Table 2 Overall summary of safety

	N (%)	Rate
Any adverse events (AE)	44 (100)	11.15
Serious AE (SAE)	9 (21)	0.31
Any treatment-related AE	27 (61)	1.70
Any treatment-related SAE	1 (2)	0.02
Death	0 (0)	0
Withdrawal due to AE	0 (0)	0

Rate events per 100 patient-weeks

Table 3 Serious adverse events (SAE)

	N (rate)
All SAE	14 (0.31)
Hemorrhagic anemia	1 (0.02)
Thrombocytopenia	1 (0.02)
Appendicitis	1 (0.02)
Grand mal convulsion	1 (0.02)
Transient ischemic attack	1 (0.02)
Epistaxis	1 (0.02)
Intracranial aneurysm	1 (0.02)
Lumbar spinal stenosis	1 (0.02)
Allergic transfusion reaction	1 (0.02)
Melena	1 (0.02)
Mouth hemorrhage[a]	1 (0.02)
Subcutaneous hematoma	1 (0.02)
Wound	1 (0.02)
Spinal compression fraction	1 (0.02)

Rate events per 100 patient-weeks

[a] Considered by the investigator to be related to romiplostim

no life-threatening adverse events, and no patients died or withdrew from the study.

A total of 50 hemorrhagic adverse events were reported in 20 patients (46 %), with a duration-adjusted rate of 1.12/100 patient-weeks. The most common hemorrhagic adverse events were contusion (0.29/100 patient-weeks), epistaxis (0.16/100 patient-weeks), purpura (0.11/100 patient-weeks), and conjunctival hemorrhage (0.09/100 patient-weeks). Three patients (7 %) had a total of 5 serious hemorrhagic adverse events; one with epistaxis and hemorrhagic anemia, one with melena and subcutaneous hematoma, and one with mouth hemorrhage.

Regarding adverse events of interest, no cases were reported of hematopoietic malignancy, myelodysplastic syndrome, thrombocytosis, or bone marrow reticulin/collagen fibrosis (bone marrow biopsies were performed at investigator discretion). A total of 14 biopsies were performed on 8 patients over a wide range of time, from before the study (1), within the first year (8), to more than 1 year up to over 2 years (5). All biopsies were negative for reticulin and collagen. However, after this data cutoff (on study day 735), one patient experienced a mild non-serious adverse event of increased reticulin that was considered by the investigator to be related to treatment with romiplostim. The only thromboembolic event was a serious adverse event of transient ischemic attack. Additionally, no patients tested positive for neutralizing antibodies to romiplostim or TPO in the antibody assays that were performed every 12 weeks.

Efficacy

Overall, 96 % of patients had a platelet response (doubling of the baseline platelet count at study entry of the previous study and platelet counts $\geq 50 \times 10^9$/L) (response rate over time shown in Fig. 3). Median platelet counts stayed above 50×10^9/L each week from week 2 onward (Fig. 4). Of the 25 patients receiving concurrent ITP therapy at baseline, all were able to reduce or discontinue that therapy: 11 (44 %) had a >25 % reduction in at least 1 concurrent therapy, 5 (20 %) had a >50 % reduction in at least 1 concurrent therapy, and 9 (36 %) discontinued all concurrent therapies. There was an overall decrease over time in the proportion of patients with bleeding events (Fig. 5). Eight patients (18 %) received rescue medications for ITP at some point during the study. These included prednisolone (6 patients), platelet transfusion (5 patients), immunoglobulins (3 patients), dexamethasone and red blood cell transfusion (each 1 patient). Details on individual patients are provided in Table 4.

