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14. ABSTRACT

We found that IGFBP2 supports the activity of AML stem cells and AML development. Using the retroviral MLL-AF9-IRES-YFP transplantation AML mouse model, we showed that IGFBP2 was expressed in normal hematopoietic cells, leukemia cells, and bone marrow stroma. The IGFBP2-null mice have decreased ability to develop AML in our AML mouse model, suggesting that the environmental IGFBP2 positively supports AML development. Consistently, IGFBP2 upregulated the expression of a number of oncogenes, and decreased the expression of certain tumor suppressor genes in AML stem cells. IGFBP2 is expressed by a variety of mouse bone marrow stromal cells, enriched in CD29+ CD44+ SSEA4+ Sca-1+ cells; these IGFBP2-producing cells are adjacent to endothelial cells in mouse BM. The environmental effect of IGFBP2 on AML stem cells is not dependent on IGF signaling. These results, together with our flow cytometry analysis of the phenotypic AML stem cells, concluded that IGFBP2, as an environmental factor, supports the activity of AML stem cells.

15. SUBJECT TERMS
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Introduction

Acute myeloid leukemia (AML) affects approximately 9,000 individuals in the United States annually. A minority of patients are cured by current therapy and the majority relapse within 5 years despite continuous treatment [1]. It was found that, the expression of a secreted protein IGFBP2 is correlated with the development of AML and acute lymphoblastic leukemia (ALL) [2-4]. The expression of IGFBP2 is significantly higher in childhood AML patients than it is in normal children [3] and its level is significantly associated with the event-free survival of the patients with AML [5]. Patients who relapsed later had higher IGFBP-2 expression [2-4]. A multivariate analysis identified IGFBP-2 mRNA expression as an independent factor for the prediction of relapse [2].

IGFBP2 is a member of the IGFBP family; this family contains at least six circulating proteins that bind IGF-1 and IGF-2 with an affinity equal or greater than that of the three IGF receptors. IGFBPs modulate the biological effects of IGFs by controlling IGF distribution, function, and activity [6]. IGFBP2 preferentially binds IGF-2 over IGF-1. IGFBP2 is expressed in the fetus and in a number of adult tissues and biological fluids [7]. It is also overexpressed in many tumors and in some cases its expression level correlates with grade of malignancy [8-10]. The level of IGFBP2 appears to be low in well-differentiated tumors but high in poorly differentiated tumors [11].

The known functions of IGFBP2 are very interesting. IGFBP2 displays IGF-dependent inhibitory effects on normal somatic growth [12]. However, several studies have demonstrated that IGFBP2 has intrinsic bioactivities that are independent of IGF-1 and IGF-2. For example, IGFBP2 binds to the cell surface [8, 13] and its binding to integrin α5 [8, 14, 15] or αv [16] influences cell mobility [8, 14, 15] and proliferation [9, 10]. IGFBP2 suppresses PTEN, stimulates telomerase activity [9], activates MMP-2 [17], and modulates MAPK and PI3K/Akt activations [9] [14]. In addition, oxidative stress leads to the uptake of IGFBP2 into the cell cytosol after 12-24 h [10, 18]. Although IGFBP2 deficient mice have decreased spleen weights and total splenic lymphocyte numbers [19], the roles of IGFBP2 in the hematopoietic system are largely unknown. We recently identified IGFBP2 as a secreted factor that supports the *ex vivo* expansion of mouse and human HSCs [20, 21].

The role of IGFBP2 in AML development is unknown. Since IGFBP2 can act as an inhibitory regulator of the IGF ligands by binding and forming a binary complex with them [12], it is possible that an increase in the concentration of IGFBP2 leads to the binding of IGFs and a decrease in the concentration of free IGFs. This may result in the attenuation of IGF signaling. However, whether IGF signaling is involved in AML pathogenesis is an open question. Alternatively, IGFBP2 may regulate AML in an IGF-independent manner. As the expression of IGFBP2 is positively correlated with human AML relapse, and our preliminary studies showing that IGFBP2 regulates HSC expansion and AML development, it is possible that AML-SCs may be a direct cell target of IGFBP2.

In this DOD supported study, we will investigate the possible roles of cell-autonomous and non-cell-autonomous regulation of AML by IGFBP2. In Aim 1, we will study the dynamic relationship between non-leukemic IGFBP2-expressing stromal cells and AML stem cells in the bone marrow. In Aim 2, we will study whether IGFBP2 has a cell-autonomous role in AML stem cells. Finally, as described in Aim 3, we will study how IGFBP2 regulates the cell fates of AML stem cells These complementary experiments will provide important insight into the role of IGFBP2 in regulation of cell fates of AML stem cells, and greatly benefit the diagnosis and treatment of AML.

Body

We successfully achieved Milestones 1 (Animal and human use approval) and Milestone 2 (Clarification of the environmental role of IGFBP2), including all sub-tasks in Task 1 and sub-task 2a in Task 2 of the originally proposed SOW. Some of the important progresses are listed below.

Task 1. Dissect the role of IGFBP2 in the potential niche of AML stem cells (AML-SCs) in the mouse AML model (months 1-20):

1a. Obtain approval of animal use and human IRB exemption (months 1-4).

All the animal protocols and exemption of human IRB were approved before the end of 2011.

1b. Determine whether IGFBP2 regulates AML-SCs in vivo (months 4-10).

While we showed that extrinsic IGFBP2 and IGFBP2 from the bone marrow (BM) environment supports the number and activity of mouse normal hematopoietic stem cells (HSCs) (see appendix 1 and 2: Huynh H, Zheng J, Umikawa M, Zhang C, Silvany R, Iizuka S, Holzenberger M, Zhang W, Zhang CC. 2011. IGF Binding Protein 2 Supports the Survival and Cycling of Hematopoietic Stem Cells. Blood 118:3236-3243; Zheng J, Umikawa M, Zhang S, Huynh H, Silvany R, Chen BPC, Chen L, Zhang CC. 2011. Ex vivo expanded hematopoietic stem cells overcome the MHC barrier in allogeneic transplantation. Cell Stem Cell 9:119-130), we developed an MLL-AF9 retrovirus transplantation mouse model as shown in the original proposal in order to study the environmental effect of IGFBP2 on AML. To this end, we transplanted MLL-AF9 retrovirus infected bone marrow cells into wild-type (WT) or IGFBP2 deficient mice. AML development in IGFBP2 deficient recipients was significantly slower than in wild-type mice (Fig. 1). We also found that AML-SCs in the IGFBP2-deficient recipients were lower than in WT recipients.

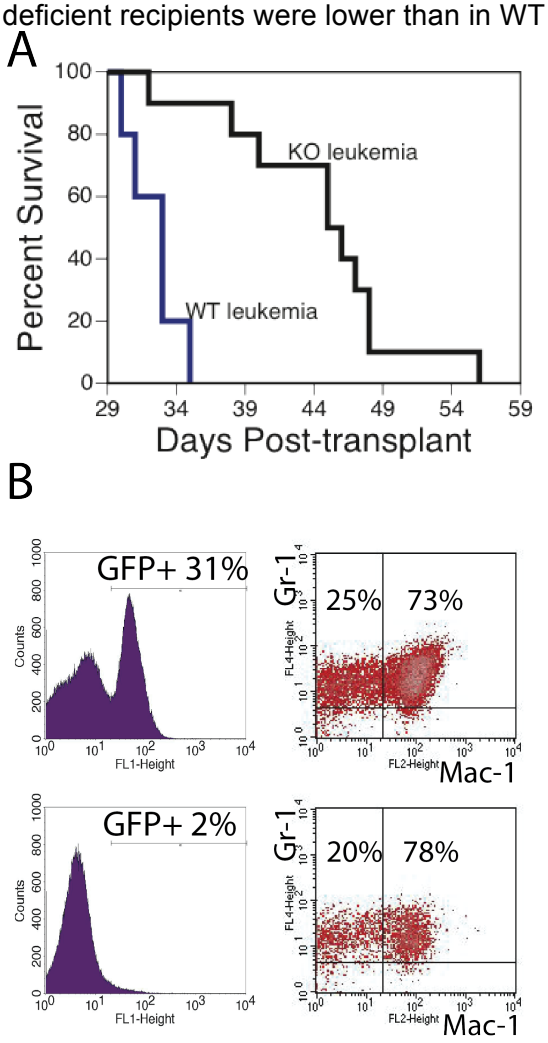

Figure 1. IGFBP2 supports AML development *in vivo* and AML-SC activity *in vitro*. (A) Loss of IGFBP2 in recipient mice leads to slower progression of acute myeloid leukemia. Survival curve of WT and IGFBP2-deficient mice receiving MLL-AF9-infected WT hematopoietic progenitors. $p < 0.05$. Data shown are from one of three independent experiments. (B) Representative flow cytometry plots showing AML development in WT mice (upper plots) and in IGFBP2-deficient mice (lower plots). While the AML development in GFP+ cells reflected by the Mac-1 and Gr-1 staining in peripheral blood remains similar in WT and IGFBP2-deficient mice, the significant increased GFP^+ percentages in WT recipients compared to IGFBP2-deficient recipients indicates that AML development in WT mice is more severe than in IGFBP2-deficient mice.

To directly test the effect of IGFBP2 on AML-SC growth, we isolated Mac-1$^+$Kit$^+$ cells from MLL-AF9 AML mice, and cultured them in the presence or absence of IGFBP2. IGFBP2 increased the growth of these AML-SCs (Fig. 2).

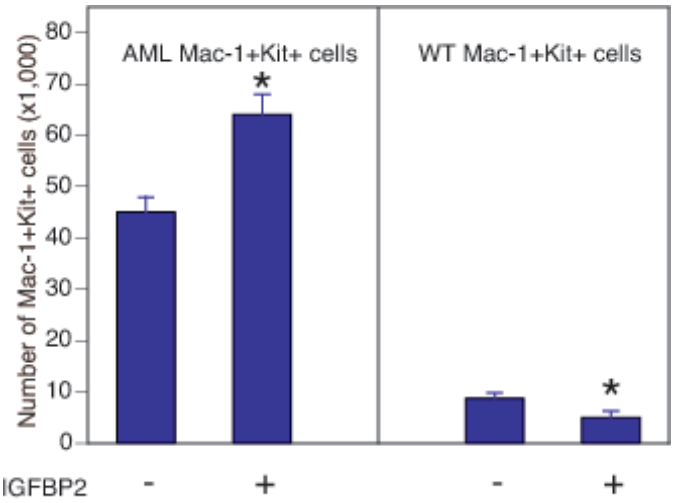

Figure 2. IGFBP2 supports ex vivo growth of AML-SCs but not wild-type myeloid progenitors. Three thousand bone marrow Mac-1$^+$Kit$^+$YFP$^+$ cells were isolated by FACS from MLL-AF9-IRES-YFP induced AML mice and cultured in SCF, IL-3, IL-6, with or without IGFBP2 (left). The same numbers of wild-type Mac-1$^+$Kit$^+$GFP$^+$ cells were isolated from control GFP mice and cultured in the same conditions (right). After 5 days, the Mac-1$^+$Kit$^+$ cells from cultures were isolated and measured by flow cytometry analysis. n = 6. *, P < 0.05.

We further measured gene expression in AML-SCs in WT and IGFBP2-deficient BM, and identified a number of genes that are regulated by the environmental IGFBP2 in AML-SCs (Fig. 3).

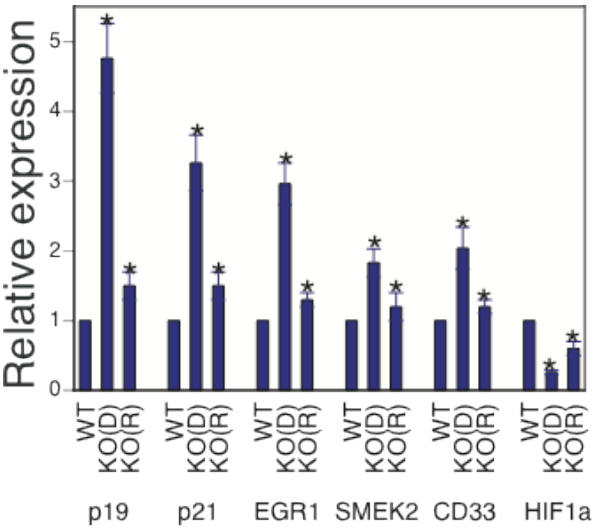

Figure 3. IGFBP2 regulates gene expression in AML-SCs. Real-time RT-PCR results were shown in AML-SCs that were isolated from WT recipient mice (WT), IGFBP2-deificient donor (KO(D)), or IGFBP2-deficient recipients (KO(R)).

In summary, we found that the IGFBP2-null mice have decreased ability to develop AML in our MLL-AF9 AML mouse model, suggesting that the environmental IGFBP2 positively supports AML development. Consistently, IGFBP2 upregulated the expression of some oncogenes and invasive genes, and decreased the expression of certain tumor suppressor and pro-apoptotic genes in AML stem cells. These results, together with our flow cytometry analysis of the phenotypic AML stem cells, concluded that IGFBP2 supports the activity of AML-SCs in the BM environment.

1c. Determine the immunophenotye of IGFBP2-producing cells in mouse bone marrow (months 4-10).

We showed that IGFBP2 was expressed in normal differentiated hematopoietic cells and bone marrow stroma, but little in HSCs (Appendix 1, Fig. 2A in Huynh H, Zheng J, Umikawa M, Zhang C, Silvany R, Iizuka S, Holzenberger M, Zhang W, **Zhang CC**. 2011). IGF Binding Protein 2 Supports the Survival and Cycling of Hematopoietic Stem Cells. Blood 118:3236-3243). Importantly, IGFBP2 is expressed significantly higher in AML-SCs than in HSCs (Fig. 4).

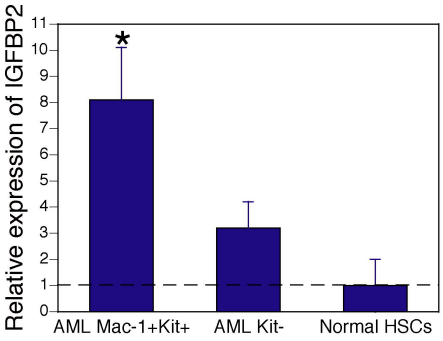

Figure 4. IGFBP2 is expressed in AML-SCs. Real-time RT-PCR results were shown in AML-SCs (AML Mac-1+Kit+), differentiated AML cells (AML Kit-), and HSCs.

We further showed that non-hematopoietic CD45$^-$ stromal cells had approximately 8-fold higher levels compared to HSCs (see appendix 1, Fig. 2A in Huynh H, Zheng J, Umikawa M, Zhang C, Silvany R, Iizuka S, Holzenberger M, Zhang W, **Zhang CC**. 2011. IGF Binding Protein 2 Supports the Survival and Cycling of Hematopoietic Stem Cells. Blood 118:3236-3243). 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 other cells types (Fig. 5).

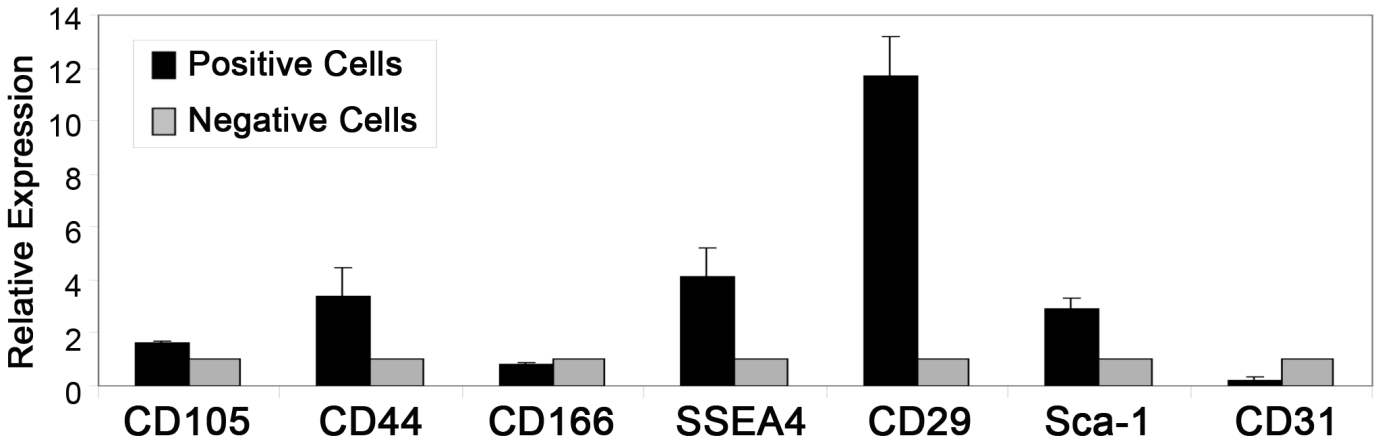

Figure 5. The expression pattern of IGFBP2 in BM stroma. Various BM CD45$^-$ stromal cell populations were isolated, and *IGFBP2* expression was determined by real-time RT-PCR (n = 2-3).

1d. Study the spatial relationship of IGFBP2-producing stromal cells with HSCs (months 4-10).

In order to study the spatial relationship of IGFBP2-expressing cells and the bone-marrow HSC niche cells, we examined mouse BM or G-CSF-mobilized spleen frozen sections. Markers that were validated in previous studies were used to confirm that IGFBP2$^+$ cells are adjacent to MECA-32$^+$ sinusoidal endothelium (as a known HSC niche [22]) in BM and in G-CSF-mobilized spleen in frozen sections (Fig. 6). As also shown in Fig. 7, the co-culture of IGFBP2-producing BM stromal cells with AML-SCs supports the proliferation of AML-SCs.

1e. Test whether IGFBP2-producing cells are mesenchymal stem cells (months 8-20).

We isolated BM stromal CD29$^+$ CD44$^+$ SSEA4$^+$ Sca-1$^+$ cells that are enriched for IGFBP2 expression, and performed a standard CFU-F assay to test whether they are enriched for mesenchymal stem cells. While the standard CFU-F assay showed that normal BM cells can produce standard CFU-F in our condition (as a positive control), these stromal IGFBP2-producing cells do not enrich CFU-F. Our results suggest that IGFBP2 –producing cells are not enriched for mesenchymal stem cells.

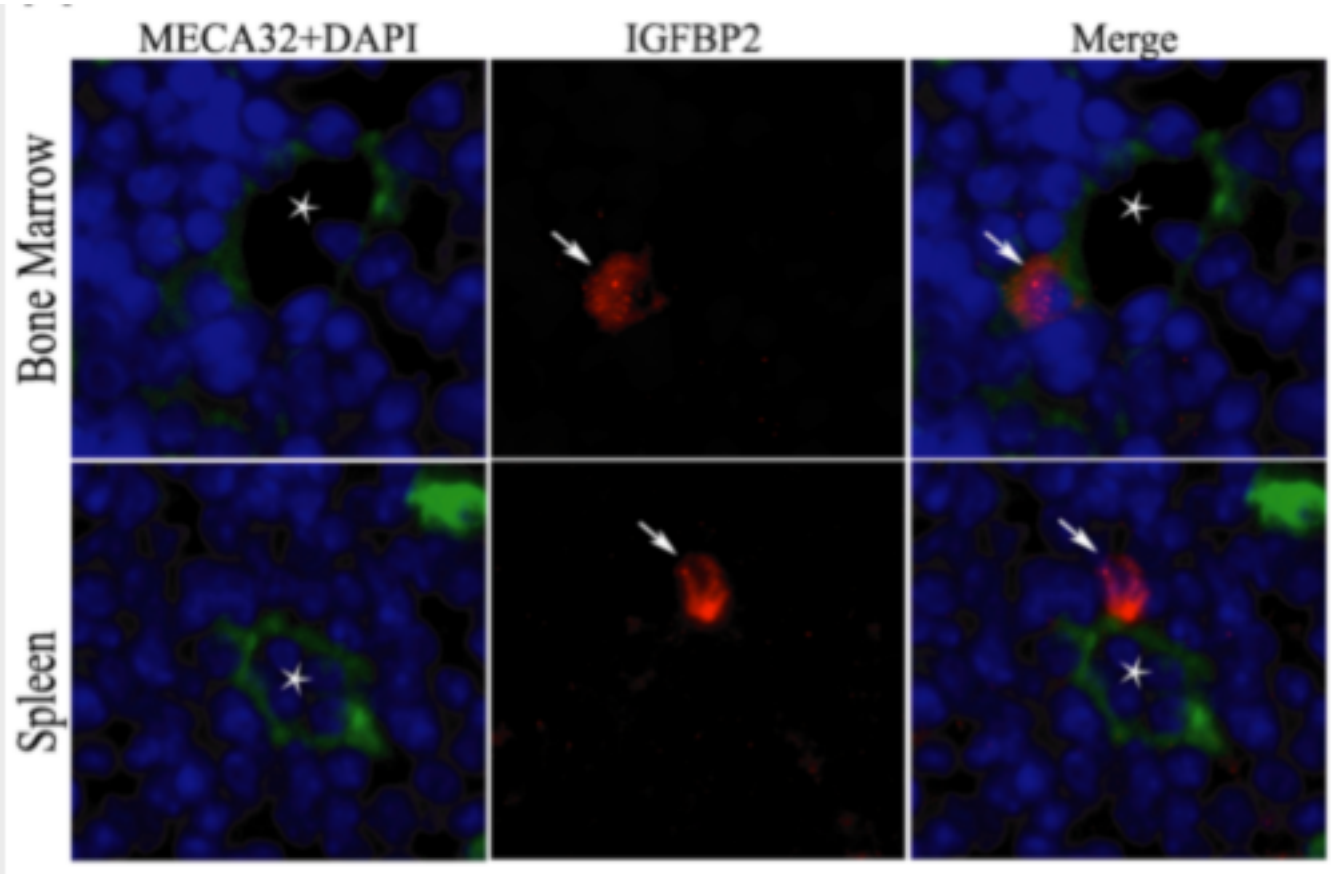

Figure 6. IGFBP2$^+$ cells are adjacent to sinusoidal endothelial cells in mouse bone marrow and in G-CSF-mobilized spleen. Bone marrow and spleen were stained to reveal IGFBP2$^+$ cells (red), Meca-32$^+$ sinusoidal endothelial cells (green), and nuclei (DAPI, blue). Twenty-nine of 35 (83%) of IGFBP2$^+$ cells were in contact with sinusoidal endothelial cells in bone marrow.

