cells (5×10^5) were transplanted into lethally irradiated CD45.2 WT or IGFBP2-null recipients for 4 months. Subsequently, 1×10^6 CD45.1 total BM cells from primary WT or IGFBP2-null recipients were cotransplanted with 1×10^5 CD45.2 total BM cells into secondary CD45.2 recipients (n = 9). Shown are donor repopulations at indicated time after transplantation. Data shown are representative of 2 independent experiments that gave similar results. (C) Different donor lineages from long-term repopulation were determined. (D) One hundred twenty CD45.2 donor $Lin^-Sca\text{-}1^+Kit^+Flk2^-CD34^-$ cells were cocultured with 360 WT or IGFBP2-null $CD45^-$ cells in STF medium containing 10% FBS for 5 days. The total mixture of cultured cells were then cotransplanted with 1×10^5 CD45.1 total BM cells into CD45.1 recipients (n = 5). Shown are donor repopulations at indicated time after transplantation. (E) One hundred and twenty CD45.2 donor $Lin^-Sca\text{-}1^+Kit^+Flk2^-CD34^-$ cells were cultured alone or cocultured with 360 IGFBP2-null $CD45^-$ cells in serum-supplemented STF medium, with or without IGFBP2, for 5 days (n = 5). Shown is donor repopulation at indicated time, representative of 2 independent experiments that gave similar results.

the WT or null recipients at 4 months after transplantation, when the hematopoietic system had reached homeostasis (supplemental Figure 3A). We found that donor-derived HSCs made up 0.014% and 0.009% of HSCs in the WT and null recipients, respectively; recapitulating the higher percentages of HSCs in untransplanted WT mice (Figure 1A-B).

Next, we conducted a secondary competitive transplantation to measure the repopulation and self-renewal of HSCs. One million of the BM cells from the primarily repopulated WT and IGFBP2-null recipient mice were collected for secondary transplantation along with 10^5 competitor cells (Figure 3A). We found that the repopulating activity of cells that originated from the primary null recipients was significantly decreased compared with those from the primary WT recipients. Over time, the secondary repopulation of original donor HSCs from the null primary recipients was ~ 25% of that of the same donor cells from the WT primary recipients (Figure 3B, see bars for 4.5 months). Original donor HSCs repopulated both myeloid and lymphoid lineages (Figure 3C). Overall these results indicate that IGFBP2 in the BM environment supports in vivo self-renewal of HSCs.

To directly test whether IGFBP2-producing BM cells support HSC expansion, we cocultured 120 CD45.2 $Lin^-Sca\text{-}1^+Kit^+Flk2^-CD34^-$ cells with 360 $CD45^-$ stromal cells isolated from the WT or IGFBP2-null mice. After 5 days, the cocultured cells were used for competitive reconstitution to measure HSC activity. As shown in Figure 3D and supplemental Figure 3B, at 7 and 20 weeks after transplant, HSCs cocultured with null $CD45^-$ stromal cells had dramatically decreased repopulation compared with those cocultured with WT $CD45^-$ cells. Next, we tested whether extrinsic IGFBP2 was responsible for the supportive effect of WT stroma. Indeed, exogenous addition of IGFBP2 to the coculture rescued the defect of the KO $CD45^-$ cells (Figure 3E). Overall, our results provided functional evidence that IGFBP2 produced by BM $CD45^-$ cells supports HSC activity.

IGFBP2 promotes the survival and cell cycling of HSCs

To identify the mechanisms by which IGFBP2 supports HSC activity, we investigated whether any of the cell fates – apoptosis, quiescence, and motility – was altered in HSCs isolated from the null mice. We first measured of the percentages of $Lin^-Sca\text{-}1^+Kit^+$ cells that underwent apoptosis in WT and null mice by flow cytometry. Although there was no significant difference in early apoptosis ($annexinV^+7\text{-}AAD^-$) between WT and IGFBP2-null HSCs, IGFBP2-null HSCs showed a significant increase in late apoptosis ($annexinV^+7\text{-}AAD^+$) compared with WT counterparts (Figure 4A). Further analysis indicates LT-HSCs in null mice had elevated apoptosis (supplemental Figure 4A). In contrast, there was no difference in apoptosis in mature hematopoietic lineages in WT and null mice (supplemental Figure 4B).