Discussion

Results of this study indicate that romiplostim administration for up to 3.5 years was well tolerated in Japanese patients with ITP. The reported incidence of adverse events did not increase over time during long-term exposure to romiplostim and were similar to those seen in other romiplostim studies, such as the long-term open-label extension in ITP patients of other ethnic origins ($N = 292$) [32]. In addition, both studies had similar proportions of patients having a platelet response (96 % in this study, 95 % in the other), similar median doses (3.8 vs. 4.0 μg/kg), and a majority of patients initiating self-injection (64 vs. 82 %). In this study, the safety and tolerability of romiplostim self-injection was generally satisfactory; however, please note that self-injection of romiplostim is not approved in Japan. During self-injection, patients continued to have regular platelet count assessments, and, if platelet counts were greater than the target range ($50–200 \times 10^9$/L), romiplostim was discontinued. This discontinuation rule applied to those who received romiplostim from a healthcare provider

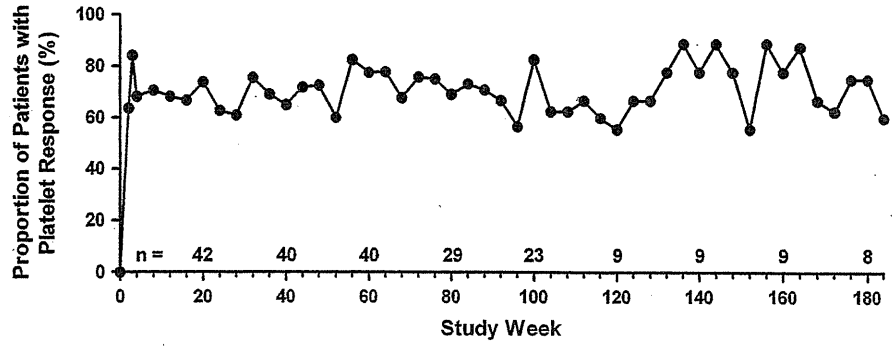

Fig. 3 Platelet response over time

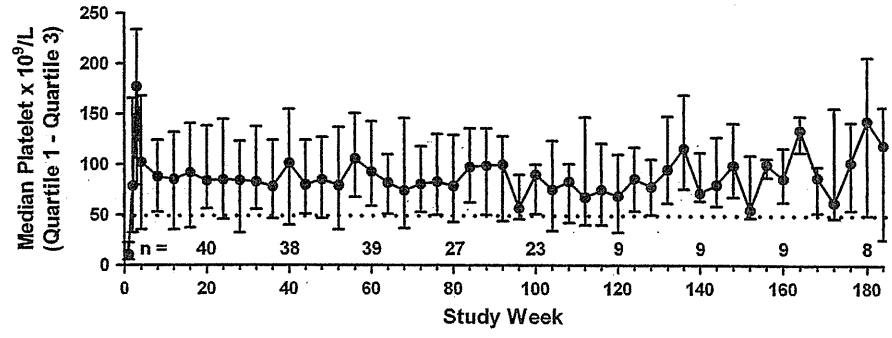

Fig. 4 Platelet count over time

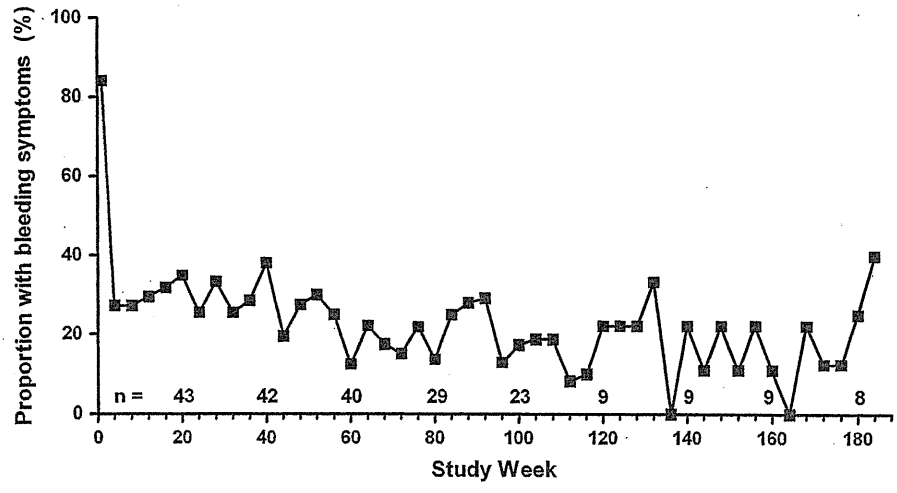

Fig. 5 Bleeding symptoms over time

as well. Concurrent ITP medications at baseline were reduced or discontinued in most patients in both studies (100 vs. 81 %). Overall, the efficacy and safety profile is consistent in these two study populations [28, 33, 34].