1f. Determine whether the environmental effect of IGFBP2 is IGF-dependent (months 12-16).

Because the C-terminus of IGFBP2 is involved in both IGF binding [23] and IGF-independent signaling [24], we sought to determine whether IGFBP2's effect on AML-SCs depended on IGF signaling. Here, we compared the AML-SC activities of IGF-IR-null donor cells in WT and IGFBP2-null recipients. We demonstrated that the IGF-IR-null HSCs repopulating ability was similar to WT counterparts with no defects in differentiation (see appendix 3, Fig 4A-B in Huynh H, Zheng J, Umikawa M, Silvany R, Xie XJ, Wu CJ, Holzenberger M, Wang Q, Zhang CC. 2011. Components of the hematopoietic compartments in tumor stroma and tumor-bearing mice. PLoS ONE 6: e18054). Next, we transplanted IGF-IR-null MLL-AF9 infected cells into WT or IGFBP2-null mice. We measured the donor AML-SCs from the WT or IGFBP2-null BM 4 months after transplantation, and found significantly fewer IGF-IR-null AML-SCs in the IGFBP2-null environment than in the WT (7.11 ± 1.28% vs. 4.59 ± 0.64%). This decrease was similar to the difference between WT AML-SC activities in the WT and IGFBP2-null environments.

Therefore, AML-SCs that were defective in IGF-IR signaling had decreased number and activity in IGFBP2-null recipients. This result can be explained by two possibilities: 1) IGFBP2 stimulates IGF-IR signaling, or 2) IGFBP2's regulation of AML-SC 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 AML-SCs is independent of IGF-IR-mediated signaling.

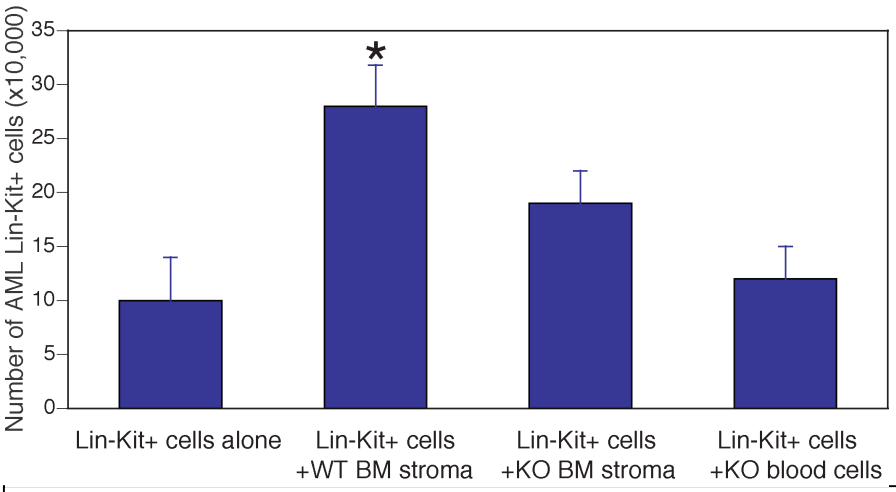

Figure 7. IGFBP2 deficient bone marrow stroma has decreased ability to support growth of AML-SCs. One thousand bone marrow $Lin^{-}Kit^{+}YFP^{+}$ cells were isolated by FACS from MLL-AF9-IRES-YFP induced AML mice and cultured alone alone (bar 1), or co-cultured with 3,000 CD45- bone marrow stromal cells isolated from CD45.2 wild-type mice (bar 2), or with the same number of stromal cells from IGFBP2 deficient mice (bar 3), or with $CD45^{+}$ $Sca\text{-}1^{-}$ differentiated hematopoietic cells from IGFBP2 deficient mice (bar 4), in IMDM supplemented with SCF, IL-3, and IL-6. After 5 days, the YFP^{+} cells from cultures were measured. n = 6, * significantly different from KO value.

Task 2. Study the cell-autonomous role of IGFBP2 in the mouse AML model (months 4-24):

2a. Study the AML development and AML-SC activity resulted from IGFBP2 deficient donor cells (months 8-12).

We showed that IGFBP2 has little cell-autonomous effect on normal HSCs, and this result is consistent with the finding that IGFBP2 is not significantly expressed in HSCs per se (see Appendix 1, Huynh H, Zheng J, Umikawa M, Zhang C, Silvany R, Iizuka S, Holzenberger M, Zhang W, Zhang CC. 2011. IGF Binding Protein 2 Supports the Survival and Cycling of Hematopoietic Stem Cells. Blood 118:3236-3243; Fig 2). However, since AML-SCs express significantly higher level of IGFBP2 than HSCs (Fig. 4), we hypothesized that IGFBP2 possibly has cell-autonomous effect on AML-SCs. To test whether IGFBP2 has cell-autonomous function on AML-SCs, we injected MLL-AF9 infected WT and IGFBP2-null donor cells into the same type of recipients. AML development was slowed in mice transplanted with KO donor cells (Fig. 8). The analysis of AML-SCs in WT and KO transplanted mice suggests that KO AML-SCs are lower as determined by flow cytometry analysis. Moreover, Cell-autonomous IGFBP2 in AML-SCs upregulates certain oncogenes and suppresses some tumor suppressors (Fig. 3). These results suggest that IGFBP2 in donor AML-SCs supports the activity of AML-SCs and AML development.

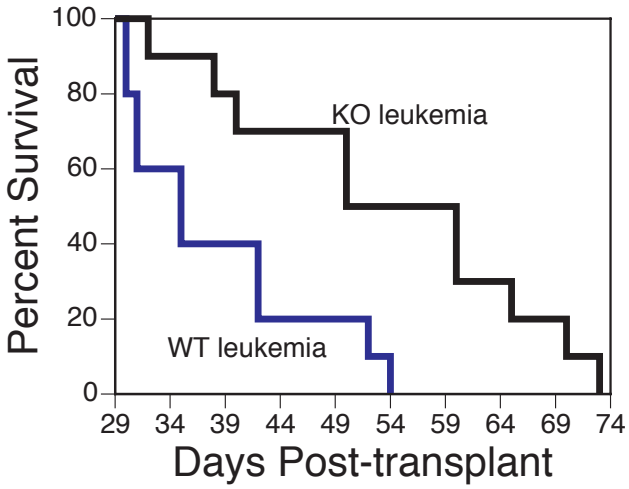

Figure 8. IGFBP2 expressed in AML-SCs supports AML development *in vivo*. Loss of IGFBP2 in donor AML-SCs leads to slower progression of acute myeloid leukemia. Survival curve of WT and IGFBP2-deficient mice receiving MLL-AF9-infected WT hematopoietic progenitors. $p < 0.05$.

Potential problems and solutions:

Since MLL-AF9 AML model is very potent, the mice develop AML symptom within 3 weeks post-transplant of the donor cells. While this model gives fast result, it would be helpful if we have another AML model that allows slower disease development. We performed in silico study of public databases of human diseases, and found that IGFBP2 is highly expressed in AML1-ETO AML patients. While we obtained an AML1-ETO retrovirus and planned to establish a mouse transplantation AML model, we was informed the inefficiency of this model to develop AML in a timely manner. Given this fact, we decided not to pursue the AML1-ETO model during the DOD supportive period. To overcome the potential problem of fast AML development of the MLL-AF9 model, we decreased the donor AML numbers for transplantation and found that we were able to slow down the AML development. This will help us to observe more significant difference in AML development between WT and IGFBP2-deficient donor cells or recipient mice.

Key Research Accomplishments

1. IGFBP2 expressed by the bone marrow environment in the mice support the activity of normal blood stem cells and AML stem cells
2. IGFBP2 is expressed by a variety of mouse bone marrow stromal cells, enriched in $CD29^+$ $CD44^+$ $SSEA4^+$ $Sca\text{-}1^+$ cells
3. IGFBP2-producing cells are adjacent to endothelial cells in mouse BM
4. IGFBP2-producing cells in the mouse BM are not enriched for mesenchymal stem cells
5. The environmental effect of IGFBP2 on AML stem cells is not dependent on IGF signaling
6. IGFBP2 is expressed on AML-SCs and has cell-autonomous effect to support AML-SC activity

Reportable Outcome

1. Peer-review publications

The Award directly supported the publication of the following paper by my lab:

a. Huynh H, Zheng J, Umikawa M, Zhang C, Silvany R, Iizuka S, Holzenberger M, Zhang W, Zhang CC. 2011. IGF Binding Protein 2 Supports the Survival and Cycling of Hematopoietic Stem Cells. Blood 118:3236-3243

b. Paper copy: Zheng J, Umikawa M, Zhang S, Huynh H, Silvany R, Chen BPC, Chen L, Zhang CC. 2011. Ex vivo expanded hematopoietic stem cells overcome the MHC barrier in allogeneic transplantation. Cell Stem Cell 9:119-130

c. Paper copy: Huynh H, Zheng J, Umikawa M, Silvany R, Xie XJ, Wu CJ, Holzenberger M, Wang Q, Zhang CC. Components of the hematopoietic compartments in tumor stroma and tumor-bearing mice. PLoS ONE. 2011 Mar 25;6(3):e18054.

2. Abstracts for meetings

a. American Society of Hematology, December 2010, Orlando, FL. IGF Binding Protein 2 Supports the Cycling of Hematopoietic Stem Cells, by HoangDinh Huynh, Junke Zheng, Masato Umikawa, Robert Silvany, and Cheng Cheng Zhang

b. American Society of Hematology, December 2010, Orlando, FL. Angptl3 regulates the homing of hematopoietic stem cells, by Masato Umikawa, Junke Zheng, Robert Silvany, and Cheng Cheng Zhang

c. Keystone Meeting of Stem Cells: January 2011, Santa Fe, NM. Ex vivo expanded hematopoietic stem cells overcome the MHC barrier in allogeneic transplantation, by Junke Zheng, and Cheng Cheng Zhang

d. Keystone Meeting of Stem Cells: March 2011, Big Sky, MT. IGF Binding Protein 2 Supports the Activity of Acute Myeloid Leukemia Stem Cells, by Junke Zheng, and Cheng Cheng Zhang

3. Degree awarded to graduate students

PhD awarded to HoangDinh Hyunh on April 15, 2011, UT Southwestern Medical Center. HoangDinh was a graduate student in my laboratory and was supported by this grant.

4. Reagents produced

Cell Line:
MSCV-IGFBP2 stable cell line

Mouse model:
IGFBP2 null MLL-AF9 AML model

5. Funding applied:

1. Cancer Prevention and Research Institute of Texas High Risk/High Reward Grant, “Metabolism of leukemia stem cells” 2012-2014
2. NIH R01, “Regulation of Hematopoietic Stem Cell Metabolism” 2012-2017

6. Employment:

Junke Zheng, PhD
Robert Silvany
Masato Umikawa, PhD
HoangDinh Hyunh
Xian-Jin Xie, PhD

Conclusion

The overall goal of the grant and the specific aims remain the same. We are making excellent progress in all the aims. We demonstrated that, 1) IGFBP2 expressed by the bone marrow environment in the mice support the activity of normal blood stem cells and AML stem cells, 2) IGFBP2 is expressed by a variety of mouse bone marrow stromal cells, enriched in $CD29^+$ $CD44^+$ $SSEA4^+$ $Sca\text{-}1^+$ cells, 3) IGFBP2-producing cells are adjacent to endothelial cells in mouse BM, 4) IGFBP2-producing cells in the mouse BM are not enriched for mesenchymal stem cells, 5) The environmental effect of IGFBP2 on AML stem cells is not dependent on IGF signaling, and 6) IGFBP2 is expressed on AML-SCs and has cell-autonomous effect to support AML-SC activity. The grant support so far resulted in publication of three peer-reviewed paper. The grant also supported our training of one graduate student, two postdoctoral researchers, and one technician. The continuous support of DOD will ensure our success in this important project.

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Appendices

1. Paper copy: Huynh H, Zheng J, Umikawa M, Zhang C, Silvany R, Iizuka S, Holzenberger M, Zhang W, Zhang CC. 2011. IGF Binding Protein 2 Supports the Survival and Cycling of Hematopoietic Stem Cells. Blood 118:3236-3243

2. Paper copy: Zheng J, Umikawa M, Zhang S, Huynh H, Silvany R, Chen BPC, Chen L, Zhang CC. 2011. Ex vivo expanded hematopoietic stem cells overcome the MHC barrier in allogeneic transplantation. Cell Stem Cell 9:119-130

3. Paper copy: Huynh H, Zheng J, Umikawa M, Silvany R, Xie XJ, Wu CJ, Holzenberger M, Wang Q, Zhang CC. Components of the hematopoietic compartments in tumor stroma and tumor-bearing mice. PLoS ONE. 2011 Mar 25;6(3):e18054.

4. Meeting abstract copy: American Society of Hematology, December 2010, Orlando, FL. IGF Binding Protein 2 Supports the Cycling of Hematopoietic Stem Cells, by HoangDinh Huynh, Junke Zheng, Masato Umikawa, Robert Silvany, and Cheng Cheng Zhang

5. Meeting abstract copy: American Society of Hematology, December 2010, Orlando, FL. Angptl3 regulates the homing of hematopoietic stem cells, by Masato Umikawa, Junke Zheng, Robert Silvany, and Cheng Cheng Zhang

6. Meeting abstract copy: Keystone Meeting of Stem Cells: January 2011, Santa Fe, NM. Ex vivo expanded hematopoietic stem cells overcome the MHC barrier in allogeneic transplantation, by Junke Zheng, and Cheng Cheng Zhang

7. Meeting abstract copy: Keystone Meeting of Stem Cells: March 2011, Big Sky, MT. IGF Binding Protein 2 Supports the Activity of Acute Myeloid Leukemia Stem Cells, by Junke Zheng, and Cheng Cheng Zhang

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

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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

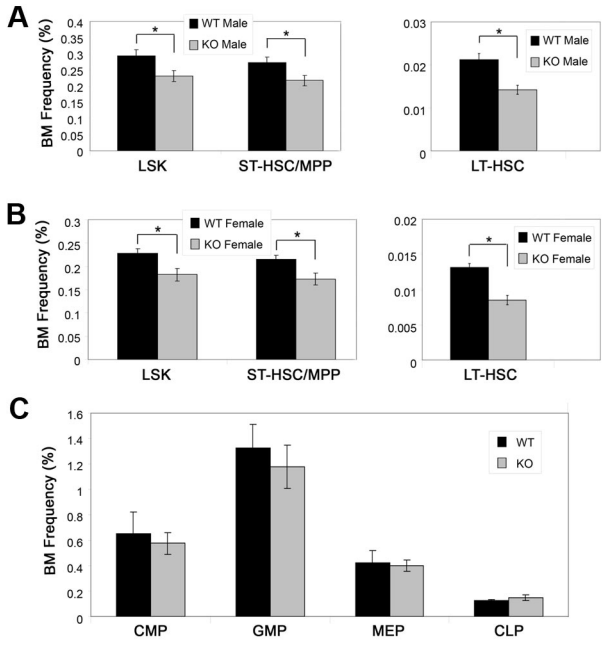

Figure 1. IGFBP2-null mice have fewer HSCs than wild-type mice. The frequencies of BM Lin$^-$Sca-1$^+$Kit$^+$ cells, ST-HSCs/MPPs as Lin$^-$Sca-1$^+$Kit$^+$Flk2$^+$CD34$^+$ cells, and LT-HSCs as Lin$^-$Sca-1$^+$Kit$^+$Flk2$^-$CD34$^-$ cells in IGFBP2-null and WT male littermates (A; n = 8) and female 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

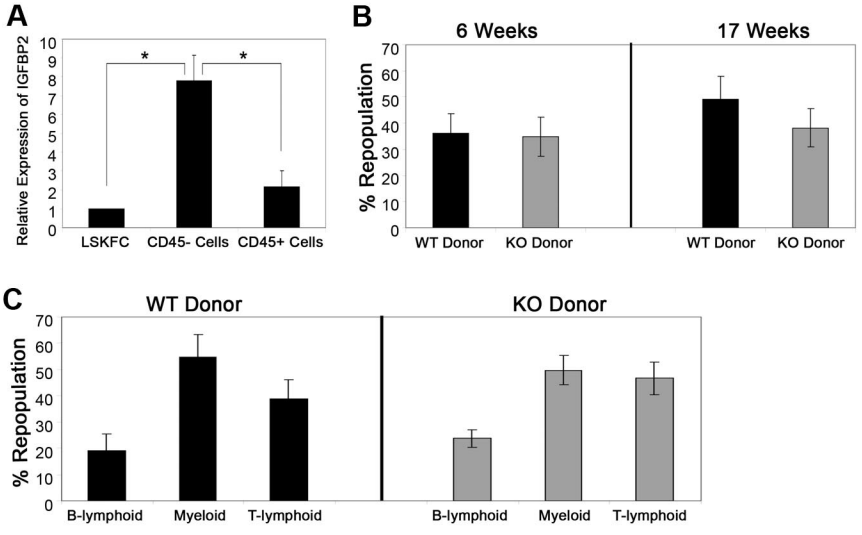

Figure 2. IGFBP2 has little cell-autonomous effect on HSCs. (A) Lin$^-$Sca-1$^+$Kit$^+$Flk2$^-$CD34$^-$, hematopoietic CD45$^+$, and nonhematopoietic CD45$^-$ stromal cells were freshly isolated from BM, and IGFBP2 gene expression was determined by real-time RT-PCR (n = 4-5). (B) Five hundred freshly 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.

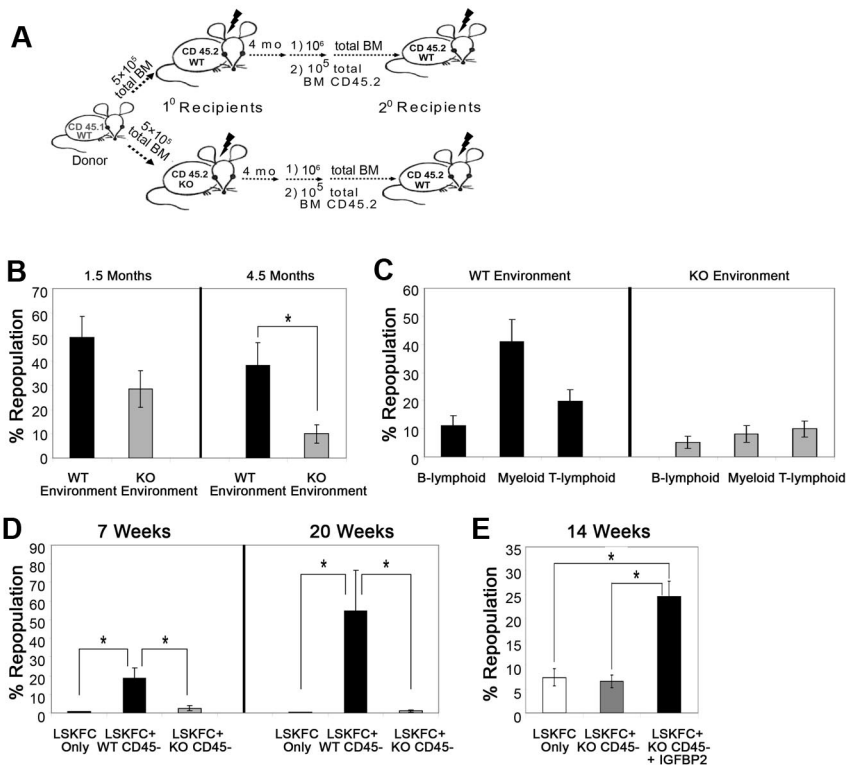

Figure 3. IGFBP2 supports the repopulation of HSCs in the BM environment. (A) A schematic representation of BM transplantation. (B) CD45.1 total BM cells (5×10^5) were transplanted into lethally irradiated CD45.2 WT or IGFBP2-null recipients for 4 months. Subsequently, 1×10^6 CD45.1 total BM cells from 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

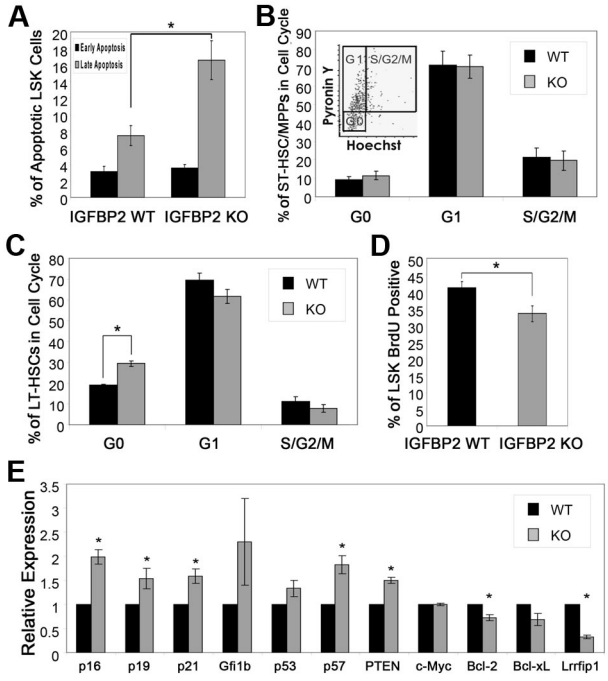

Figure 4. IGFBP2 supports the survival and cycling of HSCs in the BM. (A) Lin$^-$Sca-1$^+$Kit$^+$ cells from BM of WT or IGFBP2-null mice were analyzed for early apoptosis (annexinV$^+$7-AAD$^-$) and late apoptosis (annexinV$^+$7-AAD$^+$) markers (n = 6). (B) Cell cycle status of Lin$^-$Sca-1$^+$Kit$^+$Flk2$^+$CD34$^+$ 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).