We also compared the cell cycle status of HSCs in 7- to 8-week-old adult WT and IGFBP2-null mice. The proportion of LT-HSCs ($Lin^-Sca\text{-}1^+Kit^+Flk2^-CD34^-$) and ST-HSCs ($Lin^-Sca\text{-}1^+Kit^+Flk2^+CD34^+$) in G0 was evaluated by Hoechst 33 342 and pyronin Y staining.[34] No significant difference was observed between the cell cycle status of WT and null ST-HSCs (Figure 4B). In contrast, ~ 30% of null LT-HSCs were in G0, a significantly higher percentage than that in WT mice (20%; Figure 4C). A similar pattern was observed in 1-year-old mice for ST-HSCs (supplemental Figure 4C), and LT-HSCs (supplemental Figure 4D). Moreover, we isolated donor $Lin^-Sca\text{-}1^+Kit^+Flk2^-CD34^-$ cells from CD45.1 HSC-transplanted WT or IGFBP2-null recipient mice. LT-HSCs in null hosts were more quiescent than those from WT hosts (supplemental Figure 4E), suggesting that IGFBP2 produced by the environment increased the cycling of LT-HSCs. To further confirm that the stem cells of WT mice cycle faster than those from the IGFBP2-null mice, we analyzed BrdU incorporation into $Lin^-Sca\text{-}1^+Kit^+$ cells. Indeed, WT cells incorporated BrdU

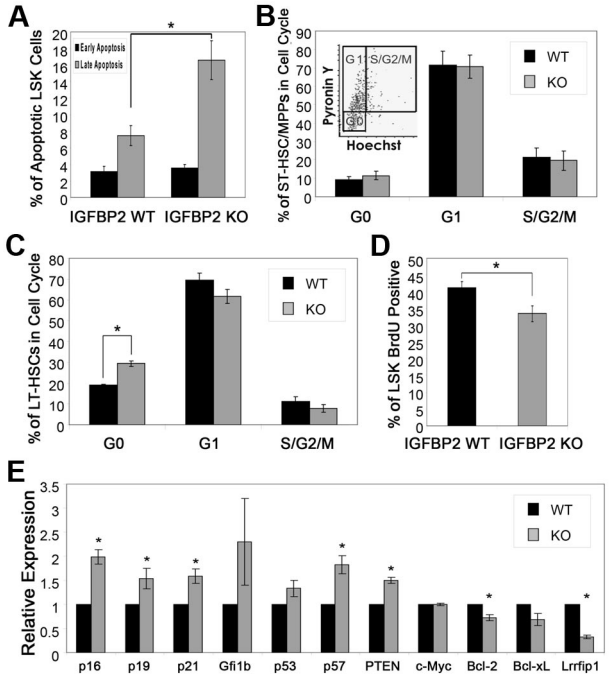

Figure 4. IGFBP2 supports the survival and cycling of HSCs in the BM. (A) Lin$^-$Sca-1$^+$Kit$^+$ cells from BM of WT or IGFBP2-null mice were analyzed for early apoptosis (annexinV$^+$7-AAD$^-$) and late apoptosis (annexinV$^+$7-AAD$^+$) markers (n = 6). (B) Cell cycle status of Lin$^-$Sca-1$^+$Kit$^+$Flk2$^+$CD34$^+$ cells and (C) Lin$^-$Sca-1$^+$Kit$^+$Flk2$^-$CD34$^-$ cells was evaluated by Hoechst 33 342 and pyronin Y staining (n = 6). (D) BrdU incorporation into Lin$^-$Sca-1$^+$Kit$^+$ cells was measured (n = 8). (E) The expression of certain growth and survival related genes in freshly isolated BM Lin$^-$Sca-1$^+$Kit$^+$ cells was evaluated by real-time RT-PCR (n = 4-6).

significantly faster than IGFBP2-null cells (41.25 ± 1.74% vs. 33.46 ± 2.39%, respectively) as shown in Figure 4D.