During the study, one thromboembolic event of transient ischemic attack was reported. The event occurred in a patient who had a history of paroxysmal atrial fibrillation, hyperlipidemia, and hyperbilirubinemia. Although the transient ischemic attack was not judged to be related to romiplostim by the investigator based on the patient's medical history, this kind of thromboembolic event should be noted and followed carefully during romiplostim treatment. Thus, the benefit:risk ratio of romiplostim should be carefully considered in patients with significant risk factors for thromboembolic events, and platelet counts should be closely monitored. Other reported serious adverse events were mouth hemorrhage, hemorrhagic anemia, thrombocytopenia, appendicitis, grand mal convulsion, epistaxis, intracranial aneurysm, lumbar spinal stenosis, allergic transfusion reaction, melena, mouth hemorrhage, subcutaneous hematoma, wound, and spinal compression fracture, each occurring at a rate of 0.02/100 patient-weeks. Eight patients received rescue medications for ITP, including prednisolone, platelet transfusion,

Table 4 Rescue medication use

Patient	Rescue medication	Number of incidents	Notes[a]
1	Prednisolone	1	
2	Platelet transfusion	2	
3	Prednisolone	3	
4	Prednisolone	2	
	IVIg	1	
5	Dexamethasone	1	
	IVIG	2	GI hemorrhage leading to anemia
	Platelet transfusion	7	One was due to epistaxis, the other 6 due to GI hemorrhage leading to anemia
	Prednisolone	2	
6	Platelet transfusion	1	Mouth hemorrhage
	Prednisolone	5	
	IVIg	3	1 was due to mouth hemorrhage
7	Platelet transfusion	22	2 were due to subcutaneous hematoma
	RBC transfusion	11	All were due to anemia
	Prednisolone	3	
8	Platelet transfusion	4	

GI gastrointestinal

[a] Unless otherwise indicated, use was for thrombocytopenia, not any other specific cause

immunoglobulin, dexamethasone and red blood cell transfusion. There were no deaths and no neutralizing antibodies to romiplostim or TPO. A total of 14 bone marrow biopsies were performed on 8 patients over a wide range of time, with no findings of bone marrow reticulin or collagen as of this data cutoff. Subsequently, there was a mild nonserious adverse event of increased reticulin considered related to romiplostim (study day 735).

A higher romiplostim dose was consistently seen with patients originally from the phase 2 study as compared with those from the phase 3 study. It was thought that this may reflect that the patients from the phase 2 study had a longer history of ITP (median 11.8 vs. 5.8 years for the phase 3 study), and hence likely more advanced disease. To explore this possibility, we performed a post hoc analysis and found that higher romiplostim doses were related to lower platelet count at study entry ($p = 0.0003$)(i.e., inversely related) and inversely related to *H. pylori* eradication prior to study start ($p < 0.0001$), and positively associated with starting dose in this extension study ($p = 0.006$). Of note, the association of ITP duration with romiplostim dose was not statistically significant ($p = 0.1$). Rather, the higher dose in patients originally in the phase 2 study was due to lower platelet count at study entry ($p = 0.01$) and higher starting dose ($p = 0.02$) compared with the phase 3 study, as per study design.

One limitation of this study is the relatively small size (44 patients). Therefore, it is difficult to make conclusions regarding different patient subgroups (such as splenectomized vs. non-splenectomized, etc.). As of this data cutoff, there have been 86 patient-years of romiplostim exposure in this extension study; as this study is ongoing, analyses at future dates will be based on longer exposure time. Another possible limitation is self-selection, as often patients who respond to a medication are more likely to enter an extension study. As a high proportion of patients from earlier studies (44/46, or 96 %) enrolled into this study, it is unlikely that selection bias influenced the results of this extension trial.

In conclusion, similarly to non-Japanese patients, long-term administration of romiplostim was well tolerated in Japanese patients with ITP, with the vast majority of patients achieving a platelet response and no new safety concerns. With the approval of romiplostim in Japan, Japanese patients with ITP will now have access to another option for second-line therapy.