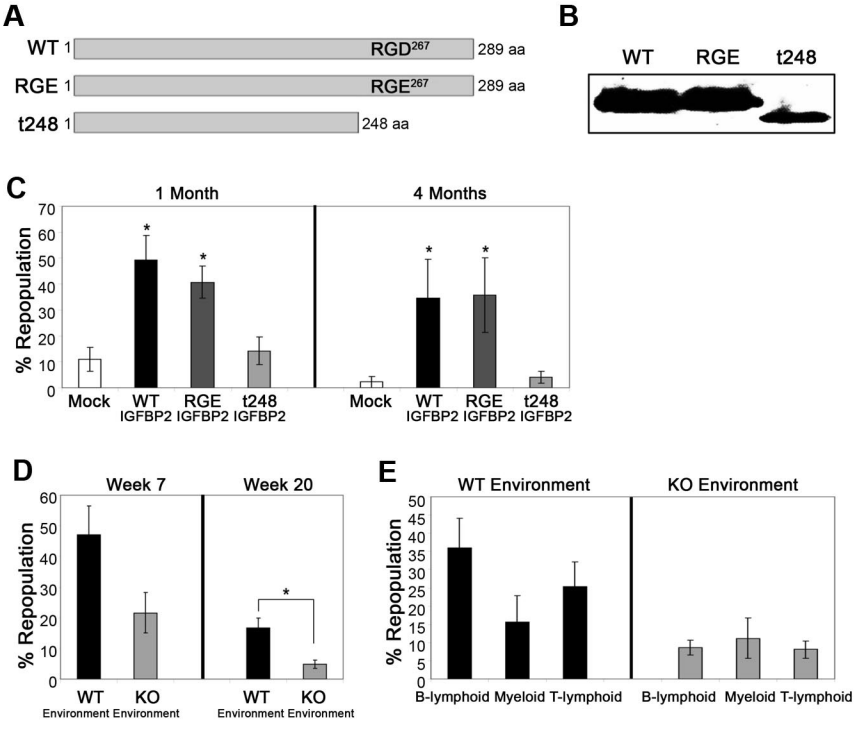

Figure 5. The C-terminus of IGFBP2 is essential for IGFBP2's HSC supportive activity. (A) Schematic representation for IGFBP2 mutants. (B) WT, RGE^{267}, and t248 IGFBP2 constructs were transfected into 293T cells and the levels of secreted IGFBP2 proteins in the media at 60 hours after transfection 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

Contribution: H.H. and C.C.Z. contributed to design, experimental performance, interpretation, and writing; J.Z., M.U., and R.S. contributed to experimental performance, interpretation, and writing; M.H. and W.Z. contributed to experimental performance and writing; and C. Z. and S.I. contributed to experimental performance.

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Cell Stem Cell
Article

Ex Vivo Expanded Hematopoietic Stem Cells Overcome the MHC Barrier in Allogeneic Transplantation

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SUMMARY

The lack of understanding of the interplay between hematopoietic stem cells (HSCs) and the immune system has severely hampered the stem cell research and practice of transplantation. Major problems for allogeneic transplantation include low levels of donor engraftment and high risks of graft-versus-host disease (GVHD). Transplantation of purified allogeneic HSCs diminishes the risk of GVHD but results in decreased engraftment. Here we show that ex vivo expanded mouse HSCs efficiently overcame the major histocompatibility complex barrier and repopulated allogeneic-recipient mice. An 8-day expansion culture led to a 40-fold increase of the allograft ability of HSCs. Both increased numbers of HSCs and culture-induced elevation of expression of the immune inhibitor CD274 (B7-H1 or PD-L1) on the surface of HSCs contributed to the enhancement. Our study indicates the great potential of utilizing ex vivo expanded HSCs for allogeneic transplantation and suggests that the immune privilege of HSCs can be modulated.

INTRODUCTION

Hematopoietic stem cells (HSCs) have been used in transplantation to treat patients with leukemia, lymphoma, some solid cancers, and autoimmune diseases (Bryder et al., 2006). In particular, allogeneic bone marrow (BM) transplantation is potentially curative for both inherited and acquired hematopoietic diseases (Gyurkocza et al., 2010). Two major problems, failure of engraftment and graft-versus-host disease (GVHD), have severely limited the progress in the field, however. Although the inclusion of donor T cells in transplantation enhances donor engraftment and has graft-versus-leukemia effects, it causes life-threatening GVHD. Transplantation of purified allogeneic HSCs diminishes the risk of GVHD but also results in decreased engraftment (Shizuru et al., 1996; Wang et al., 1997). It is not clear why most allogeneic HSCs cannot escape immune rejection and whether the allograft efficiency of HSCs can be improved. The resolution of these questions will promote the understanding of the immunology of HSCs and other stem cells and greatly improve the practice of allogeneic transplantation.

We recently developed an efficient culture system for ex vivo expansion of HSCs (Zhang and Lodish, 2008). This system is based on the use of serum-free culture medium supplemented with several growth factors including SCF, TPO, FGF-1/Flt3-L, IGFBP2, and angiopoietin-like proteins (Angptls) (Huynh et al., 2008; Zhang et al., 2006, 2008). In vivo studies suggested that Angptls are new molecular components of the microenvironment of fetal liver and adult HSCs (Chou and Lodish, 2010; Zheng et al., 2011), and Angptl1 and 2 are essential to HSC development in zebrafish (Lin and Zon, 2008, ASH 50th Annual Meeting, abstract). We and others have used this culture system to expand mouse and human HSCs for transplantation or genetic modification purposes (Akala et al., 2008; Carter et al., 2010; Chen et al., 2009; Drake et al., 2011; Heckl et al., 2011; Huynh et al., 2008; Khoury et al., 2011; Kiel et al., 2007; Stern et al., 2008; Zhang et al., 2006, 2008; Zhao et al., 2010). There are two important features of this HSC culture system: the increased number of repopulating HSCs (Huynh et al., 2008; Zhang et al., 2006, 2008) and the change of surface expression of many surface proteins (Zhang and Lodish, 2005). While the expansion of repopulating HSCs were validated by transplanting cultured HSCs into congeneic or immune-deficient mice in these previous studies, we hypothesized that ex vivo expansion of HSCs may also modulate the immunological properties of HSCs so that they possess an altered ability to cross the immune barrier upon allogeneic transplantation. To test this hypothesis, we started to compare the allograft abilities of freshly isolated HSCs and ex vivo expanded HSCs in allogeneic transplantation models.

RESULTS

Ex Vivo Expanded HSCs Have Dramatically Enhanced Allograft Ability

With a well-established mouse model for fully allogeneic transplantation (see Figure S1 available online), we compared the allograft abilities of freshly isolated and ex vivo expanded HSCs from CD45.1 C57BL/6 donors transplanted into lethally irradiated

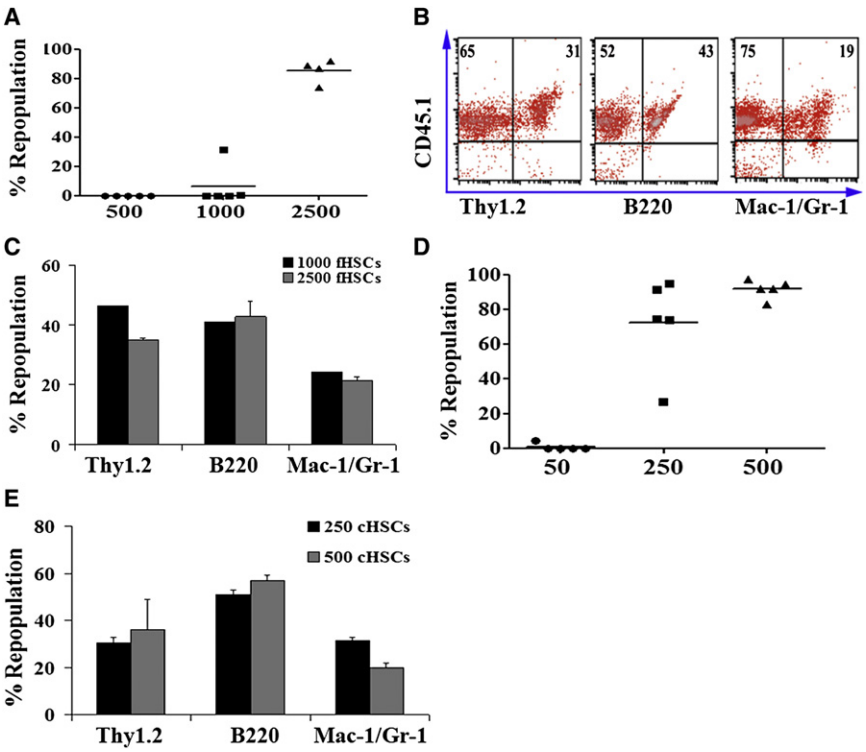

Figure 1. Ex Vivo Expanded HSCs Overcome MHC Barrier in Noncompetitive Allogeneic Transplantation

Indicated numbers of freshly isolated Lin$^-$Sca-1$^+$Kit$^+$CD34$^-$Flk2$^-$ HSCs (A–C) or their 8 day cultured progenies (D and E) from CD45.1 C57BL/6 donors were transplanted into lethally irradiated BALB/c (CD45.2) recipients without competitors (n = 4–5). HSCs were cultured in STFIA medium, which allows ex vivo expansion of HSCs (Zhang et al., 2006), and there were ~200-fold increase of total cells after 8 days of culture (with 1.16 ± 0.14 × 10^4 cultured cells derived from the input 50 cells).

(A and D) Numbers of mice with failed or successful donor engraftment after being transplanted by indicated numbers of freshly isolated HSCs or their cultured equivalents at 16 weeks posttransplant. The 0% repopulated mice included both survived and dead ones.

(B) Representative flow cytometry analysis of the multilineage repopulation of 2,500 freshly isolated HSCs at 16 weeks posttransplant.

(C and E) Multilineage contribution of indicated numbers of freshly isolated HSCs or cultured equivalent at 16 weeks posttransplant, respectively (n = 4–5).

Data are expressed as mean ± SEM. See also Figure S1.

BALB/c (CD45.2) recipients. The culture was performed in our optimized STFIA medium (Huynh et al., 2008; Zhang et al., 2006) for 8 days that allows ex vivo expansion of HSCs. Consistent with previously reported results (Shizuru et al., 1996; Wang et al., 1997), a relatively large number (1,000 or more) of freshly isolated BM Lin$^-$Sca-1$^+$Kit$^+$CD34$^-$Flk2$^-$ HSCs were needed for successful allograft (Figures 1A–1C). By striking contrast, the cultured progeny of 50 or more input equivalent HSCs were capable of achieving the same level of allograft (Figures 1D and 1E). Similar to freshly isolated HSCs, cultured HSCs were capable of multilineage differentiation in allogeneic mice (Figures 1B, 1C, and 1E) and no sign of GVHD was observed. This suggests that ex vivo expanded HSCs have enhanced allograft abilities compared with freshly isolated cells.

The above strategy may result in the death of mice when donor HSCs are not capable of engrafting recipients. To ensure that recipient mice survive after transplantation and to better quantitate the allograft abilities of different donor cells, we performed allogeneic transplantation by including competitors (Figure S1). These competitors are total BM cells freshly isolated from the same type of mice as the recipients; these cells provide short-term radio-protection and serve as internal controls but also significantly enhance the host immune rejection and increase the difficulty of donor engraftment. Figure 2 shows the result of a representative competitive allogeneic transplantation from donor C57BL/6 (CD45.1) to BALB/c (CD45.2) recipients. Although 10,000 freshly isolated CD45.1 C57BL/6 BM Lin$^-$Sca-1$^+$Kit$^+$CD34$^-$Flk2$^-$ HSCs failed to engraft into the BALB/c recipients in the presence of competitors (0%; Figure 2A, left), their cultured progenies had dramatically increased engraftment (55%; Figure 2A, right). Similar results were obtained from the measurement of major histocompatibility complex (MHC) markers of donors and recipients (Figure 2B). This allogeneic reconstitution sustained over time (Figure 2C) and the donor cells repopulated the lymphoid and myeloid lineages (Figures 2D and 2E), attesting to the engraftment of the donor long-term HSCs. Again, no sign of GVHD was observed in the transplanted mice. To test whether allogenic donor HSCs were tolerated in the host, we performed secondary transplantation by isolating BM cells from the primary recipients and transplanting them into secondary BALB/c recipients. We found that the original CD45.1 donor cells successfully repopulated secondary recipients (Figures 2F and 2G). The successful secondary transplantation indicates that the allogeneic donor HSCs were already tolerated after the primary transplantation. This result was further confirmed by the mixed lymphocyte reaction (MLR) experiment, showing that BALB/c T cells were not stimulated by the original donor-derived cells in primary transplanted mice, but reacted to the counterpart cells isolated from CD45.1 C57BL/6 mice (Figure 2H). Therefore, the competitive allogeneic transplantation (Figure 2) gave similar results as the noncompetitive allograft (Figure 1).

We further employed a third transplantation model to compare the abilities of donor HSCs before and after ex vivo expansion to engraft the allogeneic recipient mice with sublethal irradiation. Again the ex vivo expanded HSCs achieved markedly increased allograft compared to their uncultured counterparts (Figure S2A). All these results indicate a dramatic enhancement of allograft ability of HSCs after ex vivo expansion.

Moreover, to directly compare the allograft capacities of HSCs before and after ex vivo expansion, we cotransplanted freshly isolated CD45.2 C57BL/6 HSCs and ex vivo expanded progenies of CD45.1 C57BL/6 HSCs into the same BALB/c recipient

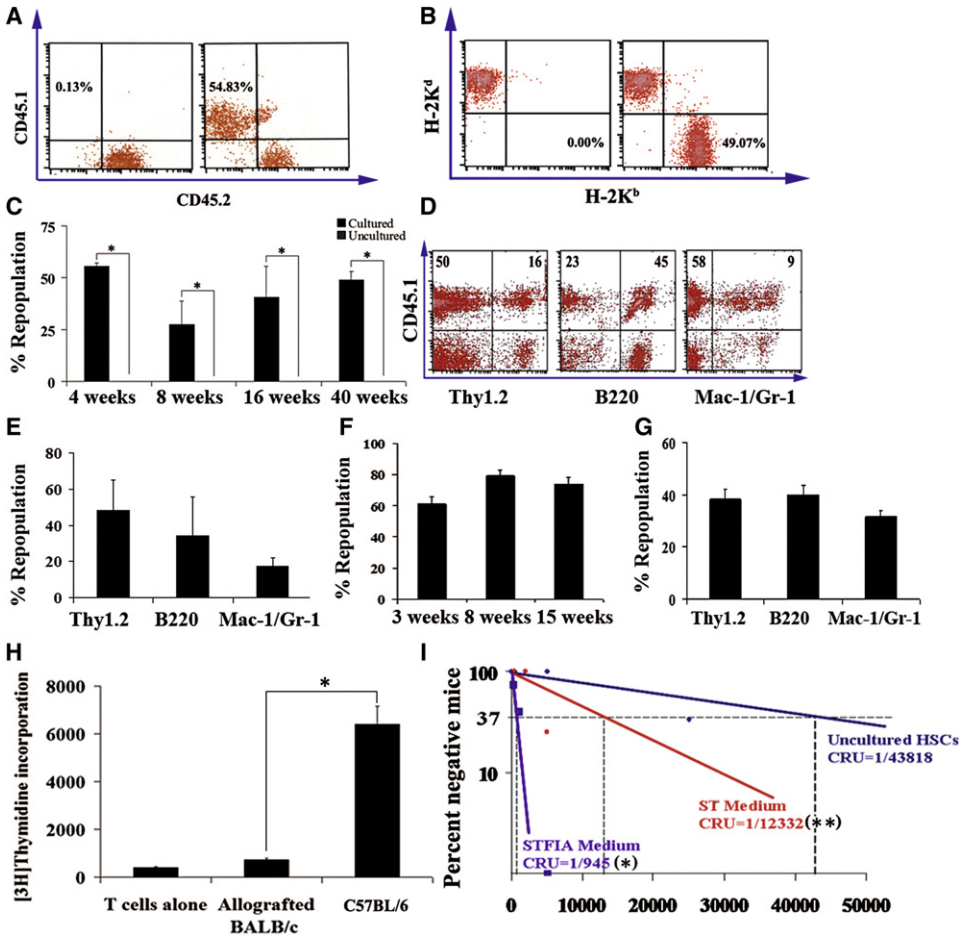

Figure 2. Ex Vivo Expanded HSCs Overcome MHC Barrier in Competitive Allogeneic Transplantation

(A–E) Freshly isolated 10,000 Lin$^-$Sca-1$^+$Kit$^+$CD34$^-$Flk2$^-$ HSCs or their 8 day cultured progenies from CD45.1 C57BL/6 donors were transplanted into lethally irradiated BALB/c (CD45.2) recipients along with 100,000 total bone marrow cells freshly isolated from BALB/c mice as competitors. HSCs were cultured in STFIA medium, which allows ex vivo expansion of HSCs. Similar results were obtained in at least two independently repeated experiments.

(A and B) Representative flow cytometry plots show that 10,000 freshly isolated donor HSCs had no engraftment (left), whereas the cultured progeny of 10,000 input donor HSCs (right) had significant engraftment (54.83% CD45.1 or 49.07% H-2K^b donor chimerism in A or B, respectively) in allogeneic recipients.

(C) Summary of donor engraftment in allogeneic recipients at 4, 8, 16, and 40 weeks posttransplant (*$p < 0.05$, n = 6).

(D and E) Multilineage contribution of cultured cells in allogeneic recipients at 16 weeks posttransplant (n = 6).

(F and G) Summary of donor engraftment at 3, 8, and 15 weeks after secondary transplantation into BALB/c mice (n = 5). Multilineage contribution of cultured cells in allogeneic recipients at 15 weeks posttransplant is shown (G).

(H) MLR assay was performed in which splenocytes from C57BL/6 mice stimulated the proliferation of BALB/c T cells (bar 3) whereas splenocytes isolated from cultured C57BL/6 donor HSCs repopulated BALB/c recipients lost the ability to stimulate the proliferation of BALB/c T cells (bar 2). *$p < 0.05$, n = 3.

(I) Comparison of the allograft abilities of freshly isolated and cultured HSCs by limiting dilution analysis. Three types of donor cells, including freshly isolated C57BL/6 CD45.1 Lin$^-$Sca-1$^+$Kit$^+$CD34$^-$Flk2$^-$, progenies after 8-day culture in ST medium (which does not support HSC expansion), and progenies after 8-day culture in STFIA medium (which supports HSC expansion), were compared. Transplantation into lethally irradiated BALB/c mice was conducted along with 100,000 total BM competitors isolated from BALB/c mice. Limiting dilution analysis was performed and L-Calc software was used to calculate the HSC frequency (*, **$p < 0.05$, compared to uncultured HSCs).

Data are expressed as mean ± SEM. See also Figures S1 and S2 and Table S1.

mice (Figure S2B). Ex vivo expanded HSCs demonstrated a clear advantage over freshly isolated HSCs in this direct competitive allograft setting (Figure S2B).

This ability of cultured HSCs to overcome the allogeneic barrier was not restricted to the use of particular allogeneic transplantation models. In addition to using C57BL/6 mice and BALB/c mice as the donor and recipient, respectively, we tested a number of other donor/recipient combinations and reached the same conclusion. For example, ex vivo expanded HSCs isolated from FVB (CD45.1) mice had much greater ability to repopulate CD45.2 C57BL/6 recipients than their freshly isolated counterparts (Figure S2C).