To examine the spontaneous mobilization of HSCs, we determined the levels of Lin$^-$Sca-1$^+$Kit$^+$ cells circulating in the peripheral blood. The level of Lin$^-$Sca-1$^+$Kit$^+$ cells in circulation of IGFBP2-null mice was higher than that in WT mice on average (supplemental Figure 4F); however, the difference was not statistically significant. To examine the homing ability of HSCs in WT and IGFBP2-null mice, we labeled and injected 1×10^7 total BM cells into each recipient via retro-orbital injection. Our analysis of recipient BM, spleen, and liver indicated that HSCs had similar homing abilities in WT and IGFBP2-null mice (supplemental Figure 4G).

Real-time RT-PCR was performed to confirm the results of apoptosis and cell cycle analyses. To obtain manageable cell numbers and reliable results for real-time RT-PCR, we used Lin$^-$Sca-1$^+$Kit$^+$ cells from WT and IGFBP2-null mice. Concordant with the phenotypic analyses, our quantitative PCR showed that antiapoptotic gene Bcl-2 was significantly decreased whereas the cell cycle inhibitors p16, p19, p21, p57, and PTEN were increased in the null Lin$^-$Sca-1$^+$Kit$^+$ cells compared with those of WT cells (Figure 4E).

Cumulatively, these data suggested that IGFBP2 supported HSC survival and cycling in LT-HSCs but not ST-HSCs. Based on this result and our observation that IGFBP2 in the BM enhances the number and repopulation of HSCs in primary and secondary transplantation, we propose that IGFBP2 supports the self-renewal of LT-HSCs.

The C-terminus of IGFBP2 is important for supporting HSC activity

It has been shown that integrin α5 binds to IGFBP2, specifically to the RGD^{267} motif. This binding can be abolished by mutating the RGD^{267} of IGFBP2 to RGE^{267}.[14,38] To test whether the RGD domain mediates the function of extrinsic IGFBP2 in HSCs, we constructed 2 IGFBP2 mutants, one with the RGD^{267} to RGE^{267} mutation and the other with 41 amino acids deleted from the C-terminus (Figure 5A).