Acknowledgments The authors would like to thank all the study investigators and study coordinators, as well as Dr. Kiyoshi Kitamura of Tokyo University who provided medical advice during the study. This study was supported by Amgen Inc and Kyowa Hakko Kirin Co., Ltd. Medical writing assistance was provided by Susanna Mac, MD/PhD, of Amgen Inc.

References

1. Rodeghiero F, Stasi R, Gernsheimer T, Michel M, Provan D, Arnold DM, et al. Standardization of terminology, definitions and outcome criteria in immune thrombocytopenic purpura of adults and children: report from an international working group. Blood. 2009;113:2386–93.
2. Cines DB, Bussel JB, Liebman HA, Luning Prak ET. The ITP syndrome: pathogenic and clinical diversity. Blood. 2009;113:6511–21.
3. Nugent D, McMillan R, Nichol JL, Slichter SJ. Pathogenesis of chronic immune thrombocytopenia: increased platelet destruction and/or decreased platelet production. Br J Haematol. 2009;146:585–96.
4. McMillan R. The pathogenesis of chronic immune thrombocytopenic purpura. Semin Hematol. 2007;44:S3–11.
5. Molineux G, Newland A. Development of romiplostim for the treatment of patients with chronic immune thrombocytopenia: from bench to bedside. Br J Haematol. 2010;150:9–20.
6. Fujimura K, Kuwana M, Kurata Y, Imamura M, Harada H, Sakamaki H, et al. Is eradication therapy useful as the first line of treatment in Helicobacter pylori-positive idiopathic thrombocytopenic purpura? Analysis of 207 eradicated chronic ITP cases in Japan. Int J Hematol. 2005;81:162–8.