Both the Increase of HSC Numbers and Expansion-Independent Characteristics Acquired during Ex Vivo Culture Contribute to the Improved Allograft Efficiency

Because we used a culture system that expands HSCs, we sought to determine the contribution of the increase of HSC

numbers during ex vivo expansion to the increased allograft ability by conducting limiting dilution analyses (Huynh et al., 2008; Zhang et al., 2006; Zheng et al., 2011). First, we used competitive syngeneic transplantation to calculate the numbers of repopulating CD45.1 C57BL/6 BM HSCs before and after ex vivo expansion. When we cultured HSCs in the optimized STFIA medium for 8 days, we obtained 11-fold (= 69/6; Table S1A, Figure S2D) expansion in the number of HSCs as determined by limiting dilution analysis in syngeneic transplantation. Next, we quantitated the allograft abilities of these HSCs before and after culture by competitive allogeneic transplantation into BALB/c mice. For freshly isolated donor HSCs, the frequency of allograftable cells was 1/43,818, whereas the frequency in those cells cultured in the STFIA medium was 1/945 of input equivalent cells, determined by the competitive allogeneic transplantation (Figure 2I; Tables S1B and S1C). This represents a ~40-fold (= 43,818/945) increase of allograft ability when cells were ex vivo expanded. Hence, in this experiment, ex vivo expansion led to 11-fold increase of HSC numbers and 40-fold increase of allograft ability. This result is concordant with previous reports that an increased number of HSCs enhances reconstitution of the hematopoietic compartment across the MHC barrier (Shizuru et al., 1996; Wang et al., 1997). Nevertheless, since the ex vivo expansion of HSCs had 40-fold increase of allograft ability and the net increase of HSC number was 11-fold, another ~4-fold increase (= 40/11) should be contributed by culture independent of expansion of HSCs.

To further determine whether culture enhances allograft ability independent of expansion, we cultured HSCs in conditions that do not support HSC expansion and used these cells for transplantation. To this end, we cultured HSCs in serum-free medium supplemented with only SCF and TPO (as ST medium; Figure 2I; Tables S1A–S1C, Figure S2D), based on previous results (Huynh et al., 2008; Zhang et al., 2006; Zhang and Lodish, 2004, 2005) and our syngeneic transplantation (Table S1A), this condition does not support HSC expansion. We determined that the allograft frequency for these cultured but unexpanded HSCs was 1/12,332 input equivalent cells (Figure 2I). This represented a ~4-fold increase (= 43,818/12,332) of allograft ability compared to freshly isolated HSCs. This number is in perfect agreement with the above estimate of a ~4-fold of increase of allograft ability by expansion-independent mechanism(s) based on comparison of results in syngeneic transplantation and allogeneic transplantation. Therefore, increase of allograft ability of HSCs does not necessarily need HSC expansion. In summary, our results indicate that both the increase of HSC numbers and expansion-independent characteristics acquired during ex vivo culture contribute to the improved allograft efficiency.

Accessory Cells Produced during Culture Does Not Contribute to the Enhanced Allograft Ability of HSCs

To identify the expansion-independent mechanism for cultured HSCs to cross the MHC barrier, we explored two possibilities: the presence of certain accessory hematopoietic or mesenchymal cells and a change of HSC immunogenicity during culture. To test the first possibility, we examined whether facilitating cells (Gandy et al., 1999; Kaufman et al., 1994), regulatory T cells (Taylor et al., 2008), or other cells produced during culture supported allograft. It has been established that unique differentiated BM populations as facilitating cells improve allogeneic reconstitution and result in donor-specific transplantation tolerance across MHC disparities (Gandy et al., 1999; Kaufman et al., 1994). The reported facilitating cells express conventional T cell components such as CD8 but are not T cells because they do not express TCR (Kaufman et al., 1994; Bridenbaugh et al., 2008). Interestingly, facilitating cells induce an increase in numbers of donor regulatory T cells (Treg) (Taylor et al., 2008), which directly facilitate allograft. Although freshly isolated HSCs do not contain $CD3^+$ cells (Figure S3A), after HSCs were cultured for 8 days in STFIA medium, approximately 0.3% of cells possessed the surface phenotype of $CD8^+CD45R^+TCR^-$ (Figure S3B), the same phenotype as the previously characterized facilitating cells (Kaufman et al., 1994). To test whether the phenotypic "facilitating cells" produced in culture supported allograft, we collected these culture-produced $CD8^+CD45R^+TCR^-$ cells by FACS and cotransplanted them with freshly isolated HSCs (1:1 as reported) (Kaufman et al., 1994) for allogeneic transplantation. We did not observe improved transplantation efficiency by including these cultured phenotypic facilitating cells, suggesting that they were not functional facilitating cells. In parallel, we were unable to detect phenotypic Treg ($FoxP3^+CD4^+CD25^+$) cells in the cultures we examined, suggesting the increased allograft of cultured cells was probably not contributed by production of Treg cells. To further test whether differentiated hematopoietic cells affected allograft, we isolated Lin^+ cells from the HSC culture and cotransplanted them with freshly isolated HSCs. These Lin^+ cells did not alter allogeneic transplantation efficiency (Figure S3C). In addition, there were no apparent adherent cells during our 8-day culture, and a CFU-F assay showed that no meshenchymal stem cells were produced from the cultured HSCs (Figure S3D). These results indicate that there is no engraftment-enhancing effect from mesenchymal stem cells. Taken together, we concluded that the accessory cells produced during the culture did not significantly contribute to increased allograft ability of ex vivo expanded HSCs.

Upregulation of CD274 during Culture Supports HSC Allograft

Next we tested the possibility that the immunogenicity of HSCs changes during culture by examining the expression of surface immune proteins, including MHC-I, MHC-II, CD274 (B7-H1 or PD-L1), CD275 (B7-H2), CD47, CD80, and CD86. The expression of these surface proteins on freshly isolated and cultured cells, as determined by flow cytometry, are summarized in Figures 3A and 3B. Almost all the freshly isolated HSCs and cultured cells expressed MHC-I and CD47, whereas very few of either population expressed MHC-II, CD275, CD80, or CD86. By contrast, there was a significant increase of surface expression of CD274 upon culture, as evidenced by an increase of $CD274^+$ cells from 61% to 88% (Figure 3A). Importantly, cultured cells contained a new population with more than 10-fold increase of CD274 expression (Figures 3C and 3D, and fold increase of CD274 staining intensity = 10,270/752 in Figure 3C). There was a greater portion of CD274-positive cells in the phenotypic cultured HSCs as $Lin^-Sca\text{-}1^+Kit^+CD48^-$ cells (Noda et al., 2008) than in differentiated cultured cells (Figure 3E), although the expression intensities of CD274 were similar in all fractions of cultured cells (Figure 3F).

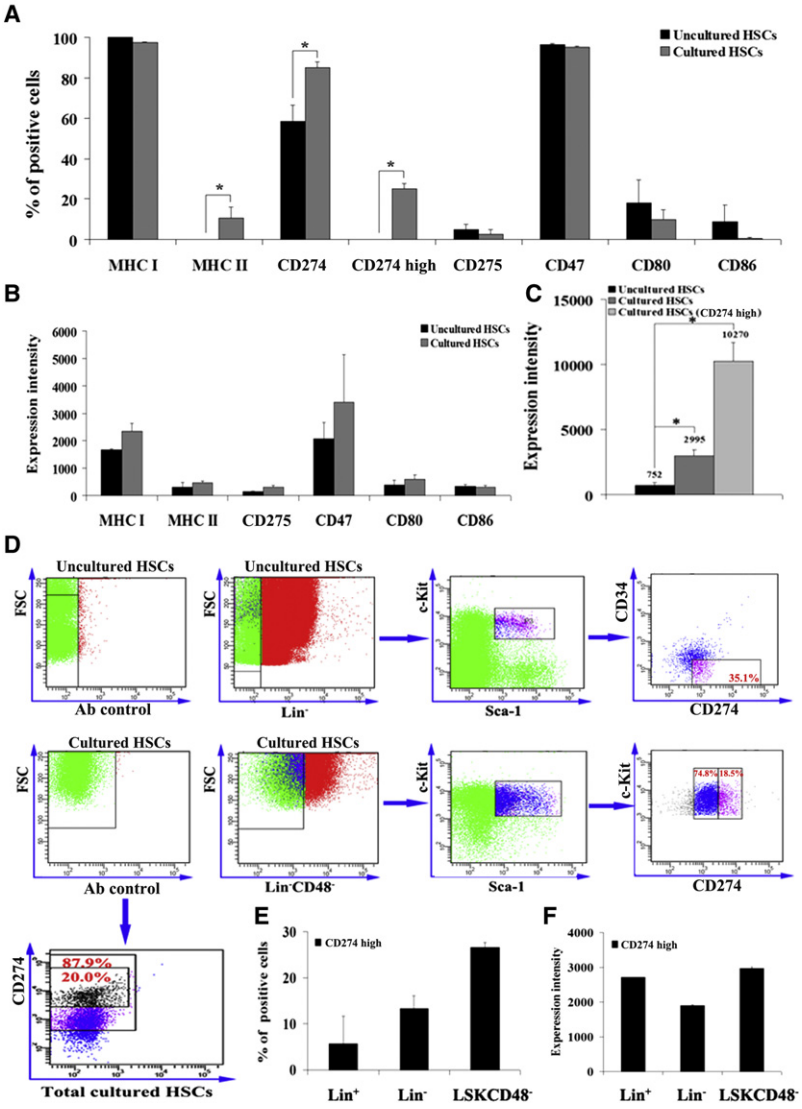

Figure 3. Altered Expression of Certain Surface Immune Molecules on Phenotypic HSCs during Culture

(A and B) A summary of the result of flow cytometry analysis of surface expression of indicated immune molecules after 8 days of culture of HSCs in STFIA medium (*$p < 0.05$, $n = 3–5$).

(C and D) CD274 surface expression was increased in cultured cells. In (C), MFI of CD274 expression determined by flow cytometry analysis in freshly isolated and cultured HSCs are shown (*$p < 0.05$, $n = 3–5$). Shown in (D) are representative flow cytometry plots indicating 43.5% freshly isolated HSCs were CD274 positive (as $CD274^{low}$), whereas 69% of cultured cells were $CD274^{low}$ and 20.0% as $CD274^{high}$. Gatings were set based on isotype controls.

(E and F) Percentages of $CD274^{high}$ cells and MFI of CD274 expression in different fractions of cultured HSCs. Data are expressed as mean ± SEM. See also Figure S3.

B7 immune proteins belong to the immunoglobulin (Ig) superfamily, with two Ig-like extracellular domains and short cytoplasmic domains. CD274 is a member of the B7 family that is expressed or induced on dendritic cells or non-antigen-presenting cells and inhibits T cell or innate activation (Francisco et al., 2010; Zou and Chen, 2008). While Figure 3D shows that CD274 might be upregulated on cultured HSCs based on phenotypic analysis, because the exact surface phenotype of cultured HSCs is not defined (Zhang and Lodish, 2005), we used the "gold standard" BM reconstitution analysis to test whether CD274 was expressed on functional HSCs and whether its level was altered upon culture. We first sorted freshly isolated BM cells into fractions negative and positive for immunostaining with antibodies against CD274. The repopulation activities of these fractions were then analyzed in the competitive syngeneic transplantation model. All the repopulating activity was within the CD274-positive fraction (Figures 4A–4C), indicating that all freshly isolated HSCs express CD274 on their surface. Since CD274 level was elevated more than 10-fold on some cultured cells (Figures 3C and 3D), we sought to determine whether surface expression of CD274 was increased on functional repopulating HSCs after culture. To this end, we fractioned the low positive and high positive cultured cells (as $CD274^{low}$ and $CD274^{high}$, respectively) followed by competitive syngeneic transplantation. The repopulating activity was found in both $CD274^{low}$ and $CD274^{high}$ fractions (Figures 4D and 4E; Figure S4). This reveals that indeed a fraction of HSCs increased their surface expression of CD274 more than 10-fold under our culture conditions. Interestingly, different culture conditions did not change the CD274 upregulation (Figure 4F), suggesting that the increase of CD274 expression was induced by general proliferation signals in culture and was independent of HSC expansion.

To determine the role of CD274 in transplantation of HSCs, we utilized mice that are deficient in CD274 (Dong et al., 2004). We showed that CD274-null mice had higher frequency of phenotypic HSCs than wild-type (WT) mice (Figure S5A), and the same number of freshly isolated CD274-null HSCs or ex vivo expanded null HSCs had slightly higher or similar long-term repopulation as WT HSCs in competitive syngeneic transplantation (Figures 5A–5D). These results suggest that CD274 per se does not significantly support the HSC activity in homeostatic and cultured conditions, concordant with the general normal phenotype of the CD274-null mice in homeostasis (Zou and Chen, 2008). By contrast, cultured CD274-null HSCs showed significantly decreased long-term repopulation in the competitive allogeneic repopulation compared to WT HSCs at 16 weeks posttransplant (Figures 5E and 5F). The deficiency of B7-H4, another B7 family immune inhibitor, did not decrease allograft efficiency at 8–16 weeks posttransplant compared to WT HSCs (Figures 5E and 5F). To further confirm that the surface CD274 on cultured HSCs facilitates allograft, we performed noncompetitive allogeneic transplantation and compared the allograft of 1,000 input equivalent WT HSCs, anti-CD274 neutralizing antibody-treated

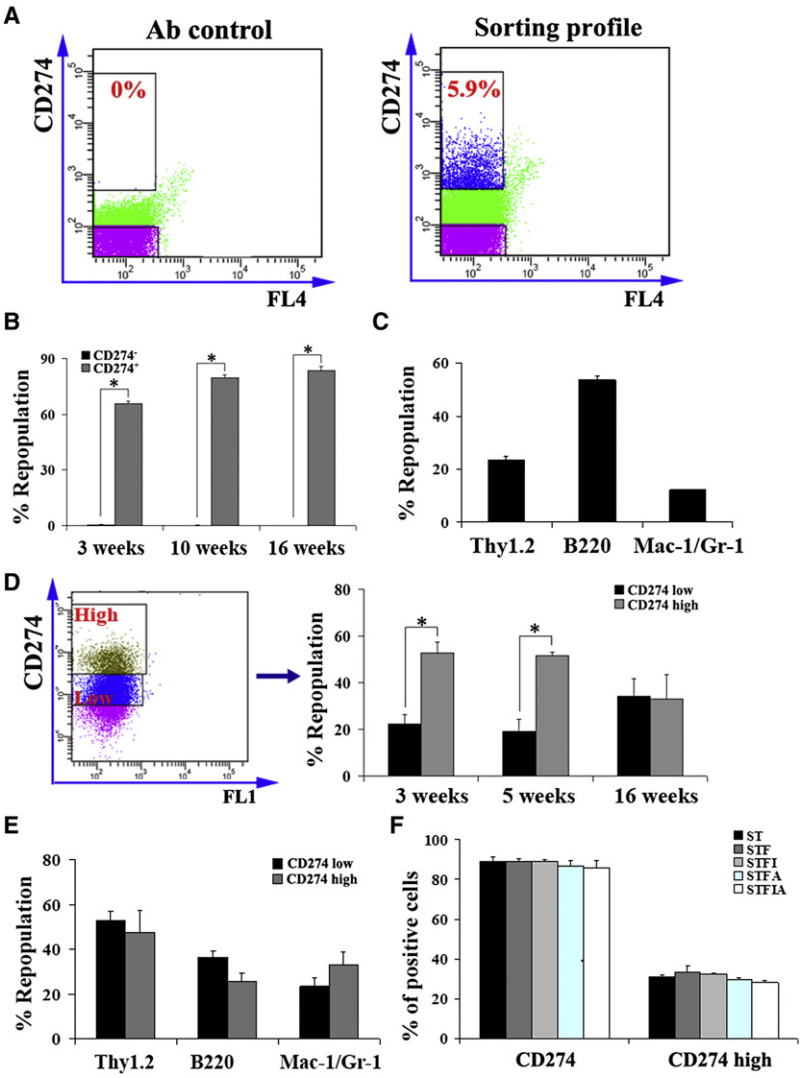

Figure 4. CD274 Is Upregulated on Repopulating HSCs during Culture

(A–C) 2 × 10^5 freshly isolated CD45.2 CD274$^+$ and CD274$^-$ BM cells were transplanted, respectively, together with 2 × 10^5 CD45.1 competitor cells into lethally irradiated congenic CD45.1 mice (*$p < 0.05$, n = 5).

(A) Gating plots of CD274$^+$ and CD274$^-$ BM cells.

(B) Peripheral blood engraftments at weeks 3, 10, and 16 after transplant.

(C) Multilineage contribution of cultured cells at 16 weeks posttransplant.

(D and E) 9.6 × 10^4 sorted cultured CD45.2 CD274^{high} and CD274^{low} total cultured cells were transplanted, respectively, together with 1 × 10^5 CD45.1 competitor cells into lethally irradiated congenic CD45.1 mice (*$p < 0.05$, n = 5).

(D) Peripheral blood engraftments at week 3, 5, and 16 after transplant.

(E) Multilineage contribution of cultured cells at 16 weeks posttransplant.

(F) All tested culture condition induces CD274 expression on the surface of HSCs (n = 3). Shown are the percentages of cells that express CD274 on their surface after 8 days of culture of HSCs in serum-free medium supplemented with SCF+TPO (ST), SCF+TPO+FGF-1 (STF), SCF+TPO+FGF-1+Angptl3 (STFA), SCF+TPO+FGF-1+IGFBP2 (STFI), and SCF+TPO+FGF-1+Angtpl3+IGFBP2 (STFIA).

Data are expressed as mean ± SEM. See also Figure S4.

WT HSCs, and CD274-null HSCs after culture (Figure 5G). Here we used ST medium (that does not support expansion of HSCs) to culture HSCs and specifically evaluate the expansion-independent effect of CD274 on HSC allograft. The 1,000 input equivalent WT HSCs engrafted 5 out of 17 recipients, whereas the anti-CD274 neutralizing antibody treated WT HSCs or the cultured CD274-null HSCs lost donor allograft activity (Figure 5G). Therefore, the deletion of CD274 or treatment with a CD274 neutralizing antibody abrogated the ability of cultured but unexpanded HSCs to cross the MHC barrier. A MLR experiment confirmed that, whereas cultured WT HSCs significantly inhibited allogeneic T cell activation, cultured CD274-null HSCs did not exhibit this inhibitory effect (Figure 5H). Anti-PD-1 was capable of decreasing the late apoptosis of activated T cells cocultured with precultured HSCs (Figure S5B). These results led us to conclude that CD274, a ligand known to inhibit T cell responses, is induced on cultured HSCs and possibly some differentiated cells; PD-1-mediated apoptosis of host T cells is one mechanism by which cultured HSCs overcome the MHC barrier in allograft.

While many studies demonstrated direct evidence that CD274 impedes T cell functions, it was reported that CD274 can also suppress the activation of innate immune cells (Yao et al., 2009). We performed a further experiment to distinguish the possible involvement of T cell-mediated immune response and innate immunity in the cultured HSC-enabled allograft. To this end, we cultured WT and CD274-null HSCs in the STFIA medium, followed by transplantation into sublethally irradiated SCID BALB/c mice (2.5 Gy). These recipient mice do not have functional T cells or B cells but do have normal NK cells. If WT and CD274-null HSCs do not have difference in repopulation in these mice, it would indicate that CD274 mainly works through suppressing allogeneic T cell activation but not innate immunity. Indeed we did not observe difference in allograft abilities of cultured WT and null HSCs in these mice (Figure 5I). Therefore, consistent with previous studies showing that CD274 suppresses T cell-mediated allo-rejection (Francisco et al., 2010; Zou and Chen, 2008), our result suggests that upregulation of CD274 on cultured cells including HSCs inhibited allogeneic T cell response.