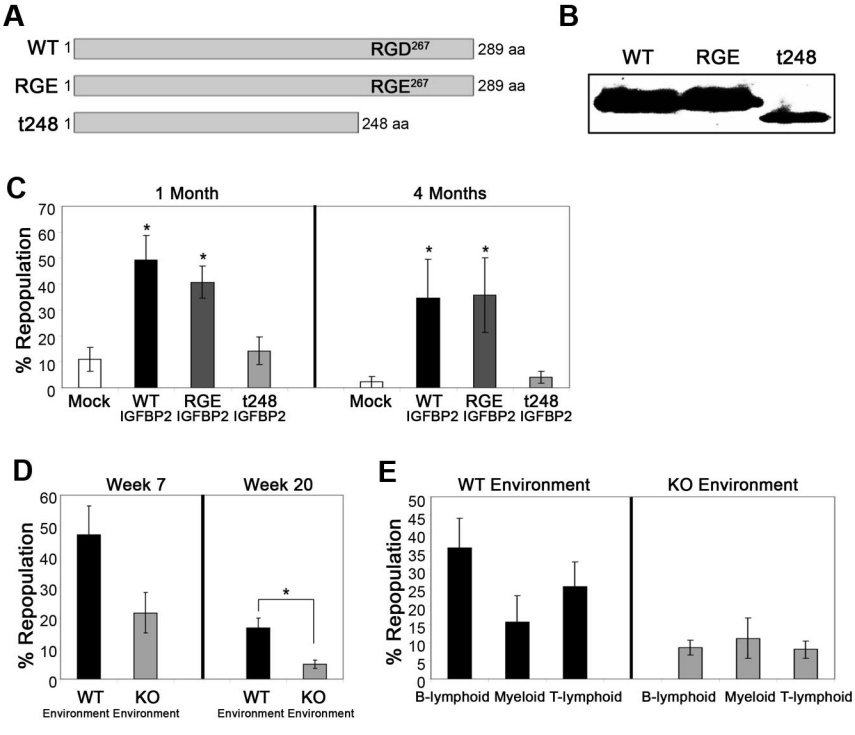

Figure 5. The C-terminus of IGFBP2 is essential for IGFBP2's HSC supportive activity. (A) Schematic representation for IGFBP2 mutants. (B) WT, RGE^{267}, and t248 IGFBP2 constructs were transfected into 293T cells and the levels of secreted IGFBP2 proteins in the media at 60 hours after transfection were evaluated by Western blot. (C) Normalized amounts of the WT and mutant IGFBP2 in the conditioned media (~ 500 ng/mL) were added to STF medium, and then 20 CD45.1 donor Lin$^-$Sca-1$^+$Kit$^+$Flk2$^-$CD34$^-$ cells were cultured for 10 days. The cultured cells were cotransplanted with 1×10^5 CD45.2 total BM cells into CD45.2 recipients (n = 6). The data shown are representative of 2 independent experiments that gave similar results. (D) Total IGF-IR-null fetal liver cells (1×10^5) were transplanted into lethally irradiated WT or IGFBP2-null recipients. After 4 months, 1×10^6 total donor BM cells from primary WT or IGFBP2-null recipients were cotransplanted with 1×10^5 CD45.1 total BM cells into secondary CD45.1 recipients (n = 5). (E) Different donor lineages from long-term repopulation were determined. Representative data from 2 independent experiments that gave similar results are shown.

We overexpressed these constructs in 293T cells. The WT or mutant IGFBP2 was secreted into the conditioned medium (Figure 5B). A normalized amount of this conditioned medium in STF medium was used to culture HSCs for 10 days as described.[6] The abilities of these IGFBP2 variants to support HSC growth were evaluated by transplanting the cultured cells into lethally irradiated recipient mice in a competitive reconstitution analysis. The donor repopulating activities were analyzed at 1 and 4 months after transplanted. RGE IGFBP2 supported HSCs activities equally as well as WT IGFBP2 (Figure 5C), suggesting that the RGD domain does not mediate IGFBP2's effect on HSC expansion. In striking contrast, the truncated IGFBP2 was unable to support the expansion of ST or LT repopulating HSCs (Figure 5C). Therefore, the C-terminal region of IGFBP2 is important for supporting HSC function.

Because the C-terminus of IGFBP2 is involved in both IGF binding[39] and IGF-independent signaling,[40] we sought to determine whether IGFBP2's effect on HSCs depended on IGF signaling. Here, we compared the repopulation of IGF-IR-null donor cells in WT and IGFBP2-null recipients. Because IGF-IR-null is lethal at birth, we used IGF-IR-null fetal liver cells to reconstitute mice. Using competitive reconstitution analysis, we demonstrated that the IGF-IR-null HSCs repopulating ability was similar to WT counterparts with no defects in differentiation (data not shown). Next, we transplanted 1×10^5 IGF-IR-null fetal liver cells into lethally irradiated WT or IGFBP2-null mice. We measured the donor stem cells ($Lin^-Sca\text{-}1^+Kit^+Flk2^-CD34^-$) from the WT or IGFBP2-null BM 4 months after transplantation, and found significantly fewer IGF-IR-null HSCs in the IGFBP2-null environment than in the WT (supplemental Figure 5). This decrease was similar to the difference between WT HSC repopulation in the WT and IGFBP2-null environments (compare supplemental Figures 3,5).