7. Kurata Y, Fujimura K, Kuwana M, Tomiyama Y, Murata M. Epidemiology of primary immune thrombocytopenia in children and adults in Japan: a population-based study and literature review. Int J Hematol. 2011;93:329–35.
8. Provan D, Stasi R, Newland AC, Blanchette VS, Bolton-Maggs P, Bussel JB, et al. International consensus report on the investigation and management of primary immune thrombocytopenia. Blood. 2009;115:168–86.
9. Neunert C, Lim W, Crowther M, Cohen A, Solberg L Jr. The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia. Blood. 2011;117: 4190–207.
10. Arnold DM, Bernotas A, Nazi I, Stasi R, Kuwana M, Liu Y, et al. Platelet count response to *H. pylori* treatment in patients with immune thrombocytopenic purpura with and without *H. pylori* infection: a systematic review. Haematologica. 2009;94:850–6.
11. Arnold DM, Stasi R. Does *Helicobacter pylori* eradication therapy result in a platelet count improvement in adults with immune thrombocytopenic purpura regardless of *H pylori* infection? ASH evidence-based review. Hematology Am Soc Hematol Educ Program. 2008;2008:31–2.
12. Kuwana M, Ikeda Y. *Helicobacter pylori* and immune thrombocytopenic purpura: unsolved questions and controversies. Int J Hematol. 2006;84:309–15.
13. Suzuki T, Matsushima M, Masui A, Watanabe K, Takagi A, Ogawa Y, et al. Effect of *Helicobacter pylori* eradication in patients with chronic idiopathic thrombocytopenic purpura-a randomized controlled trial. Am J Gastroenterol. 2005;100: 1265–70.
14. Tsumoto C, Tominaga K, Okazaki H, Tanigawa T, Yamagami H, Watanabe K, et al. Long-term efficacy of *Helicobacter pylori* eradication in patients with idiopathic thrombocytopenic purpura: 7-year follow-up prospective study. Ann Hematol. 2009;88:789–93.
15. McMillan R. Therapy for adults with refractory chronic immune thrombocytopenic purpura. Ann Intern Med. 1997;126:307–14.
16. Stasi R, Provan D. Management of immune thrombocytopenic purpura in adults. Mayo Clin Proc. 2004;79:504–22.
17. Nomura S, Dan K, Hotta T, Fujimura K, Ikeda Y. Effects of pegylated recombinant human megakaryocyte growth and development factor in patients with idiopathic thrombocytopenic purpura. Blood. 2002;100:728–30.
18. Rank A, Weigert O, Ostermann H. Management of chronic immune thrombocytopenic purpura: targeting insufficient megakaryopoiesis as a novel therapeutic principle. Biologics. 2010;4:139–45.
19. Nplate (romiplostim), European Public Assessment Report summary. 2009.
20. Nplate™ (romiplostim) Prescribing Information. Amgen, Inc., Thousand Oaks, CA. 2011.
21. Nplate™ (romiplostim) Prescribing Information. European Medicines Agency. Amgen, Inc., Thousand Oaks, CA. 2011.
22. Romiplate prescribing information. 2011; http://www.mhlw.go.jp/stf/shingi/2r985200000136yg-att/2r98520000001372i.pdf.
23. Burroughs VJ, Maxey RW, Levy RA. Racial and ethnic differences in response to medicines: towards individualized pharmaceutical treatment. J Natl Med Assoc. 2002;94:1–26.
24. Matthews HW. Racial, ethnic and gender differences in response to medicines. Drug Metabol Drug Interact. 1995;12:77–91.
25. Kumagai Y, Fujita T, Ozaki M, Sahashi K, Ohkura M, Ohtsu T, et al. Pharmacodynamics and pharmacokinetics of AMG 531, a thrombopoiesis-stimulating peptibody, in healthy Japanese subjects: a randomized, placebo-controlled study. J Clin Pharmacol. 2007;47:1489–97.
26. Shirasugi Y, Ando K, Hashino S, Nagasawa T, Kurata Y, Kishimoto Y, et al. A phase II, open-label, sequential-cohort, dose-escalation study of romiplostim in Japanese patients with chronic immune thrombocytopenic purpura. Int J Hematol. 2009;90:157–65.
27. Shirasugi Y, Ando K, Miyazaki K, Tomiyama Y, Okamoto S, Kurokawa M, et al. Romiplostim for the treatment of chronic immune thrombocytopenia in adult Japanese patients: a double-blind, randomized Phase III clinical trial. Int J Hematol. 2011;94: 71–80.
28. Kuter DJ, Bussel JB, Lyons RM, Pullarkat V, Gernsheimer TB, Senecal FM, et al. Efficacy of romiplostim in patients with chronic immune thrombocytopenic purpura: a double-blind randomised controlled trial. Lancet. 2008;371:395–403.
29. Gupta S, Indelicato SR, Jethwa V, Kawabata T, Kelley M, Mire-Sluis AR, et al. Recommendations for the design, optimization, and qualification of cell-based assays used for the detection of neutralizing antibody responses elicited to biological therapeutics. J Immunol Methods. 2007;321:1–18.
30. Mason S, La S, Mytych D, Swanson SJ, Ferbas J. Validation of the BIACORE 3000 platform for detection of antibodies against erythropoietic agents in human serum samples. Curr Med Res Opin. 2003;19:651–9.
31. Mire-Sluis AR, Barrett YC, Devanarayan V, Koren E, Liu H, Maia M, et al. Recommendations for the design and optimization of immunoassays used in the detection of host antibodies against biotechnology products. J Immunol Methods. 2004;289:1–16.
32. Kuter DJ, Bussel JB, Newland A, Wasser JS, Lyons RM, George JN, et al. Long-term efficacy and safety of romiplostim treatment of adult patients with chronic immune thrombocytopenia (ITP): final report from an open-label extension study. ASH Annu Meet Abstr. 2010;116:68.
33. Bussel JB, Kuter DJ, Pullarkat V, Lyons RM, Guo M, Nichol JL. Safety and efficacy of long-term treatment with romiplostim in thrombocytopenic patients with chronic ITP. Blood. 2009;113: 161–71.
34. Kuter DJ, Rummel M, Boccia R, Macik BG, Pabinger I, Selleslag D, et al. Romiplostim or standard of care in patients with immune thrombocytopenia. N Engl J Med. 2010;363:1889–99.