Ex Vivo Expanded HSCs Can Cure the HSC Defective Disease by Allogeneic Transplantation

To test whether ex vivo expanded HSCs can be used to cure genetic diseases, we ex vivo expanded allogeneic HSCs and transplanted these cells into homozygotic DNA-PK 3A/3A knockin mice, in which three phosphorylation sites (Thr2605, Thr2634, and Thr2643) of DNA-PK were eliminated (Zhang et al., 2011). These mice have defective HSC self-renewal during

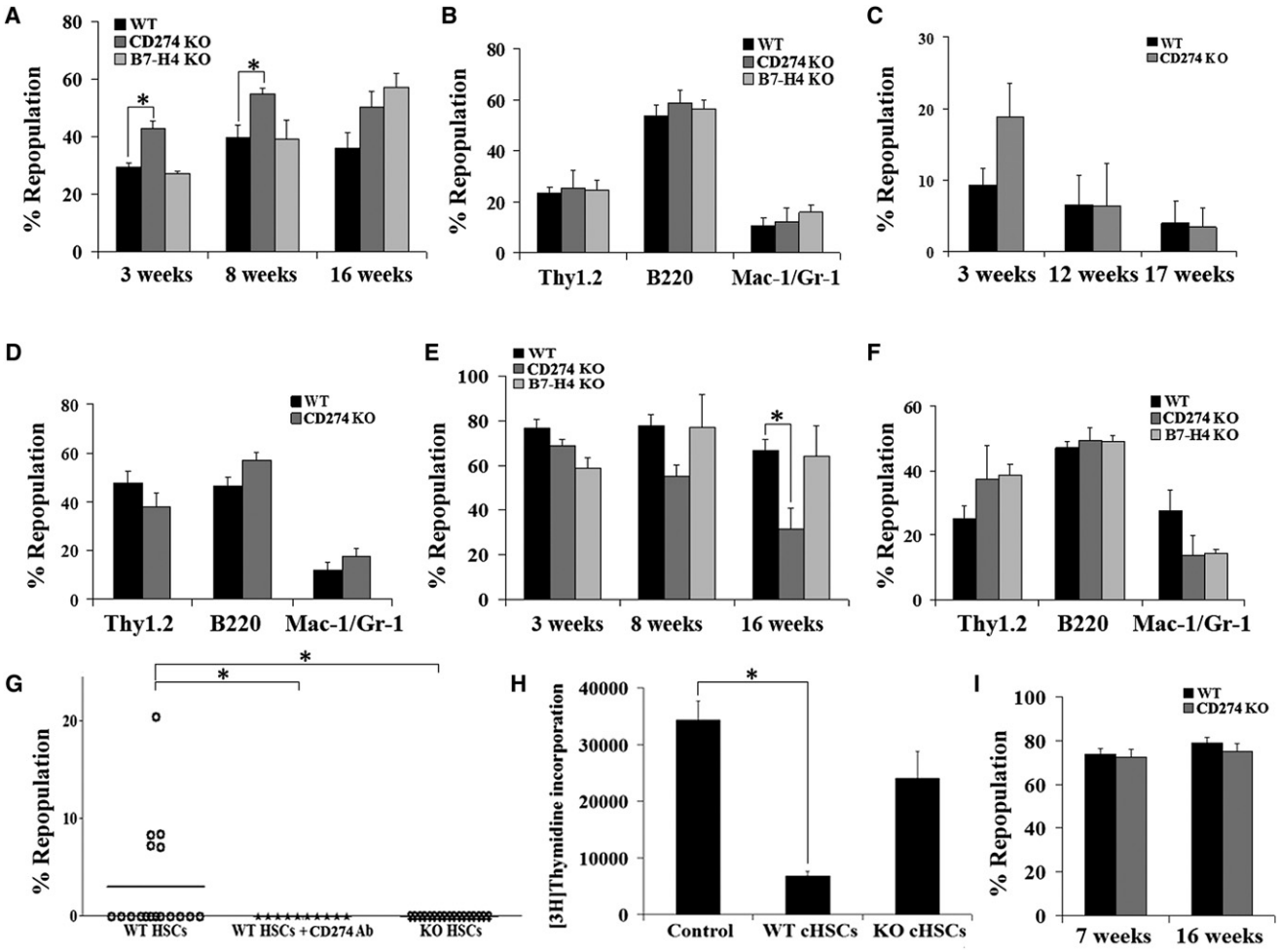

Figure 5. Elevated CD274 Expression on Cultured HSCs Is Critical to Cross MHC Barrier in Allogeneic Transplantation

(A and B) CD274 does not affect repopulation of freshly isolated HSCs in syngeneic transplantation. Freshly isolated 1 × 10^5 BM cells from WT or CD274-null CD45.2 C57BL/6 donors were transplanted into lethally irradiated CD45.1 C57BL/6 syngeneic recipients with 100,000 CD45.1 C57BL/6 total BM competitors (*$p < 0.05$, n = 5).

(A) Donor repopulation at 3, 8, and 16 weeks posttransplant.

(B) Multilineage contribution of cultured cells at 16 weeks posttransplant.

(C and D) CD274 does not affect repopulation of cultured HSCs in syngeneic transplantation. Cultured progenies of 100 $Lin^-Sca\text{-}1^+Kit^+CD34^-Flk2^-$ HSCs from WT or CD274-null CD45.2 C57BL/6 donors were transplanted into lethally irradiated CD45.1 C57BL/6 syngeneic recipients with 100,000 CD45.1 C57BL/6 total BM competitors (n = 5). Cells were cultured in STFIA medium.

(C) Donor repopulation at 3, 12, and 17 weeks posttransplant.

(D) Multilineage contribution of cultured cells at 17 weeks posttransplant.

(E and F) CD274 enhances repopulation of cultured HSCs in competitive allogeneic transplantation. Cultured progenies of input equivalent 10,000 $Lin^-Sca\text{-}1^+Kit^+CD34^-Flk2^-$ HSCs from CD45.2 C57BL/6 donors were cotransplanted with 100,000 freshly isolated BALB/c (CD45.2) BM cells into lethally irradiated BALB/c (CD45.2) recipients (*$p < 0.05$, n = 5). Cells were cultured in STFIA medium. This is an experiment representing two independent experiments that gave similar results.

(E) Donor engraftment at 3, 8, and 16 weeks posttransplantation.

(F) Multilineage contribution of cultured cells at 16 weeks posttransplant.

(G) CD274 enhances repopulation of cultured HSCs in noncompetitive allogeneic transplantation. Cultured progenies of input equivalent 1,000 $Lin^-Sca\text{-}1^+Kit^+CD34^-Flk2^-$ WT or CD274-null HSCs were transplanted into lethally irradiated BALB/c (CD45.2) recipients without competitors. ST medium was used in culture. Shown is donor engraftment at 16 weeks posttransplant (*$p < 0.05$, n = 10–17).

(H) MLR assay was performed in which cultured WT HSCs abrogated the proliferation of allogeneic T cells (bar 2) whereas cultured CD274-null HSCs were unable to do so (bar 3). *$p < 0.05$, n = 3.

(I) CD274 enhances repopulation of cultured HSCs through inhibition of T cell response. Cultured progenies of input equivalent 5,000 $Lin^-Sca\text{-}1^+Kit^+CD34^-Flk2^-$ WT or CD274-null HSCs were transplanted into sublethally irradiated (2.5 Gy) SCID-BALB/c (CD45.2) recipients. Cells were cultured in STFIA medium. Shown are donor engraftments at 7 and 16 weeks posttransplantation (n = 5).

Data are expressed as mean ± SEM. See also Figure S5.

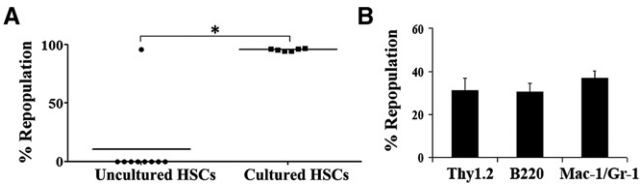

Figure 6. Culture of Allogeneic WT HSCs Rescues Lethal Phenotype of DNA-PK Knockin Mice

Freshly isolated 10,000 $Lin^-Sca\text{-}1^+Kit^+CD34^-Flk2^-$ HSCs or their 8-day cultured progenies from CD45.1 FVB donors were transplanted into lethally irradiated C57BL/6/129 CD45.2 knockin mutation at DNA-PKcs T2605 phosphorylation cluster recipients at postnatal day 12 (*$p < 0.05$, $n = 6–9$). HSCs were cultured for 8 days in STFIA medium that allows ex vivo expansion of HSCs. When competitors were used, freshly isolated 2,000,000–4,000,000 $Sca\text{-}1^-$ bone marrow cells isolated from FVB mice were cotransplanted.

(A) Donor engraftment at 16 weeks posttransplant.

(B) Multilineage contribution of cultured cells in rescued DNA-PK knockin mice at 16 weeks posttransplant.

Data are expressed as mean ± SEM.

development and normally die around 1 month after birth (Zhang et al., 2011). Figure 6A shows the result of transplantation of WT FVB (CD45.1) donor into DNA-PK knockin mice of the CD45.2 C57BL/6/129 background. Whereas freshly isolated $Lin^-Sca\text{-}1^+Kit^+CD34^-Flk2^-$ allogeneic HSCs transplanted with 2–4 × 10^6 $Sca\text{-}1^-$ helper cells (which made the total number of transplanted cells the same as or more than the number of cultured cells transplanted) engrafted only one out of nine recipients, their cultured progeny successfully engrafted and rescued all recipients. The rescued mice had almost 100% donor reconstitution and lymphoid, myeloid, and erythroid lineages were repopulated at 4 months posttransplantation (Figure 6B). Our result demonstrated that ex vivo expanded HSCs can be successfully used in fully nonmatched allogeneic transplantation to rescue the lethal phenotype of genetically mutated mice.

CD274 Is Induced on Human HSCs upon Culture

It is important to know whether a similar alteration of CD274 occurs on human HSCs upon culture. To this end, we determined the expression of CD274 on freshly isolated and cultured human cord blood HSCs. While only ~10% of freshly isolated human $Lin^-CD34^+CD38^-CD90^+$ cells express CD274 on their surface, the $CD274^+$ population increased to more than 50% after culture (Figures 7A–7E). MLR analysis showed that the elevated CD274 expression on cultured human cord blood HSCs indeed suppressed the proliferation of allogeneic T cells, and this ability was abrogated by the anti-CD274 neutralizing antibody treatment (Figure 7F). When we cultured human cord blood HSCs followed by transplantation into immune-deficient NOD/SCID/gamma(c)(null) (NSG) mice, we observed a stimulating effect of Angptl5 on HSC expansion as previously reported (Figure S6; Drake et al., 2011; Khoury et al., 2011; Zhang et al., 2008). Nevertheless, this enhanced ability to engraft NSG mice was not affected by anti-CD274 neutralizing antibody (Figure S6). This result is similar to what we observed in allograft in SCID BALB/c mice (Figure 5I), suggesting that human CD274 suppresses allogeneic T cell activation but not innate immunity. The upregulation of CD274 on cultured human HSCs may enable these stem cells to possess an enhanced allograft ability.

DISCUSSION

In this study, we demonstrated that ex vivo expanded HSCs more efficiently overcame MHC barriers and repopulated allogeneic recipient mice than freshly isolated HSCs. As measured by limiting dilution analysis, there was a 40-fold increase in the allograft ability of HSCs cultured for only 8 days compared to that of the freshly isolated HSCs. To identify the underlying mechanisms, we found that both increased numbers of HSCs and cultured-induced elevation of expression of the immune inhibitor CD274 on the surface of HSCs contributed to the enhanced allograft efficiency. As a proof of principle that ex vivo expanded HSCs can be used to cure genetic diseases in allogeneic recipients, we used ex vivo expanded allogeneic HSCs for transplantation and successfully rescued the lethal phenotype of DNA-PK knockin mice.

We used three models of allogeneic transplantation: noncompetitive transplantation into lethally irradiated recipients, competitive transplantation into lethally irradiated recipients, and noncompetitive transplantation into sublethally irradiated recipients. Whereas the first model was well-established and allows fewer numbers of donor cells for engraftment, it may result in the mouse death if donor HSCs cannot repopulate recipients. The second and third models ensure the survival of all recipients and better mimic the human transplantation scenario in which reduced intensity conditioning is often applied. Nevertheless, because of the enhanced host immune rejection, more than 10-fold of freshly isolated allogeneic donor HSCs are needed for successful engraftment in these models. This also underscores the importance of the increased number and MHC matching of donor HSCs in the clinical setting.

Our findings may shed new light on allogeneic transplantation of human HSCs into patients, which cannot be appropriately modeled by xenograft into immune-deficient mouse recipients. Two major problems, failure of engraftment and GVHD, have limited the progress in allogeneic transplantation. A strategy that significantly improves donor engraftment and reduces the risk of GVHD compared to current practice is needed. Transplantation of freshly isolated allogeneic HSCs indeed decreases the risk of GVHD but results in much lower engraftment (Shizuru et al., 1996; Wang et al., 1997). Here we show that ex vivo expanded mouse HSCs possess two advantages: increased HSC numbers and the enhanced immune feature to evade host rejection, therefore having dramatically enhanced allogeneic engraftment. Importantly, similar to freshly isolated HSCs (Shizuru et al., 1996; Wang et al., 1997), no sign of GVHD was observed after allogeneic transplantation of ex vivo expanded HSCs. This is expected because the condition of our (or other) HSC culture supports expansion of HSCs, along with production of differentiated myeloid but not much lymphoid cells. The culture thus does not seem to produce the source cells including T cells that may cause GVHD. Therefore, ex vivo expanded mouse HSCs appear to be an appropriate cell source to solve the problems of allogeneic transplantation in the mouse model. Based on these results of mouse HSCs and the elevation of CD274 on cultured human HSCs, we propose that ex vivo expansion of human HSCs may benefit the practice of allogeneic transplantation for patients. This would apply to nonmatched or low-matched donor human cord blood, BM, or mobilized

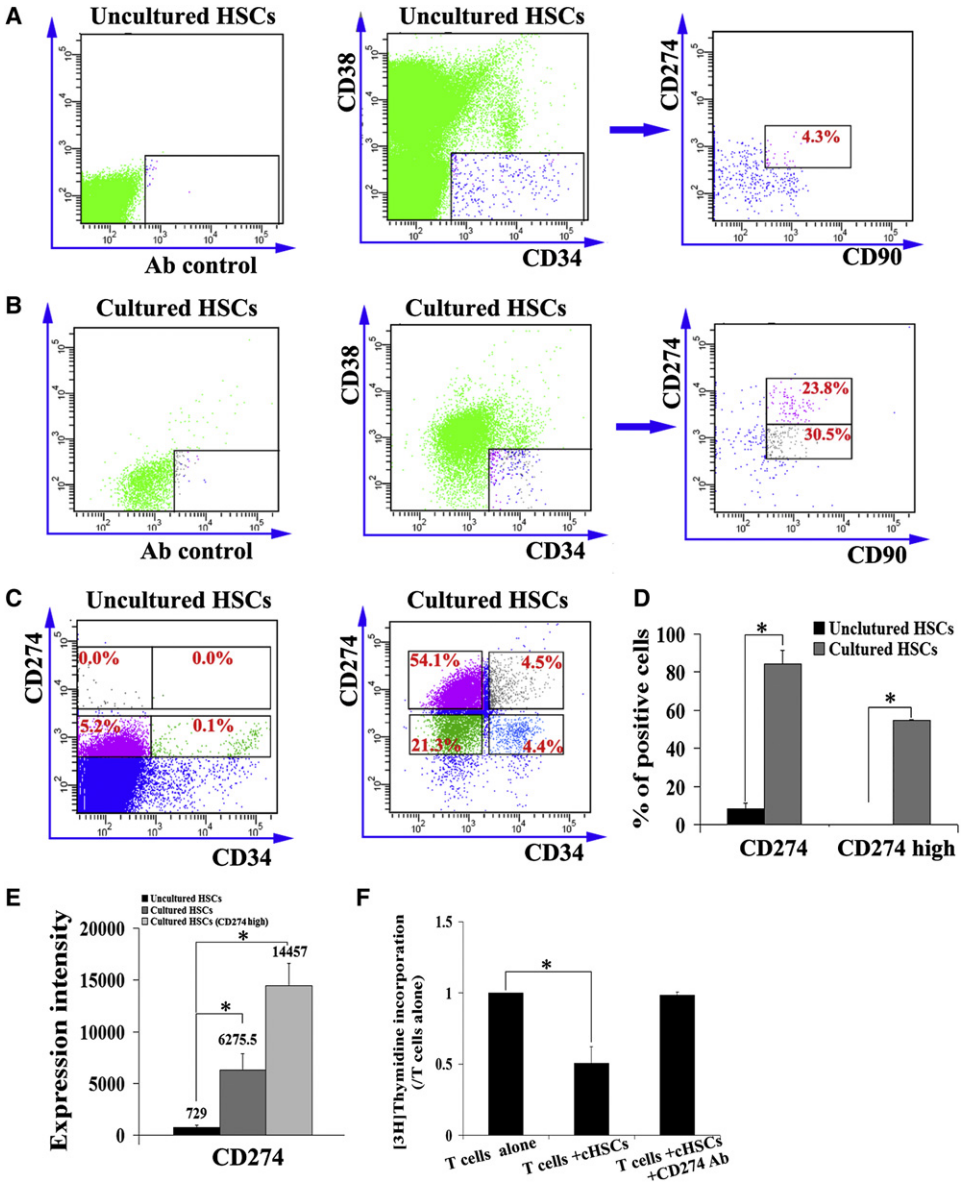

Figure 7. CD274 Is Upregulated in Cultured Human HSCs

Human cord blood Lin$^-$CD34$^+$CD38$^-$CD90$^+$ cells were cultured in serum-free medium supplemented with SCF, TPO, and Flt3-L for 8 days.

(A and B) Representative plots of CD34$^+$CD38$^-$CD90$^+$ cells that express CD274 on their surface before (A) and after (B) culture. Gatings were set based on isotype controls.

(C) Representative plot of CD34 and CD274 high or low staining in cultured human cells.

(D) Summary of percentages of CD274$^+$ and CD274^{high} cells in uncultured and cultured human cells (*$p < 0.05$, $n = 5$).

(E) MFI of CD274 expression determined by flow cytometry analysis in freshly isolated and cultured human cells (*$p < 0.05$, $n = 5$).

(F) MLR assay was performed in which cultured human cord blood HSCs abrogated the proliferation of allogeneic T cells (bar 2) whereas CD274 antibody reversed the inhibitory effect of cultured human cord blood HSCs (bar 3). *$p < 0.05$, $n = 6$.

Data are expressed as mean ± SEM. See also Figure S6.

peripheral blood HSCs. If donor human HSCs can be expanded in culture and engraft nonmatched or low-matched patients without GVHD, this strategy will possibly lead to an ultimate solution to problems in allogeneic transplantation.

It is known that some adult stem cells, such as mesenchymal and amnion stem cells, but not embryonic stem cells, are capable of avoiding rejection through production of immunosuppressive molecules and can be used in intra- and even interspecies transplantation (Salem and Thiemermann, 2010; Swijnenburg et al., 2008). Here we demonstrated that the immune inhibitor CD274 is expressed on freshly isolated HSCs and that its expression dramatically increased upon culture. Interestingly, CD274 does not appear to significantly affect the repopulation of long-term HSCs before and after culture as

determined by syngeneic transplantation, suggesting that its main role is not regulation of the regular activity of HSCs, but modulation of immunological properties of these cells. This was confirmed by the result that the deletion of CD274 or treatment with a CD274-neutralizing antibody abrogated the ability of cultured but unexpanded HSCs to cross the MHC barrier. CD274 was shown in previous studies to be expressed on activated immune cells and parenchymal cells and in immune-privileged sites such as eyes and placenta (Francisco et al., 2010; Zou and Chen, 2008). CD274 is also selectively expressed by various cellular components in the tumor microenvironment, where it inhibits tumor-specific T cell immunity by inducing T cell apoptosis and delay rejection (Zou and Chen, 2008). Here we provided an example suggesting that HSCs possess the ability to evade the rejection of the acquired immune system by regulating the expression of their own surface immune inhibitor such as CD274. Besides HSCs, the elevation of CD274 on hematopoietic progenitors produced during culture also might have contributed to the enhanced allograft. However, it is interesting to note that more differentiated Lin^+ cells elicit no effect, although they also express CD274. This may be contributed by the different cellular locations of HSCs/progenitors and more differentiated hematopoietic cells home after transplantation. It therefore will be interesting to study where the T cell-mediated immune response occurs for allogeneic transplanted HSCs in the future. In addition, it is noteworthy that CD274 may not be the only immune-suppressor acts on the HSC allograft. This is because that, although CD274-null HSCs behave much worse in allogeneic transplantation than their WT counterparts, they still possess a certain ability for allogeneic engraftment. Consistent with the elevation of the expression of immune inhibitor CD274 upon culture, costimulatory molecules such as CD80 and CD86 lost their expression on some cells after culture. All these observations clearly indicate that ex vivo culture significantly modulates the immunogenicity of stem cells. The identification of additional immune molecules whose alterations can regulate allograft will enable the complete resolution of the issue of immune rejection in allogeneic transplantation.

While our study suggests that the upregulation of CD274 on cultured cells including HSCs inhibited allogeneic T cell response, a related example is surface expression of CD47, which enables HSCs and leukemia cells to evade innate macrophage phagocytosis (Jaiswal et al., 2009). Based on these results, we hypothesize that all homeostatic HSCs express low levels of surface immune suppressors, and the levels of these suppressors can be induced by stress or immune signals. These immune suppressors may thus modulate HSC immunogenicity and, therefore, contribute to the "immune privilege" of HSCs. This regulatable immune privilege should be advantageous to HSCs, because it may allow these important stem cells to rapidly adjust to altered environment or to protect them from the excessive immune activation and even potential autoimmune disorder. Whether the expression of CD274 on HSCs or cancer cells can be regulated in vivo and its biological significance warrants further investigation.

Furthermore, we speculate that a common mechanism exists for regulation of expression of immune inhibitory signals in some other types of stem cells—similar to that in tumor cells. The expression and regulation of immune inhibitors on stem cells per se may allow these cells to survive an unexpected immune attack. It will be interesting to study the immunology of stem cells by investigating the roles of surface immune molecules on embryonic stem cells, induced pluripotent stem cells, other adult stem cells, and cancer stem cells.

In summary, our study demonstrated the great benefits of ex vivo expansion of HSCs for overcoming problems in allogeneic transplantation and revealed the importance of an immune inhibitor on the surface of HSCs. This work should shed new light on understanding the immunology of HSCs and other stem cells and may lead to development of novel strategies for successful allogeneic transplantation of human patients.