We then performed secondary competitive BM transplantation 4 months after primary transplantation similar to the experiment shown in Figure 3A. The donors from the WT environment repopulated more efficiently than the donors from IGFBP2-null environment in the short-term ($47.11 \pm 9.28\%$ vs $21.59 \pm 6.64\%$) and long-term ($16.61 \pm 3.37\%$ vs $4.89 \pm 1.41\%$) as shown in Figure 5D. Donors from both environments had normal differentiated lineages (Figure 5E). Therefore, HSCs that were defective in IGF-IR signaling had decreased repopulation in IGFBP2-null recipients. This result can be explained by 2 possibilities: (1) IGFBP2 stimulates IGF-IR signaling, or (2) IGFBP2's regulation of HSC activity is independent of IGF-IR signaling. Because we found that, as demonstrated by numerous other studies, IGFBP2 in fact blocks the binding of IGF to IGF-IR (not shown), the first possibility does not exist. Our result thus suggests that the environmental effect of IGFBP2 on HSCs is independent of IGF-IR–mediated signaling.

Discussion

Previously, we showed that IGFBP2 stimulated ex vivo expansion of mouse and human HSCs.[6,7] Here, we demonstrated that, even though IGFBP2 does not have a significant cell-autonomous effect on HSCs, it supports HSC repopulation as an extracellular factor in the bone marrow in vivo. In principle, the homeostasis of HSCs is regulated by self-renewal, apoptosis, differentiation, and mobility. Our data showed that the decreased number and activity of HSCs in IGFBP2-null mice is because of increased apoptosis and slower cycling of the HSCs. Furthermore, we found that the C-terminus but not the RGD domain of IGFBP2 was essential to its HSC supportive activity, and that the effect of IGFBP2 on HSCs was independent of IGF-IR mediated signaling.

To our knowledge, this is the first demonstration that IGFBP2 supports survival and cycling of HSCs in vivo. The increased repopulation in secondary transplant suggests that increased cycling involves increased self-renewal. Indeed, HSCs from the IGFBP2-null mice have decreased expression of antiapoptotic molecule Bcl-2 and increased levels of multiple cell cycle inhibitors including p21, p19, p16, p57, and PTEN. This is consistent with the report that IGFBP2 suppresses PTEN expression[41] and supports the survival and expansion of glioma cells,[13] glioma cancer stem cells,[17] and epidermal progenitors.[42] This is also in accord with the report that CCN3/NOV, a protein containing an IGFBP domain, supports expansion of human cord blood HSCs,[43] and that IGFBP2 may be important for supporting the activity of fetal liver HSCs.[44] Whether IGFBP2 has similar effects on differentiated blood cells is unclear. In fact we observed opposite trends in HSCs and in certain CFUs in IGFBP2-null mice, suggesting either that the effects of IGFBP2 on HSCs and differentiated blood cells are different or that the increases in certain CFUs are caused by a compensatory effect. If the first possibility holds true, IGFBP2 may inhibit HSC differentiation, concordant with previous reports that IGFBP inhibits the differentiation of adipose progenitors.[45,46] These possibilities need to be investigated in the future.

A major question in IGFBP biology is whether the effect of IGFBP is IGF-dependent, and whether the IGFBP2 acts in an extracellular or intracellular manner. We found that HSCs express little IGFBP2 so it should not play a significant cell-autonomous role. Our results suggest that the effect is extrinsic and independent of the IGF signaling receptor IGF-IR. First, IGFBP2 supported ex vivo expansion of HSCs in serum-free medium that did not contain IGFs,[6] and extrinsic IGFBP2 rescued the defects of IGFBP2-null BM stroma in supporting the repopulation of cocultured HSCs. Second, different deletion mutants of extrinsic recombinant IGFBP2 showed different effects. In particular, we found that the C-terminus of IGFBP2 is important for HSC function. The C-terminus of IGFBP2 is known to exist as a native fragment in vivo and bind to cell surface, trigger MAP activation, and stimulate cell growth.[40] Third, the expression of some genes essential for survival and cycling is significantly altered in IGFBP2-null HSCs. Lastly, IGF-IR-null donor HSCs did not differ from WT HSCs in repopulation activity in IGFBP2-null recipient mice. It has been shown that both extrinsic and intrinsic IGFBP2 binds to integrin $\alpha5\beta1$[9,14]; although our data suggested that integrin $\alpha5\beta1$ may not mediate IGFBP2's extrinsic effect on HSC function/expansion, it is still possible that IGFBP2 binds to other surface receptor(s) on HSCs. Recently, Zhu et al showed that several IGFBPs, including IGFBP4 and IGFBP2, are inhibitors of canonical Wnt signaling in cardiomyocytes by binding to Frizzled 8 receptor and LDL receptor-related protein 6.[22] Fleming et al used DKK1-transgenic mice to show that Wnt activation in the niche is required to enforce HSC quiescence and to limit their proliferation.[35] Therefore, the regulation of Wnt target genes by extrinsic IGFBP2 and the quiescent phenotype of IGFBP2-null HSCs suggest that IGFBP2 may also modulate Wnt signaling in HSCs. Other unidentified surface receptor(s) for IGFBP2 may also exist. Further investigation will clarify this issue.