EXPERT OPINION

New approaches to expand hematopoietic stem and progenitor cells

Taito Nishino, Mitsujiro Osawa & Atsushi Iwama†
†*Chiba University, Graduate School of Medicine, Department of Cellular and Molecular Medicine, Chiba, Japan*

Introduction: Hematopoietic stem cells (HSCs) are defined by their capacity to self-renew and to differentiate into all blood cell lineages, and are currently the foundation of HSC transplantation therapy. A variety of methods have recently been explored to find a way to expand hematopoietic stem and progenitor cells (HSCs/PCs) *ex vivo* in order to improve the efficiency and outcome of HSC transplantation.

Areas covered: Recent studies of HSCs/PCs have led to the development of new ways to detect and purify HSCs/PCs and have also revealed several intrinsic and extrinsic factors that control the molecular signals fundamental to self-renewal and differentiation of HSCs. These findings have provided new approaches for expanding HSCs/PCs *ex vivo* utilizing protein factors and small-molecule compounds (SMCs) and have also demonstrated promising outcomes in clinical trials.

Expert opinion: Although further technical innovation is still needed, elucidation of the whole picture of signaling pathways critical to HSCs/PCs and manipulation of such pathways by SMCs could establish efficient, cost-effective, riskless and robust methods for *ex vivo* expansion of HSCs/PCs. With these efforts, more sophisticated HSC transplantation would be possible in the near future.

Keywords: cord blood, epigenetic regulation, *ex vivo* expansion, hematopoietic stem cells, small-molecule compounds

Expert Opin. Biol. Ther. [Early Online]

1. Introduction

Hematopoietic stem cells (HSCs) are the best characterized among adult stem cells and stand at the forefront of stem cell research and clinical applications. HSCs are defined as cells possessing the ability to reproduce themselves, an ability termed self-renewal, and the ability to differentiate into all of the necessary blood cells, including the erythroid, myeloid and lymphoid lineages [1-4]. Due to these characteristics, HSCs can be used as cell therapy agents (grafts) in the remedy of high-risk hematological malignancies and diseases of blood-forming cells and immune systems [5-7].

There are currently three different sources of HSCs for hematopoietic stem cell transplantation (HSCT): umbilical cord blood (CB), bone marrow (BM) and mobilized peripheral blood (PB). Compared with other sources of HSCs, CB has several clinical advantages, including rapid and convenient availability from numerous CB banks, less stringent criteria for HLA matching, lower incidence of severe graft-versus-host disease (GVHD) without compromising graft-versus-leukemia effects, lower risk of viral transmission and absence of risk for donors. However, the limited dose of hematopoietic stem and progenitor cells (HSCs/PCs) provided in a single CB unit results in a higher incidence of graft failure and delayed recovery

informa
healthcare

10.1517/14712598.2012.681372 © 2012 Informa UK, Ltd. ISSN 1471-2598

Article highlights.

- Hematopoietic stem cells (HSCs) have attracted attention as representative of stem cells for biological research and therapy, and recent studies have revealed specific biomarkers to identify HSCs and several regulators that govern proliferation, self-renewal and maintenance of HSCs.
- *Ex vivo* expansion of hematopoietic stem and progenitor cells (HSCs/PCs) is a sought-after means to improve HSC transplantation therapy using cord blood (CB) and has been developed on the basis of the above-described studies.
- Preliminary results of HSC expansion in clinical settings are promising, but in-depth studies are fundamental to establish the real value of these new approaches.
- Small-molecule compounds (SMCs) are becoming more common as tools for HSC expansion and hold promise to make a difference in this field.

This box summarizes key points contained in the article.

of neutrophils and platelets. Delayed neutrophil recovery leads to higher risk of bacterial and fungal infections, which are primarily associated with transplant-related mortality in the early phase after CB transplantation [8-10]. To overcome the shortcomings of current CB transplantation, various attempts have recently been made to expand human CB HSCs/PCs in cultures *ex vivo* to acquire a greater number of transplantable HSCs/PCs [11-13]. To date, most *ex vivo* culture systems have employed soluble cytokines, including stem cell factor (SCF), Fms-like tyrosine kinase 3 ligand (FL) and thrombopoietin (TPO), but have not yet resulted in sufficient expansion in clinical trials. By contrast, more recent approaches using not only these cytokine combinations, but also key factors for the self-renewal and maintenance of HSCs have led to clinically potential *ex vivo* expansion of HSCs/PCs.