EXPERIMENTAL PROCEDURES

Mouse HSC Culture

Indicated numbers of BM $Lin^-Sca\text{-}1^+Kit^+$ $CD34^-Flk\text{-}2^-$ cells were isolated from 8- to 12-week-old mice and 150–200 of them were plated into each well of a U-bottom 96-well plate (3799; Corning) with 200 μl of the indicated medium. STFIA medium was defined as Stemspan serum-free medium (StemCell Technologies) supplemented with 10 μg/ml heparin, 10 ng/ml mouse SCF, 20 ng/ml mouse TPO, 10 ng/ml human FGF-1, 100 ng/ml IGFBP2, and 500 ng/ml Angptl3 as described (Huynh et al., 2008), which was used in Figure 2 experiments. In repeated experiments and experiments described in other figures, we refer STFIA medium as the same above medium except with serum-free conditioned medium collected from Angptl2-transfected 293T cells as described (Zhang et al., 2006) (that contains both IGFBP2 [Huynh et al., 2008] and Angptl2) to replace recombinant IGFBP2 and Angptl3. This Angptl2-supplemented medium worked equivalently and reproducibly supported HSC expansion in all experiments. ST medium was defined as Stemspan supplemented with 10 μg/ml heparin, 10 ng/ml mouse SCF, and 20 ng/ml mouse TPO. STF medium was ST medium supplemented with 10 ng/ml human FGF-1. STFA medium was STF medium supplemented with 500 ng/ml Angptl3. STFI medium was STF medium supplemented with 100 ng/ml IGFBP2. Unless otherwise described, cells were cultured for 8 days at 37°C in 5% CO_2 and the normal level of O_2. The culture duration of 8 days was shorter than that we described in previous studies (Huynh et al., 2008; Zhang et al., 2006). Because a substantially more number of cells were needed for allogeneic transplantation, we plated 150–200 HSCs per well in our experiments, instead of 20 HSCs described previously (Huynh et al., 2008; Zhang et al., 2006) for congeneic transplantation. We typically observed a ~200-fold increase of total number of cells after 8 days of culture. Therefore, a 100 input cells produced $2.32 \pm 0.28 \times 10^4$ total cells after 8 days of culture. This 8-day culture thus allowed us to harvest cells from the culture wells before the expanded cells exhausted the medium. For the purpose of transplantation, we pooled cells from at least 10 culture wells before the indicated numbers of cells were transplanted into each mouse. Flow cytometry analysis was performed to confirm multilineage reconstitution as we described (Simsek et al., 2010; Zheng et al., 2011). Calculation of CRUs in limiting dilution experiments was conducted with L-Calc software (StemCell Technologies) (Huynh et al., 2008; Simsek et al., 2010; Zheng et al., 2011).

Mouse HSC Allogeneic Transplantation

For allogeneic transplantation without competitors, the indicated numbers of mouse donor cells before or after culture were injected intravenously via the retro-orbital route into each of a group of 6- to 9-week-old recipient mice immediately after irradiation with a lethal dose of 9 or 9.5 Gy for BALB/c or 10 Gy for C57BL/6 mice. Sublethal irradiation of BALB/c mice in Figure S2A and of SCID-BALB/c mice in Figure 5I were performed at a dose of 7.5 Gy and 2.5 Gy, respectively. For competitive allogeneic transplantation, the indicated mouse donor cells before or after culture were mixed with $1–2 \times 10^5$ (as indicated) freshly isolated competitor bone marrow cells before transplantation. When indicated, 1,000,000 bone marrow cells collected from primary recipients were used for the secondary transplantation into lethally irradiated BALB/c mice. The antibody blocking treatment was conducted by incubating

cultured HSCs with 50 μg/ml CD274 neutralizing antibody (Cat# 16-5982-81, eBioscience) for 2 hr followed by washing before transplantation.

Mixed Lymphocyte Reaction

MLR was performed similarly as we described (Curiel et al., 2003). In brief, for mouse MLR in Figure 5H, BALB/c splenocyte CD90.2$^+$ T cells were plated in 96-well plate (flat-bottom) precoated with 1 μg/ml anti-CD3, followed by coculture with 8 days precultured irradiated C57BL/6 HSCs. For human MLR in Figure 7F, peripheral blood CD3$^+$ cells were plated in the presence of 2.5 μg/ml anti-CD3 and cocultured with 8 days precultured irradiated allogeneic cord blood HSCs. Proliferation was measured at day 3 of incubation at 37°C and 5% CO_2 following pulsing with [3H]TdR with a liquid scintillation counter. When indicated, 50 μg/ml anti-CD274 neutralizing antibody (Cat# 16-5983-82) was used to treat the cultured cells for 2 hr.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and one table and can be found with this article online at doi:10.1016/j.stem.2011.06.003.

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Components of the Hematopoietic Compartments in Tumor Stroma and Tumor-Bearing Mice

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Abstract

Solid tumors are composed of cancerous cells and non-cancerous stroma. A better understanding of the tumor stroma could lead to new therapeutic applications. However, the exact compositions and functions of the tumor stroma are still largely unknown. Here, using a Lewis lung carcinoma implantation mouse model, we examined the hematopoietic compartments in tumor stroma and tumor-bearing mice. Different lineages of differentiated hematopoietic cells existed in tumor stroma with the percentage of myeloid cells increasing and the percentage of lymphoid and erythroid cells decreasing over time. Using bone marrow reconstitution analysis, we showed that the tumor stroma also contained functional hematopoietic stem cells. All hematopoietic cells in the tumor stroma originated from bone marrow. In the bone marrow and peripheral blood of tumor-bearing mice, myeloid populations increased and lymphoid and erythroid populations decreased and numbers of hematopoietic stem cells markedly increased with time. To investigate the function of hematopoietic cells in tumor stroma, we co-implanted various types of hematopoietic cells with cancer cells. We found that total hematopoietic cells in the tumor stroma promoted tumor development. Furthermore, the growth of the primary implanted Lewis lung carcinomas and their metastasis were significantly decreased in mice reconstituted with IGF type I receptor-deficient hematopoietic stem cells, indicating that IGF signaling in the hematopoietic tumor stroma supports tumor outgrowth. These results reveal that hematopoietic cells in the tumor stroma regulate tumor development and that tumor progression significantly alters the host hematopoietic compartment.

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Introduction

Solid tumors are composed of cancerous and non-cancerous cells. The non-cancerous cells, including endothelial cells, hematopoietic cells, fibroblasts, myofibroblasts, pericytes, and mesenchymal stem cells, collectively form the cancer stroma or microenvironment [1,2]. These stromal cells come from the local environment or from bone marrow (BM) via the circulation system and appear to provide important support for cancer cell growth and metastasis [1,2]. For instance, BM-derived cells are recruited to the cancer site to stimulate outgrowth of tumors and form angiogenic and pre-metastatic niches for cancer growth [3,4,5]. Stromal fibroblasts and mesenchymal stem cells also play critical roles in angiogenesis and metastasis, respectively [6,7]. However, the exact compositions and functions of the microenvironment that surround solid cancer are still largely unknown. Since a tumor cannot develop without the parallel expansion of a tumor stroma, the lack of understanding of this cancer microenvironment has severely hampered cancer research and the development of effective therapeutic approaches.

There is ample evidence that certain differentiated hematopoietic cells, including macrophages, T cells, and mast cells, are incorporated into the tumor microenvironment [1,2]; however, a systematic investigation of the composition of the hematopoietic compartment of the tumor stroma has not been carried out. Hematopoiesis in vertebrates is a hierarchically organized developmental process in that highly specialized differentiated cells, including progenitors, precursors, and different lineages of blood cells, originate through an ordinate maturation program from the primitive hematopoietic stem cells (HSCs) [8]. Of these cells, HSCs are defined by their ability to self-renew and to differentiate into all blood cell types, whereas various progenitors posses much more limited self-renewal capacity and differentiation potential. In adults, HSCs mainly reside in BM; a small fraction also circulate in the blood stream and can be found in extramedullary organs including spleen and liver [8,9]. The flow

cytometry-based surface phenotype analysis and various functional assays, including the BM reconstitution analysis, remain the assays of choice for the analysis of the presence and activities of various hematopoietic cell types [8].

We sought to determine the composition and function of hematopoietic cells in tumor stroma and to determine whether tumor development affects the hematopoietic compartment of a tumor-bearing host. These studies are of fundamental importance to our understanding of the basic molecular and cellular mechanisms of tumor pathogenesis. A more complete understanding of the tumor microenvironment will make possible novel types of anti-tumor therapy.

Results

Various hematopoietic populations exist in tumor stroma

Using the Lewis lung carcinoma (LL2) implantation mouse model, we characterized the hematopoietic compartment in the tumor stroma. Figures 1A–C and Table 1 show the results of staining for hematopoietic cell surface antigens in dissociated tumor masses arising after the subcutaneous injection of 10^6 LL2 cells into C57BL/6 CD45.1 host mice at various time points post-implantation. The composition of hematopoietic cells in tumor stroma differed from that from host peripheral blood (PB) or BM (Table 1), indicating that there exists a unique hematopoietic

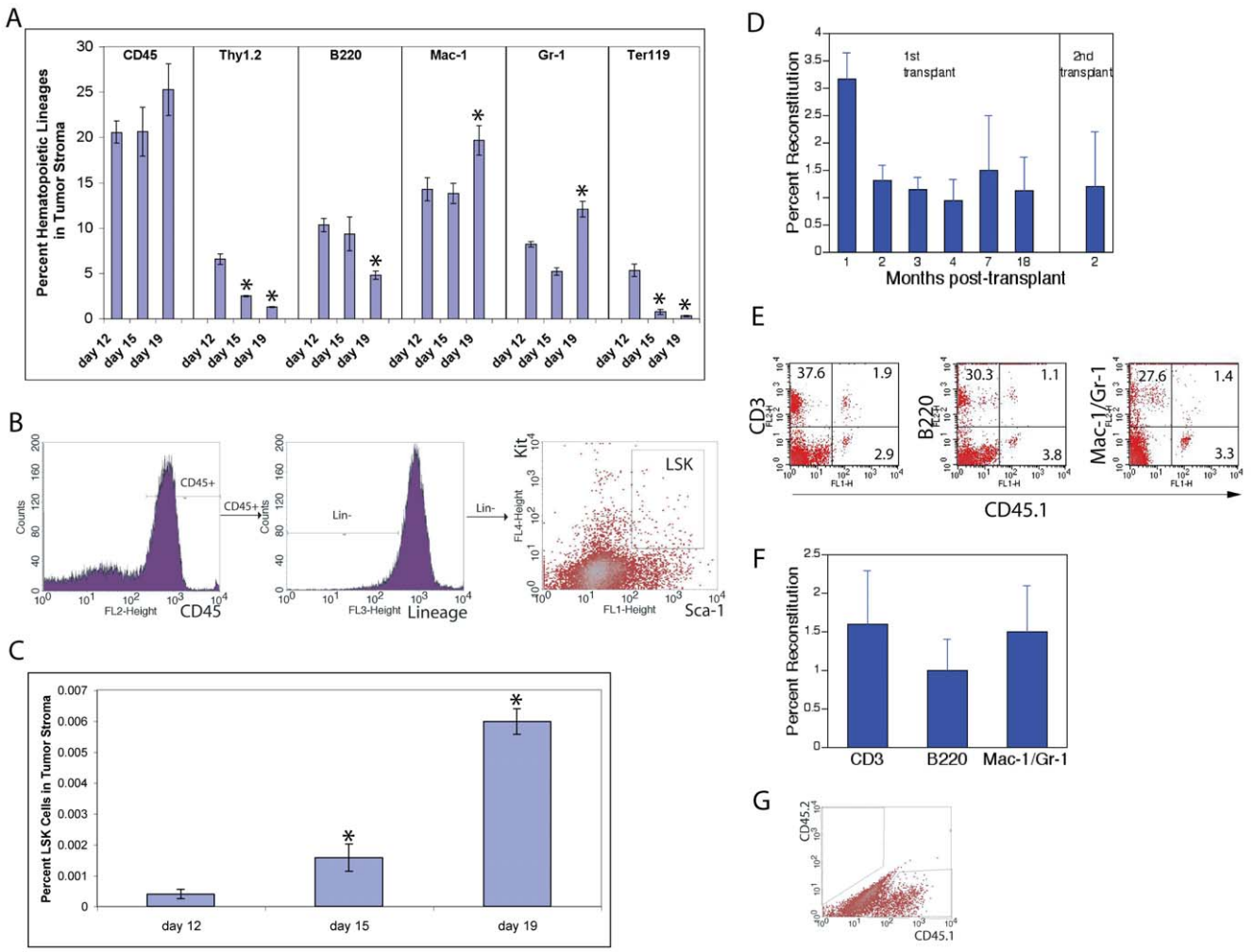

Figure 1. Analysis of the hematopoietic compartment of LL2 tumor stroma. Fig. 1A–C show the results of flow cytometry staining for hematopoietic cell surface antigens in dissociated tumor masses arising after the subcutaneous injection of 10^6 LL2 cells into C57BL/6 CD45.1 host mice at indicated days post-implantation. (A) Flow cytometry analysis of hematopoietic cells and their major lineages in LL2 tumor stroma at day 12, 15, and 19 after implantation as total hematopoietic cells (CD45), T cells (Thy1.2), B cells (B220), myeloid cells (Mac-1 and Gr-1), and erythroid cells ($Ter119^+$) (n = 5). * significantly different from day 12 values. (B) Representative flow cytometry plots showing that $CD45^+Lin^-Sca\text{-}1^+Kit^+$ (LSK) cells exist in LL2 tumor stroma. (C) Frequencies of $CD45^+Lin^-Sca\text{-}1^+Kit^+$ cells in LL2 tumor stroma at day 12, 15, and 19 after implantation (n = 5). * significantly different from day 12 values. (D) HSCs in the LL2 tumor stroma have long-term repopulation ability and repopulate secondary recipients. Lethally irradiated CD45.2 congenic mice were injected with 1×10^5 CD45.2 bone marrow competitor cells and 1×10^6 CD45.1 cells, isolated from the LL2 tumor mass. Shown are the repopulation activities of hematopoietic populations derived from the cancer stroma in long-term reconstitution (n = 6) and secondary reconstitution experiments (n = 5). (E–F) Donor repopulation in T lineage (CD3), B lineage (B220), and myeloid lineage (Mac-1/Gr-1) in peripheral blood in the experiment described in panel D at 7 months post-transplant (n = 6). (G) Hematopoietic cells in tumor stroma originate from host BM. 1,000,000 CD45.1 donor BM cells were transplanted into lethally irradiated CD45.2 C57BL/6 mice. At 4 months post-transplant, 10^5 LL2 tumor cells were subcutaneously implanted into these mice. Three weeks later, flow cytometry was used to characterize the hematopoietic cells in tumor stroma. A representative flow cytometry plot shows 100% of the tumor stromal $CD45^+$ cells were $CD45.1^+$.
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Table 1. Repopulating hematopoietic stem cells and differentiated hematopoietic cells exist in LL2 tumor stroma.

1	2	4	5	6	7	8	9	10
	$CD45^+$ %	$Thy1.2^+$ %	$B220^+$ %	$Mac\text{-}1^+$ %	$Gr\text{-}1^+$ %	$Ter119^+$ %	CFU-GM (per 1 million cells)	$CD45^+Lin^-$ $Sca\text{-}1^+Kit^+$ %
Tumor stroma	25.3±2.8	1.3±0.1	4.8±0.4	19.7±1.6	12.1±0.9	0.3±0.1	3.5±0.5	0.006±0.001
Tumor peripheral blood	98.7±2.4	11.7±1.9	13.1±2.2	74.5±3.7	66.4±4.5	15.0±5.6	N/D	0.009±0.002
Tumor bone marrow	77.0±2.0	0.9±0.2	5.5±2.5	70.5±3.8	61.1±4.7	19.4±4.2	2962±82	0.2±0.02
Normal peripheral blood	99.3±2.2	30.7±9.7	39.5±2.7	26.5±12.2	19.3±10.7	28.9±3.6	N/D	0.0009±0.0005
Normal bone marrow	69.7±2.9	2.8±0.2	20.7±1.3	44.0±4.4	41.5±4.9	32.5±2.5	1425±32	0.08±0.05
Normal muscle	1.6±0.2	0.8±0.1	0.1±0.01	0.2±0.01	0.5±0.04	0.1±0.01	0±0	0.0±0.0

Data from tumor mice were obtained from dissociated tumor masses arising after the subcutaneous injection of 10^6 LL2 cells into C57BL/6 CD45.1 host mice at day 19 post-implantation (n = 5).

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compartment in tumor stroma. The percentage of hematopoietic cells in the LL2 tumor stroma modestly increased from 20.6% at day 12 to 25.3% at day 19 after tumor implantation (Fig. 1A). The relative composition of myeloid cells ($Mac\text{-}1^+$ and $Gr\text{-}1^+$) increased whereas that of lymphoid cells (T cells as $Thy1.2^+$ and B cells as $B220^+$) and erythroid cells ($Ter119^+$) in this compartment decreased over time (Fig. 1A).

We also measured the existence of hematopoietic progenitors and phenotypic HSCs in the tumor stroma. The number of progenitors for granulocytes and monocytes (CFU-GM) was low, with 3.5 per 1,000,000 tumor cells at day 19 post-implantation (Table 1). Interestingly, in the LL2 tumor stroma, we found phenotypic HSCs, measured as $CD45^+Lin^-Sca\text{-}1^+Kit^+$ cells (Fig. 1B). The flow cytometry analyses indicate that there were few Lin^-Kit^+ cells in the tumor stroma, concordant with a previous report [3]. The average frequency of these phenotypic HSCs increased over time from 0.0004% at day 12, to 0.0016% at day 15, and to 0.006% at day 19 (Fig. 1C). In particular, the frequency of phenotypic HSCs detected in the tumor stroma (0.006±0.001%) at day 19 was 1/13 or 1/33 of that in BM of normal mice or tumor-bearing mice respectively (0.08±0.05 and 0.20±0.02% respectively) (Table 1). Since the tumors were perfused, and the cell composition including the percentage of HSCs in tumor stroma was quite different from that in peripheral blood, our result indicates that phenotypic HSCs reside in tumor stroma and are not contaminant from blood.

Since the surface phenotype of HSCs in extramedullary organs can be different from that of BM HSCs [9], we used BM reconstitution analysis, the "gold standard" for measuring HSC repopulating activity, to determine whether functional HSCs existed in the tumor stroma. As we performed before [10,11,12, 13,14,15,16], we examined the HSC activity of the donor cells in competitive reconstitution analyses, in which lethally irradiated recipient mice were co-transplanted with both the donor cells to be tested and WT BM competitors. The competitor cells serve as an internal control and as a supply of hematopoietic cells until the transplanted stem cells can generate sufficient mature lymphoid and myeloid cells for survival. Donor and recipient mice are genetically identical except for the CD45 surface protein that is found on nucleated peripheral blood cells and that is not involved in hematopoiesis or stem cell activity; donor cells carried the marker CD45.1, while recipient mice and supportive cells expressed CD45.2. An extremely stringent 18-month competitive reconstitution analysis and a secondary transplantation showed that the tumor stroma contained long-term repopulating HSCs (Fig. 1D). These cells were capable of repopulating both lymphoid and myeloid lineages in long-term reconstitution analysis (Fig. 1E–F), attesting that these are functional HSCs. The relative low repopulation of the donor cells in all lineages compared to BM competitors suggests these HSCs in the tumor stroma have weak engraftment to the recipient BM. Nevertheless, our data, for the first time to our knowledge, demonstrated that functional HSCs exist in tumor stroma.

To determine whether the hematopoietic cells in tumor stroma originated from the BM or from the local environment, we transplanted CD45.1 C57BL/6 donor BM cells into lethally irradiated CD45.2 C57BL/6 mice. At 4 months post-transplant, the recipient BM was completely repopulated by the donor CD45.1 cells, whereas peripheral tissues contained mostly CD45.1 cells with certain CD45.2 cells as reported [9]. We implanted LL2 tumor cells subcutaneously into these mice. Three weeks later, we isolated tumors and used flow cytometry to characterize the hematopoietic cells in stroma. We found that all of the tumor stromal $CD45^+$ cells were $CD45.1^+$ (Fig. 1G). Accordant with previous studies [3], we concluded that hematopoietic cells in the LL2 stroma are derived from BM.