The coculture of HSCs and BM stroma showed that IGFBP2-null stroma had dramatically decreased ability to support HSC expansion, suggesting that IGFBP2 regulates HSCs in the local BM microenvironment. We know of the existence of at least 2 BM HSC

niches: the endosteal niche and vascular niche.[47] Recently, it was demonstrated that Nestin-expressing mesenchymal stem cells represent a unique niche.[48] IGFBP2-null mice have male-specific defects in osteoblasts,[25,26] but we found that the decreased HSC numbers and activities in IGFBP2-null mice are gender independent. Therefore the environmental effect of IGFBP2 may not come from osteoblasts in the BM. In fact, the expression of IGFBP2 in BM stroma suggests that mesenchymal stromal cells may be an important source of IGFBP2. Because IGFBP2 is overexpressed by the AKT-activated but not MAPK-activated endothelial cells and is essential for the ability to support expansion of HSCs,[24] and we did not detect high IGFBP2 level in BM $CD45^{-}CD31^{+}$ cells, we speculate that these $CD45^{-}CD31^{+}$ BM cells used in our analysis were not enriched for the HSC-supportive activated endothelial cells. New markers that allow identification of different functional types of endothelium will help the study in the future. Furthermore, in the BM, in addition to its direct effect to HSCs, IGFBP2 may support HSC activity indirectly. For example, IGFBP2 can inhibit the differentiation of adipocyte precursors and decrease fat accumulation.[46] Consistently, we observed IGFBP2-null mice were more obese than WT mice (data not shown). Because fat cells in the BM negatively regulate HSC activity,[49] it is possible that the increased fat in IGFBP2-null BM contributes to decreased HSC activity.

What is the relationship between quiescence and apoptosis in IGFBP2-null HSCs? While we are not certain if these 2 fates are independent events, there is a possibility that apoptosis causes compensatory quiescence of IGFBP2-null HSCs. For these HSCs, if apoptosis continues over time, the HSCs pool should be exhausted as the mice age. However, our data suggest that their HSC levels remained constant as the mice get to 1 year of age. Thus, we speculate that IGFBP2-null HSCs become more quiescent as a secondary effect to counteract the apoptosis. This is supported by previous reports that apoptosis led to quiescence of cells and slowed cell death.[50] Either way, our results indicate that it is not necessary that more quiescent HSCs have higher repopulation rates. Similarly, $AKT1^{-/-}AKT2^{-/-}$ HSCs are more quiescent but have lower repopulation efficiencies than WT HSCs.[51]

In addition to its expression during embryonic development and in normal adulthood, IGFBP2 is overexpressed in many tumors and its expression appears to correlate with the grade of malignancy.[9-11] The level of IGFBP2 is low in well-differentiated tumors but high in poorly differentiated tumors.[12] In the hematopoietic system, a lower IGFBP2 level is associated with the survival of patients with acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL)[28,29] and the expression of IGFBP2 is an independent factor for the prediction of relapse of AML and ALL.[28,30-32] Our previous data showed that the presence of IGFBP2 in the medium, together with other growth factors, resulted in significant expansion of mouse and human HSCs ex vivo.[6,7] The in vivo data shown here suggests that IGFBP2 directly or indirectly promotes self-renewal and survival of HSCs. Therefore it is reasonable to speculate that IGFBP2 also plays a role in supporting the activity of certain leukemia stem cells.

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Authorship

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