HSC numbers dramatically increase in tumor-bearing mice

Next we sought to determine whether the host hematopoietic compartment was affected by tumor growth. Total cellularity and total hemtopoietic cell counts of tumor-bearing mice did not significantly change over time (Fig. 2A–B). However, in both BM and PB of tumor-bearing mice we observed significantly increased percentages of myeloid ($Mac\text{-}1^+$ and $Gr\text{-}1^+$) cells but decreased lymphoid ($Thy1.2^+$ and $B220^+$) and erythroid ($Ter119^+$) cells as a function of time post-implantation (Fig. 2C–D). This trend is similar to what occurred in the hematopoietic lineages in tumor stroma. In addition, we analyzed the frequencies and numbers of hematopoietic progenitors and phenotypic HSCs in the tumor-bearing mice and healthy controls. CFU-GM increased approximately 2-fold whereas CFU-E and CFU-Pre-B decreased at least 50% in tumor-bearing mice compared to normal mice (Fig. 2E), concordant with the total increase of myeloid cells and decrease of

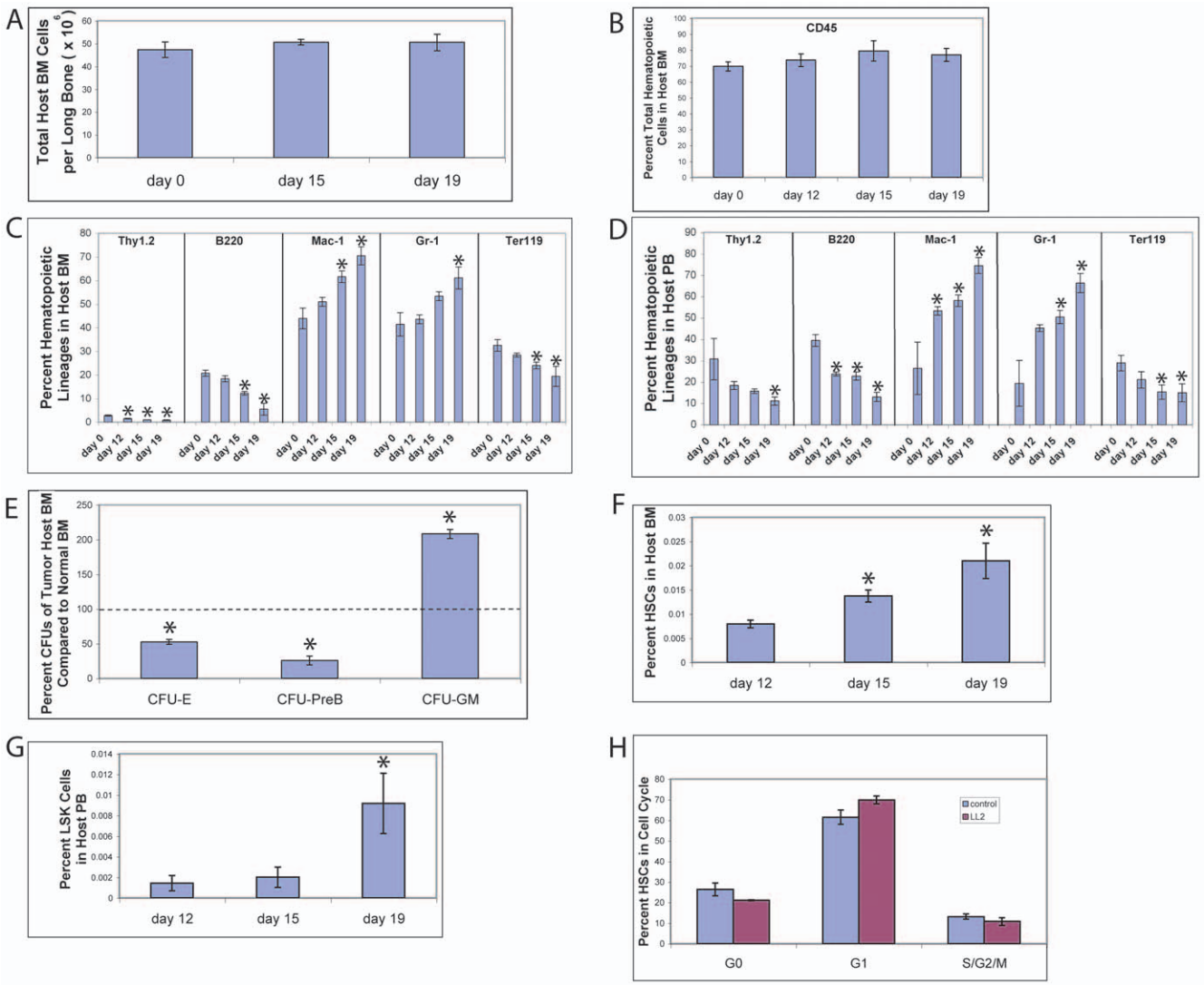

Figure 2. Analysis of the hematopoietic compartment of the LL2 tumor-bearing mice. (A–G) Total BM cells (A), total hematopoietic cells in BM (B), hematopoietic lineages in BM (C), hematopoietic lineages in PB (D), hematopoietic progenitors in BM (E), $CD45^+Lin^-Sca\text{-}1^+Kit^+CD34^-FLK2^-$ cells in BM (F), and $CD45^+Lin^-Sca\text{-}1^+Kit^+$cells in PB (G) at indicated days in C57BL/6 CD45.1 host mice before and after the subcutaneous injection of 10^5 LL2 cells were analyzed by flow cytometry or colony assays (n = 5). * significantly different from day 0 or normal values (for panel B–E), or from day 12 values (for panel F–G). (H) The cell cycle status of BM HSCs in tumor-bearing mice at day 19 post-implantation does not significantly differ from that of counterparts in normal mice. HSCs as $Lin^-Sca\text{-}1^+Kit^+Flk2^-CD34^-$ cells were stained with Hoechst 33342 and pyronin Y, and analyzed for cell cycle stage (n = 5).
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lymphoid and erythroid cells. The BM and PB enriched phenotypic HSCs as $Lin^-Sca\text{-}1^+Kit^+CD34^-Flk\text{-}2^-$ or $Lin^-Sca\text{-}1^+Kit^+$ cells increased over time. Numbers of BM HSCs increased about 3-fold and PB HSCs increased 7-fold at day 19 relative to numbers on day 12 (Fig. 2F–G). This suggests that the presence of a tumor induces *in vivo* expansion of HSCs and progenitors in the BM, which leads to increased HSCs and progenitors in the circulation. Consistent with this result, the tumor-bearing mice display splenomegaly, with 2-fold increase of spleen size and weight compared to healthy controls (data not shown).

Because numbers of HSCs in the tumor-bearing mice significantly increased, we tested whether the cell cycle of HSCs in the host BM changed. As shown in Figure 2H, there was no significant changes in fractions of cells in given cell cycle stages in HSCs in the BM of tumor-bearing mice at day 19 compared to normal mice, suggesting that the tumor did not significantly alter the quiescence of host BM HSCs at the late stage of cancer development.

Co-implanted hematopoietic cells from tumor stroma promote tumor development

We developed an assay to compare the abilities of different hematopoietic populations to collaborate functionally with tumor cells to affect the tumor development. In this assay, we use FACS to isolate a certain population of hematopoietic cells and mix them with a fixed number of LL2 cancer cells prior to implantation into the C57BL/6 mice. The kinetics of tumor growth was determined to evaluate the tumor-promoting ability of the co-implanted hematopoietic cells. In the experiment summarized in Figure 3A, we co-implanted 1×10^5 LL2 cells with 1×10^4 CD45.1 total BM cells or with enriched normal BM HSCs as $Lin^-Sca\text{-}1^+Kit^+$ cells subcutaneously into C57BL/5 CD45.2 host mice. During the 3-

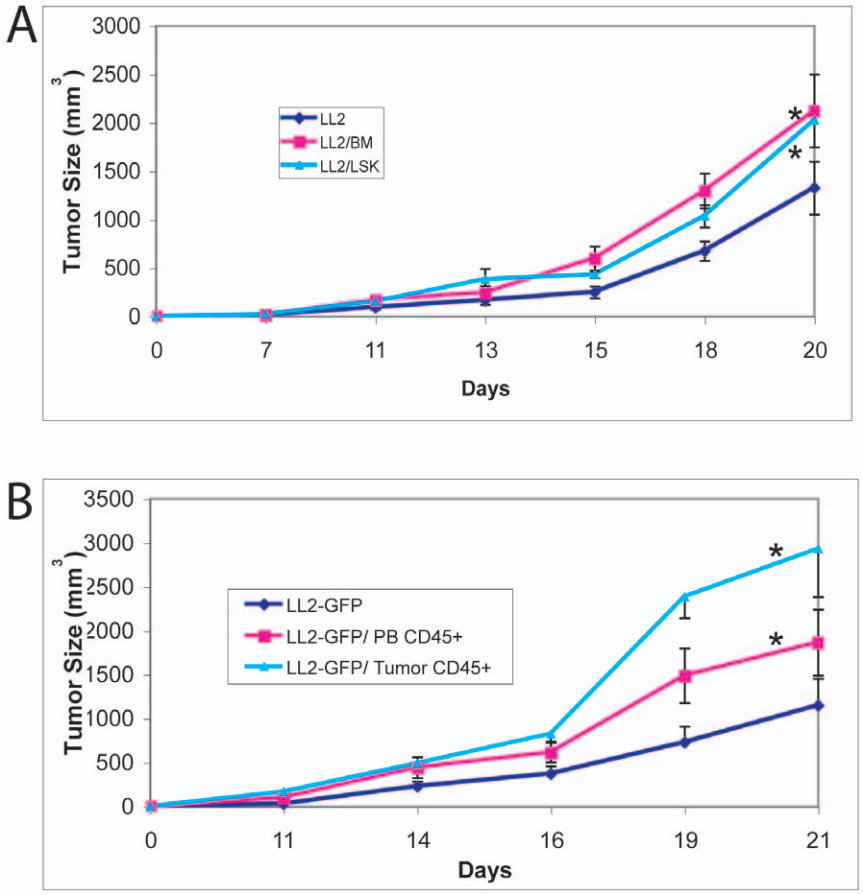

Figure 3. Tumor stromal hematopoietic cells stimulate LL2 tumor growth. (A) Co-implanted HSCs or total BM cells stimulate the growth of primary LL2 tumor. 100,000 GFP-marked LL2 cells were co-implanted with with 1×10^4 CD45.1 total BM cells or enriched normal bone marrow HSCs (as $Lin^-Sca\text{-}1^+Kit^+$ cells) subcutaneously into C57BL/6 CD45.2 host mice (n = 8). During the 3-week period of analysis, the size of the primary tumor was measured. (B) Tumor stromal hematopoietic cells stimulate tumor growth. 100,000 tumor stromal $CD45^+$ cells or PB cells from tumor-bearing mice were collected by FACS and co-implant with 10^5 LL2 cells into healthy mice (n = 5). Shown are the sizes of the primary tumors. Tumor growth curves for different experimental groups were compared using the Generalized Estimating Equations (GEE) method with AR(1) correlation structure. * significantly different from LL2-GFP growth curve, ** significantly different from LL2-GFP and LL2/PB $CD45^+$ growth curves, $p<0.05$.
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week period of analysis, the tumor size was measured. These data show that both total BM cells and the enriched HSC population positively regulated tumor growth. It is noteworthy that the co-implantation of total BM cells with LL2 cells led to similar level of tumor size as the same number of transplanted LSK cells. Clearly, differentiated hematopoietic cells did promote tumor progression. This suggests that additional HSCs in the tumor local environment do not necessarily further promote tumor growth.

We further determined the effect of hematopoietic cells isolated from the tumor stroma on cancer development. Because it was technically difficult to isolate the low frequent $Lin^-Sca\text{-}1^+Kit^+$ cells from these tumors, we isolated hematopoietic cells from LL2 tumor stroma or host PB as a control and co-injected them with GFP^+LL2 tumor cells into secondary mice. We found that, although the host PB $CD45^+$ cells stimulated LL2 tumor growth compared to LL2 cells alone, $CD45^+$ cells isolated from previously existing tumor stroma had significantly increased ability to enhance LL2 tumor growth than these PB $CD45^+$ cells (Fig. 3B, $p<0.05$). This result suggests that 1) hematopoietic cells in tumor stroma enhance tumor growth, 2) hematopoietic cells from tumor stroma are different from those from PB in promoting tumor growth, not unexpectedly as the compositions of these two sources of cells are different (Table 1), and 3) tumor stroma has certain "educating" effect on hematopoietic cells that leads to tumor development.

IGF-IR expressed on cells from hematopoietic stroma is important for tumor development and metastasis

To further validate the pathological effect of the hematopoietic cells in the tumor stroma, we studied the role of IGF signaling in these hematopoietic compartment. We previously showed that the the receptor for IGF-2 is expressed on the surface of all HSCs [10]. IGF type I receptor (IGF-IR) is the signaling receptor for insulin-like growth factor 1 and 2 (IGF-1 and IGF-2). The IGF pathway has been reported to play important roles in the development of a range of malignancies, including both non-small cell lung cancer and small cell lung cancer (see review [17]). For example, elevated plasma levels of IGF-1 and single nucleotide polymorphisms within the IGF axis are associated with an increased risk of lung cancer [17]. The activation of IGF-IR facilitates malignant transformation and the majority of IGF-2 transgenic mice develop lung cancer by 18 months of age [17]. Of note is that these studies were focused on the activity of IGF-IR that is expressed on cancer cells. We sought to test whether IGF-IR expressed by the hematopoietic stroma plays any role in the development of the LL2 tumors.

Although IGF-IR$^{-/-}$ mice die after birth [18,19], we were able to collect IGF-IR$^{-/-}$ HSCs from the fetal liver. Then we reconstituted recipient mice with IGF-IR$^{-/-}$ or wild-type fetal liver HSCs. Four months later, the recipients were fully repopulated by the donor IGF-IR$^{-/-}$ or wild-type HSCs, and we implanted LL2 cancer cells into these mice. The IGF-IR$^{-/-}$ HSCs had no apparent defect in engrafting the recipient mice (Fig. 4A), nor did they have a noticeable skew in lineage differentiation compared to HSCs from wild-type mice (Fig. 4B). Nevertheless, LL2 tumors grew significantly more slowly in these IGF-IR$^{-/-}$ HSC reconstituted mice than in mice engrafted with wild-type HSCs (Fig. 4C, $p<0.05$), accompanying with dramatically decreased metastasis to the lung (Fig. 4D). This experiment suggests that the lack of IGF signaling in the hematopoietic compartment of tumor stroma hampers the solid tumor development. This novel result complements the conventional view that IGF signaling in tumor cells *per se* is important for cancer development.

Discussion

Since cancer pathogenesis involves a concerted interplay between the tumor and the microenvironment, it is desirable to elucidate the roles of tumor stroma in tumor development. In this study, we sought to determine the composition and potential function of hematopoietic cells in the stroma of solid tumors and in tumor-bearing mice. To this end, we used an LL2 implantation tumor model. Cells of the LL2 line are advantageous because they produce tumors in syngeneic C57BL/6 mice, which are ideal for quantitating HSCs by reconstitution analysis based on congenic CD45.1 and CD45.2 markers. Because LL2 cells are syngeneic with their hosts, their tumorigenicity can proceed in the presence of a fully competent host immune system. This is especially important in a study of the hematopoietic compartment of the tumor stroma and the tumor-bearing host. Moreover, LL2 cells, when injected subcutaneously, can form lung metastases. This makes it possible to compare the effects of different stromal components on regulation of potential tumor cell migration.

Here we provide evidence that functional hematopoietic progenitors and HSCs exist in tumor stroma. Although the very low frequency of HSCs in tumor stroma makes them impossible to observe by immunohistochemistry, we were able to detect these cells using flow cytometry analysis and the "gold standard" BM reconstitution assay. To our knowledge, this was the first demonstration that functional HSCs exist in tumor stroma. It is apparent that these tumor stromal HSCs are not contaminants from PB: this was assured by our approach to isolate hematopoietic cells from perfused tumors and was also attested by the demonstration of different frequencies of various hematopoietic populations in tumor stroma and PB. Nevertheless, these tumor stromal hematopoietic cells do originate from BM and may be recruited to tumor sites from PB through inflammatory signals. Our result is supported by the emerging evidence showing that HSCs or progenitors can themselves home to sites of inflammation

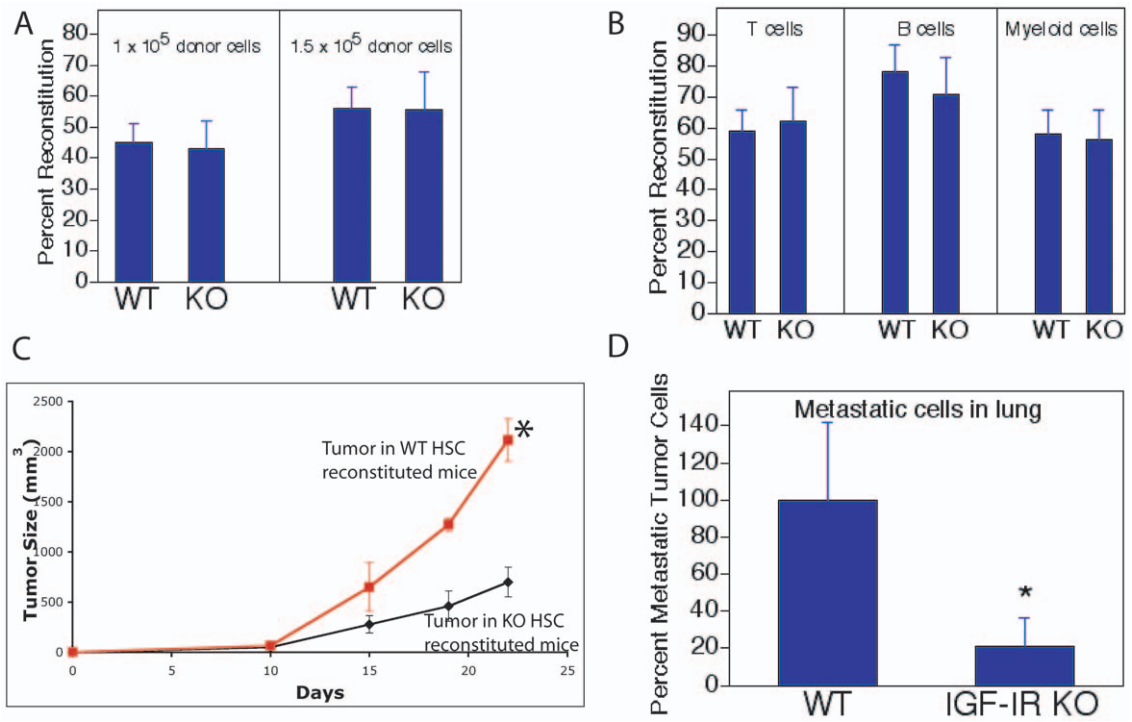

Figure 4. Mice with the IGF-IR$^{-/-}$ tumor stromal HSCs had slower tumor development in the LL2 implantation model than mice reconstituted with wild-type HSCs. (A) C57BL/6 CD45.2 E15.5 IGF-IR$^{-/-}$ fetal liver HSCs had similar engraftment as wild-type HSCs in CD45.1 recipient mice in a competitive repopulation analysis at 4 months post-transplant (n = 7). CD45.1 total BM cells were used as competitors. (B) IGF-IR$^{-/-}$ HSCs had similar differentiation into T, B, and myeloid lineages as wild-type HSCs at 4 month post-transplant (n = 7). (C) Mice with the IGF-IR$^{-/-}$ tumor stromal HSCs had slower tumor growth in the LL2 implantation model. Shown is a representative result of three independent experiments of tumor growth measured after 1×10^6 LL2 cells were implanted subcutaneously into IGF-IR$^{-/-}$ or wild-type HSCs reconstituted mice at 4 month post-transplant (n = 5). * significantly different from the KO growth curve, $p<0.05$. (D) Mice with the IGF-IR$^{-/-}$ tumor stromal HSCs had dramatically decreased GFP$^+$ LL2 tumor cell metastasis in lung compared to those reconstituted with wild-type HSCs. Results shown were from pooled data from three experiments (in each experiment n = 3–5). Tumor growth curves for different experimental groups were compared using the Generalized Estimating Equations (GEE) method with AR(1) correlation structure. * significantly different from wild-type value, $p<0.05$.
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to rapidly produce cells that are essential for the immune response [20]. Concordant with the idea that tumors release certain endocrine signals that change the representation of stem cells or progenitors in other tissues or organs [3], we showed that the tumor-bearing mice had 3-fold and 7-fold increases in of BM and PB HSCs, respectively. Similarly, during tumor development, the percentage of faster proliferating myeloid cells (including myeloid progenitors) increased and that of slower growing lymphoid cells decreased over time in host BM and PB. In summary, the whole process appears to be as follows. Tumor-produced hormone signals reach BM, thus BM HSCs increase. The increased proliferation of BM HSCs exceeds the capacity of HSC microenvironment leading to HSC mobilization and increases in numbers of PB HSCs. These BM-derived hematopoietic cells including HSCs are eventually recruited to tumor and become the source of hematopoietic cells in tumor stroma. A better understanding of the alteration of the hematopoietic compartment in host and tumor stroma during tumor progression may lead to new strategies for cancer treatment. For instance, the effective retention of BM HSCs in their BM niche or block of the migration of BM-derived hematopoietic cells in cancer patients should negatively control the cancer development.

Is the existence of non-cancerous cells in tumor stroma a consequence or a cause of tumor development? So far numerous lines of prior evidence already indicate that stromal cells play important roles in tumor progression. Endothelial cells recruited to the tumor mass support neovascularization. BM-derived cells have been shown to support tumor outgrowth and form pre-metastatic niches [3,4,5]. Stromal fibroblasts and mesenchymal stem cells also support in angiogenesis and metastasis, respectively [6,7]. Our studies provide further evidence that tumor stromal hematopoietic cells regulate the tumor growth and metastasis. We found that the co-implantation of normal HSCs with cancer cells in this tumor model promoted tumor growth. Since total BM cells had similar ability to stimulate co-implanted tumor growth as LSK cells, HSCs may not directly affect tumor growth; instead, it may be hematopoietic cells that differentiate in the tumor microenvironment that play a critical role in tumor outgrowth. Nevertheless, it seems that a combination of multiple lineages of hematopoietic cells instead of a certain single lineage is advantageous to promoting tumor development. When we co-implanted individual lineage cells such as $CD3^+$ (T cells), $B220^+$ (B cells), $Mac\text{-}1^+$ (monocytes), $Gr\text{-}1^+$ (granulocytes), or $Ter119^+$ cells (red blood cells) with LL2 cells, we found their tumor-promoting effects were not as potent as LSK cells or total BM cells (data not shown). This suggests that the full lineage spectrum that is derived from HSCs locally may have the strongest activity in tumor promotion. Consistent with this view, we showed that previously "educated" tumor stromal total hematopoietic cells had greater ability to support tumor growth than circulating hematopoietic cells.

The IGF ligands IGF-1 and IGF-2 bind to their common signaling receptor IGF-IR and initiate a variety of signaling events. It is known that IGF-IR regulates cell growth, survival, adhesion, and motility [17]. Our previous work demonstrated that all normal HSCs express the receptor for IGF-2 and that IGF-2 stimulates *ex vivo* expansion of these normal HSCs [10]. In the present study, we found that tumor stromal HSCs are originally derived from BM. Although $IGF\text{-}IR^{-/-}$ HSCs do not appear to have overt defects in hematopoietic engraftment and differentiation, the incorporation of $IGF\text{-}IR^{-/-}$ hematopoietic cells into the tumor stroma significantly hampered tumor growth and metastasis. This suggests that IGF-IR may regulate certain cell fates (division, differentiation, migration, or apoptosis) or activities (such as immune response) of hematopoietic cells under inflammation stress in the unique tumor stromal environment. Overall our results reveal that, in addition to its direct role in cancer survival and growth, IGF signaling in tumor stroma is also important for solid cancer development. These results do not contradict but rather complement the conventional view that IGF signaling is important for cancer development.

Questions regarding the repopulating hematopoietic cells in tumor stroma still remain. For example, what is the mechanism for LL2 stroma to maintain the primitive status of a small fraction of HSCs? One possibility is that HSCs are supported by the growth factors, cytokines, chemokines, or membrane proteins produced by the tumor stromal environment. As we previously demonstrated, certain tumor cells can secrete proteins to support HSCs *ex vivo* [14]. It is therefore reasonable to assume that, while the LL2 stromal environment largely supports HSC differentiation *in vivo*, it possesses a certain capacity to maintain a very small number of primitive HSCs possibly for its own sake. A systematic study of the effects of the tumor stroma-produced factors on cell fates of HSCs will be necessary to find the answer. Another question is, what is the exact phenotype of HSCs in tumor stroma? Is it the same as that of BM HSCs? We need to emphasize that, although we identified the existence of $CD45^+Lin^-Sca\text{-}1^+Kit^+$ cells (as the phenotype of enriched BM HSCs) in the LL2 stroma, we are uncertain if this represents the actual phenotype of these HSCs. That is why we used the "gold standard" reconstitution analysis to confirm the existence of functional repopulating HSCs in this study. Given the fact that HSCs change their surface phenotype in stressed conditions, culture, or extramedullary tissue such as in the liver [9,11], future investigations coupling FACS-based cell fractionation with BM reconstitution analysis will be needed to clarify this issue. Other questions include, whether our observation of hematopoietic compartments in the LL2 tumor stroma can be applied to other tumor models, metastatic cancers, and even human cancers. Since one of the key features of hematopoietic cells is their ability to migrate and access to various tissues and organs, we hypothesize that this ability may contribute to the formation of clusters of hematopoietic cells that have been "educated" by the primary tumor to serve as metastatic microenvironment in distant locations. It thus will be interesting to study the relationship between hematopoietic cells in the primary cancer and the stroma of the metastatic cancer. It will also be important to determine the hematopoietic compositions of human tumor stroma.

Materials and Methods

Ethics Statement

All animal experiments were performed with the approval of UT Southwestern Committee on Animal Care (APN# 2007-0068).

Cell lines, animals, and tumor implantation and measurement

Murine Lewis lung carcinoma (LL2) cells were obtained from the ATCC and cultured under standard conditions. Retroviral MSCV-GFP was introduced into LL2 cells to produce stable GFP^+ LL2 cells. C57BL/6 CD45.2 and CD45.1 mice were purchased from the Jackson Laboratory or the National Cancer Institute. $IGF\text{-}IR^{-/+}$ mice as previously described [19] were in pure C57BL/6 background. All animals were maintained at the University of Texas Southwestern Medical Center animal facility and animal experiments were performed with the approval of UT Southwestern Committee on Animal Care. Tumor cells were injected subcutaneously into mice and mice were maintained for about 3 weeks. Tumor size was measured on the flanks of live mice using calipers; volume was calculated as (length of tumor)×(width of tumor)^2/2. To analyze lung metastasis, entire lungs were

harvested and single cell suspensions were prepared by collagenase treatment. GFP^+ LL2 cells originating from the distant implanted tumor were counted by flow cytometry analysis. The flow cytometry result was confirmed by counting GFP^+ surface foci of the harvested lung under a dissecting microscope.

Preparation of hematopoietic cells from tumors

Mice were perfused with cold PBS and primary tumors were removed and chopped with a McIlwain Tissue Chopper (Mickle Laboratory Engineering Company). The tissue was washed with PBS and then placed in collagenase-dispase medium (Liver Digest Medium, Invitrogen) at 37°C for 90 min as we described previously [21]. Cells passed through a 70-μm strainer were used for further flow cytometry analysis or sorting.

Flow cytometry

BM and PB cells were isolated from 5–8 week old C57BL/6 mice. $Lin^-Sca\text{-}1^+Kit^+$ or $Lin^-Sca\text{-}1^+Kit^+CD34^-Flk\text{-}2^-$ cells were isolated by staining with a biotinylated lineage cocktail (anti-CD3, anti-CD5, anti-B220, anti-Mac-1, anti-Gr-1, anti-Ter119, and anti-7-4; Stem Cell Technologies) followed by streptavidin-PE/Cy5.5, anti-Sca-1-FITC, and anti-Kit-APC, and anti-CD34-PE and anti-Flk-2-PE if necessary. To analyze hematopoietic lineages and repopulation of mouse HSCs, mouse peripheral blood cells were collected by retro-orbital bleeding, followed by lysis of red blood cells and staining with anti-CD45.2-FITC, and anti-CD45.1-PE, and anti-Thy1.2-PE (for T-lymphoid lineage), anti-B220-PE (for B-lymphoid lineage), anti-Mac-1-PE, anti-Gr-1-PE (cells co-staining with anti-Mac-1 and anti-Gr-1 were deemed to be of the myeloid lineage), or anti-Ter119-PE (for erythroid lineage) monoclonal antibodies. All antibodies were from BD Pharmingen. The "percent reconstitution" shown in figures was based on the staining results of anti-CD45.2 and anti-CD45.1. In all cases flow cytometry analysis of hematopoietic lineages was also performed to confirm multilineage reconstitution as we described [14,15,16].

Hematopoietic colony assays

$CD45^+$ cells from LL2 tumor stroma or normal BM cells were resuspended in IMDM with 2% FBS and were then seeded into methylcellulose medium M3334 (StemCell Technologies) for CFU-E, M3434 (StemCell Technologies) for CFU-GM, or M3630 (StemCell Technologies) for CFU-Pre-B assays, according to the manufacturer's protocols and as described [16].

HSC cell cycle analysis

The cell cycle analysis with Hoechst and pyronin Y staining was performed as described [16]. Briefly, the $Lin^-Sca\text{-}1^+Kit^+Flk2^-CD34^-$ cells were collected in Hank's buffered salt solution medium containing 10% FBS, 1 g/liter glucose, and 20 mM Hepes (pH 7.2). Cells were washed, Hoechst 33342 (20 μg/ml, Invitrogen) was added, and cells were incubated at 37°C for 45 min after which pyronin Y (1 μg/ml, Sigma) was added. Cells were incubated for another 15 min at 37°C, washed, and resuspended in cold PBS. Samples were immediately analyzed by flow cytometry (BD Biosciences, FACSAria).

Competitive reconstitution analysis

The indicated numbers of mouse CD45.2 or CD45.1 donor cells were mixed with 1 or 2×10^5 freshly isolated CD45.1 or CD45.2 competitor BM cells, and the mixture were injected intravenously *via* the retro-orbital route into each of a group of 6–9 week old CD45.1 or CD45.2 mice previously irradiated with a total dose of 10 Gy. For secondary transplantation, $CD45.1^+$ cells were collected from primary recipients and 10^6 cells were injected with 10^5 CD45.2 competitors into lethally irradiated secondary recipient mice. To measure reconstitution of transplanted mice, peripheral blood was collected at the indicated times post-transplant and the presence of $CD45.1^+$ and $CD45.2^+$ cells in lymphoid and myeloid compartments were measured [14,16].

Statistical analysis

Data are expressed as mean ± SEM. Tumor growth curves for different experimental groups were compared using the Generalized Estimating Equations (GEE) method with AR(1) correlation structure. Tumor sizes among different experimental groups were also compared at each time points using t-test. SAS 9.1.3 was used for the analysis. Data were considered statistically significant if $p<0.05$.

Author Contributions

Conceived and designed the experiments: HH JZ CCZ. Performed the experiments: HH JZ CCZ MU QW RS CJW. Analyzed the data: XX HH JZ CCZ. Contributed reagents/materials/analysis tools: MH. Wrote the paper: HH JZ QW CCZ.

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IGF Binding Protein 2 Supports the Cycling of Hematopoietic Stem Cells

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Previously we identified IGFBP2 as an extrinsic factor that supports ex vivo expansion of hematopoietic stem cells (HSCs). The role of IGFBP2 in HSCs and cancer is very intriguing. IGFBP2 can bind to insulin-like growth factor (IGF) ligands and displays IGF-dependent growth inhibitory effects on many cell types. On the other hand, IGFBP2 is capable of stimulating growth of certain cancer cells, and is overexpressed in many cancer patients and its expression is correlated with cancer progression. Here we sought to study the role of IGFBP2 in regulation of the activity of normal HSCs. We showed that IGFBP2 was expressed in differentiated hematopoietic cells and bone marrow stroma but not in HSCs. Consistent with its gene expression pattern, IGFBP2-/- HSCs had similar repopulation activity as their wild-type counterparts. By contrast, when we transplanted HSCs into IGFBP2-/- or wild-type recipient mice, we found decreased in vivo repopulation of HSCs in primary and secondary transplanted IGFBP2-/- recipients, suggesting that the environmental IGFBP2 positively supports HSC activity. Further co-culture of HSCs with IGFBP2-/- or wild-type bone marrow stromal cells indicated that IGFBP2 produced by bone marrow stroma indeed supports HSC expansion. Consistently, HSCs in IGFBP2-/- mice showed decreased frequency and cell cycling, and had upregulated expression of cell cycle inhibitors of p21, p16, and p19. To determine whether IGFBP2's effect on HSCs depends on IGF signaling, we compared the repopulation of donor cells deficient for the IGF type I receptor in wild-type and IGFBP2-/- recipients. These HSCs that are defective in IGF signaling still have decreased repopulation in IGFBP2-/- recipients, suggesting that the environmental effect of IGFBP2 on HSCs is independent of IGF signaling. To identify the functional domain of IGFBP2 in regulation of HSC activity, we constructed IGFBP2 with mutated RGD domain or deleted c-terminus and used the mutant IGFBP2 proteins in ex vivo culture of HSCs. We found that the c-terminus of IGFBP2 is essential to support HSC activity. We are currently in the process of identifying the potential receptor of IGFBP2 on HSCs. In summary, we found that IGFBP2 supports the cycling of normal HSCs, and this effect is independent of IGF signaling. Our study is important in revealing the relationship among environmental cues and cell fates of stem cells and opens up a new avenue in investigation of the roles of IGFBP2 in stem cells and cancer.

Angiopoietin-Like 3 Regulates the Activity of Hematopoietic Stem Cells in Extramedullary Organs

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Angiopoietin-like proteins (Angptls) are a seven-member family of secreted glycoproteins that share sequence homology with angiopoietins. It is known that several members of the Angptl family including Angptl3 support ex vivo expansion of hematopoietic stem cells (HSCs). However, the physiological role of Angptls in the hematopoietic system is not well known. Here we show that Angptl3 is expressed by both bone marrow stromal cells and HSCs. To study the intrinsic effect of Angptl3 in mouse HSCs, we isolated the same number of HSCs from wild-type and Angptl3-null mice and performed reconstitution analysis. Adult bone marrow Angptl3-null HSCs showed decreased repopulation compared to wild-type HSCs, suggesting that Angptl3 has cell-autonomous effect on HSC activity. By contrast, HSCs isolated from liver of the null mice had enhanced HSC repopulation activity than their wild-type counterparts. To study whether this effect is caused by difference in homing, we injected CFSE labeled wild-type HSCs and Angptl3 null HSCs into lethally irradiated mice, and checked the homing to bone marrow, spleen, and liver. While homing of these two types of cells to bone marrow or spleen was not significantly different, Angptl3 null HSCs homed better to the liver than the wild-type HSCs. Our result suggests that Angptl3 is important for the retention of HSCs in the bone marrow, and the absence of Angptl3 leads HSCs to move to extramedullary organs such as liver.

Ex vivo expanded hematopoietic stem cells overcome the MHC barrier in allogeneic transplantation

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Success of allogeneic transplantation is severely hampered by low levels of donor engraftment and high risks of graft-*versus*-host disease (GVHD). Transplantation of purified allogeneic blood stem cells (HSCs) diminishes the risk of GVHD, but also results in decreased engraftment. Here we show that *ex vivo* expanded HSCs efficiently overcame the major histocompatibility complex (MHC) barrier and engrafted allogeneic recipient mice. As measured by limiting dilution analysis, there was a 40-fold increase in the allograft ability of the 8-day-cultured HSCs compared to that of the freshly isolated HSCs. We found that both increased numbers of HSCs and cultured-induced elevation of expression of the immune inhibitor on the surface of HSCs contributed to the enhancement. B7-H1, an immune inhibitor that is expressed on freshly isolated HSCs, was significantly upregulated on cultured HSCs. The use of B7-H1-deficient HSCs or HSCs treated with a B7-H1 neutralizing antibody abrogated the ability of cultured but unexpanded HSCs to cross the MHC barrier. To test whether cultured HSCs can be used to cure genetic diseases in allogeneic recipients, we used *ex vivo* expanded allogeneic HSCs for transplantation and successfully rescued the lethal phenotype of DNA-PK knock-in mice. Our study suggests that HSCs can be manipulated to regulate their immune privilege and this will likely lead to development of new strategies for successful allogeneic transplantation for human patients.

IGF Binding Protein 2 Supports the Activity of Acute Myeloid Leukemia Stem Cells

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Acute myeloid leukemia (AML) is the most common acute leukemia affecting adults, and its incidence increases with age. Despite continuous treatment, the majority of the patients relapse within 5 years. It has been recently suggested that cancer stem cells, including AML stem cells, are essential for tumor initiation and relapse. IGFBP2 is a secreted factor we identified that support ex vivo expansion of HSCs. The role of IGFBP2 in AML and many other types of cancer is very intriguing. IGFBP2 can bind to insulin-like growth factor (IGF) ligands and displays IGF-dependent growth inhibitory effects on many cell types. On the other hand, IGFBP2 is capable of stimulating growth of certain cancer cells, and is overexpressed in many cancer patients and in some cases its expression correlates with grade of malignancy. Some of the growth-stimulatory effects of IGFBP2 are shown to be independent of IGF signaling [2]. Consistently, IGFBP2 is expressed at significantly higher levels in AML patients than in healthy people, and the expression of IGFBP2 is an independent factor for the prediction of relapse of AML. We have established the retroviral MLL-AF9-IRES-YFP transplantation AML mouse model. We showed that IGFBP2 was expressed in normal hematopoietic cells, leukemia cells, and bone marrow stroma. The IGFBP2-/- mice have decreased ability to develop AML in our AML mouse model, suggesting that the environmental IGFBP2 positively supports AML development. Furthermore, the AML cells isolated from the primarily transplanted IGFBP2-/- mice have decreased ability to develop AML in serial transplanted mice. Consistently, IGFBP2 upregulated the expression of a number of oncogenes and invasive genes, and decreased the expression of a number of tumor suppressor and pro-apoptotic genes in AML stem cells. These results, together with our flow cytometry analysis of the phenotypic AML stem cells, concluded that IGFBP2 supports the activity of AML stem cells. To determine whether IGFBP2's effect on AML stem cells depends on IGF signaling, we compared the leukemia development of donor cells deficient for the IGF type I receptor in wild-type and IGFBP2-/- recipients. These AML cells that are defective in IGF signaling still have decreased cancer development, suggesting that the environmental effect of IGFBP2 on AML stem cells is independent of IGF signaling. To our knowledge, this project is the first mechanistic study of the role of IGFBP2 in AML development and AML-SC activity. Our study is important in revealing the relationship among environmental cues, cell fates of leukemia stem cells, and leukemia development and relapse. It will significantly advance our understanding of the AML pathogenesis, and pave the way for the development of novel strategies that treat human leukemia.

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Graduate research with Professor David J. Shapiro in expression of human estrogen receptor (ER) in bacterial and mammalian systems, mechanisms of HMG-1 enhanced ER binding to the estrogen response element, and estrogen receptor-dependent signaling pathway and apoptosis.

2000-2006: Postdoctoral Fellow, Whitehead Institute for Biomedical Research.
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2007	Michael L. Rosenberg Scholar in Biomedical Research, UT Southwestern
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4. Prion and stem cells

Teaching:

2007 Lecture on Hematopoietic Stem Cells in Advances in Germ and Stem Cell Biology
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2009 Lecture on stem cells and dormancy in Immunology
2009 Lecture on Leukemia and the Discovery of Cancer Stem Cells in Cancer Biology II (GD 5096-02)
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2009 Lecture on Endocrinology in School of Health Sciences
2009 Cells & Organelles Thread Discussion Group Leader
2009 Journal club "Stem Cells", Integrative Biology Graduate Program

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2010 Cells & Organelles Thread Discussion Group Leader
2010 Co-facilitator for small group discussion, Convergence Learning Community Day
2010 Journal club "Stem Cells", Integrative Biology Graduate Program
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UTSW Service:

2009 Faculty Search Committee, Department of Physiology
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2011 Faculty Senate, Department of Physiology, UTSW

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2007 Grant reviewer, American Institute of Biological Sciences (AIBS), New Jersey Commission on Science and Technology (NJCST): Stem Cell Initiative
2008 ad hoc grant reviewer, Cancer Research UK Program
2008 Grant reviewer, New York State Stem Cell Program (NYSTEM)
2009 Grant reviewer, NIH Challenge Grant Special Emphasis Panel/Scientific Review Group 2009/10 ZRG1 VH-D (58) R
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2010 ad hoc grant reviewer, Molecular Research Council, UK
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2011 Chair, Stem Cell Tissue Engineering, 27th Southern Biomedical Engineering Conference, Arlington, TX

Invited Talk:

2004 Biotechnology Process Engineering Center Industrial Consortium and Advisory Board Meeting, MIT, Cambridge, MA
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2006	American Society for Cell Biology Summer Meeting: Stem Cell Niche, Boston, MA
2007	Job talks in 8 institutions: UTSW, UCSD, UCLA, CSHL, Upenn, NYU, etc
2008	Children's Hospital Oakland Research Institute, CA
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Manuscripts submitted or in preparation:

1) Jinhui Shen, Ramesh Saxena, Shwetha Kamath, Jenie Borchardt, **Cheng Cheng Zhang**, Liping Tang. Multipotent stem cells recruited in response to peritoneal dialysis. Submitted

2) Chu, Pak Yan, Li, Zhihong, Bari, Sudipto, Xiubo, Fan, Huang, Jenny, Prasath, Arun, Ang, Justina ML, Virshup, David, Lodish, Harvey F., **Zhang, Chengcheng**, Chiu Ngar Chee, Gigi Lim, Sai Kiang, Hwang, William Ying Khee. Mesenchymal stromal cell co-culture enhances ex vivo cultures of human cord blood in a viability supporting, contact-dependent process involving the rescue of cells from early apoptosis. Submitted

3) Junke Zheng, Masato Umikawa, Changhao Cui, Jiyuan Li, Xiaoli Chen, Chaozheng Zhang, HoangDinh Hyunh, Robert Silvany, Sally Ward, **Cheng Cheng Zhang**. Cloning of the receptor for angiopoietin-like proteins. Submitted

4) Tan C, Wang Q, **Zhang CC**. Anthrax Biomarker Induced Optical- and Electrochemical-Responses Based on Terbium Complex Covalently Loaded Single-Walled Carbon Nanotube. Submitted

5) Kocabas F, Zheng J, Simsek T, deBerardinis RJ, **Zhang CC**, Sadek HA. Unique Metabolic Footprint of Human Hematopoietic Stem Cells. In preparation

6) Junke Zheng, Ralph T Bottcher, Reinhard Fassler, **Cheng Cheng Zhang**. Profilin-1 is required for retention of normal hematopoietic and leukemia stem cells in the bone marrow. In preparation

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Patents:

Zhang CC and Lodish HF. 2010. Cultured hematopoietic stem cells and method for expansion and analysis thereof. *U.S. patent 7767453B2*

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American Heart Association
Extrinsic control of cell fates of hematopoietic stem cells

PR093256 (Zhang) 2010-2013
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