In this article, the authors review the methods to identify HSCs and the attempts at *ex vivo* expansion of HSCs/PCs using transcriptional factors, cytokines, growth factors and niche cells that control the self-renewal, differentiation and homing of HSCs. The authors also focus on recent approaches employing small-molecule compounds (SMCs) and discuss the challenges and feasibility in this field.

2. Detection and purification of HSCs/PCs

Transplantation assays have proven to be excellent experimental models to evaluate HSCs: true HSCs can be assessed by the ability to reconstitute the entire hematopoietic system and to permanently and stably give rise to the three major blood lineages, myeloid cells, T-lymphocytes and B-lymphocytes, following transplantation into myeloablative recipients. Meanwhile, the development of technologies using combination of fluorescence-activated cell sorter (FACS) and multi-staining procedure of monoclonal antibodies has remarkably improved the efficiency of the identification and isolation of HSCs. With respect to mouse HSCs, the $CD34^{low/-}$c-$Kit^+Sca\text{-}1^+$Lineage-marker$^-$ ($CD34^-KSL$) cell population is a very rare cell population (0.004% of the mouse BM) that is highly enriched in HSCs, with single cell transplantations reconstituting hematopoiesis in more than one out of three recipients [14]. In addition, the $CD150^+CD48^-CD41^-$Lineage-marker$^-$ cell population has recently been reported to be highly enriched for HSCs in mice [15,16].

Human HSC activity can be evaluated by xenotransplantations into immunodeficient mouse strains such as non-obese diabetic/severe combined immunodeficiency (NOD/SCID), NOD/Shi-scid/IL-$2R\gamma^{null}$ (NOG) and NOD/SCID gamma (NSG) [17-20]. The cells that possess long-term reconstitution and differentiation capacity in the immunodeficient mouse are referred to as SCID-repopulating cells (SRCs) and are regarded as the closest cells to human HSCs. After xenotransplantion of human hematopoietic cells into immunodeficient mice by intravenous injection, SRCs engraft in the mouse BM and give rise to human $CD45^+$ cells throughout the lifetime of the recipient. Using this assay, the frequency of SRCs in human CB, BM and mobilized PB was reported to be 1 in 9.3×10^5, 1 in 3×10^6 and 1 in 6×10^6, respectively [21]. The frequency of SRCs in CB is higher compared with those in BM and mobilized PB, but the absolute number of SRCs from CB is much lower since a smaller volume of blood can be obtained [22]. The lower absolute number of SRCs from CB as compared with other sources might account for the delayed recovery of hematopoiesis after transplantation.

The most important cell surface marker for human HSCs/PCs is CD34, an L-selectin ligand that is expressed on CB and BM cells at a rate of < 5% [23]. Human $CD34^+$ cells have been demonstrated to have engraftment potential clinically in numerous autologous and allogeneic transplantation therapies [24]. By contrast, $CD34^-$ cells have been reported to engraft in NOD/SCID mice using the intra-BM injection (IBMI) technique, giving rise to $CD34^+$ HSCs *in vitro* and *in vivo*, which suggests that a more primitive cell resides within the $CD34^-$ cell fraction [25,26]. Engraftment with $CD34^-$ cells has only been achieved by direct IBMI [27], suggesting that $CD34^-$ primitive hematopoietic cells have greater difficulty in homing to hematopoietic niches than $CD34^+$ cells. To enrich human HSCs, cells expressing lineage markers are depleted in a pre-enrichment step. Lineage-depleted (Lin^-) $CD34^+$ cells are further enriched for HSCs using the cell surface marker CD38; the frequency of SRCs in $Lin^-CD34^+CD38^-$ cells was reported to be 1/617 [28]. Other approaches include positive selection of cells that express specific hematopoietic markers, such as Thy-1 (CD90), c-Kit (CD117) and CD133 [29,30]. Majeti *et al.* demonstrated that the $Lin^-CD34^+CD38^-CD90^+CD45RA^-$ CB fraction contains HSCs at a frequency as high as 1 in 10 cells [31]. Cellular metabolic activity also offers an indication of stemness; the fraction highly excreting Hoechst 33342 (Ho) and rhodamine 123 (Rho) dyes has higher HSC ability [32,33].

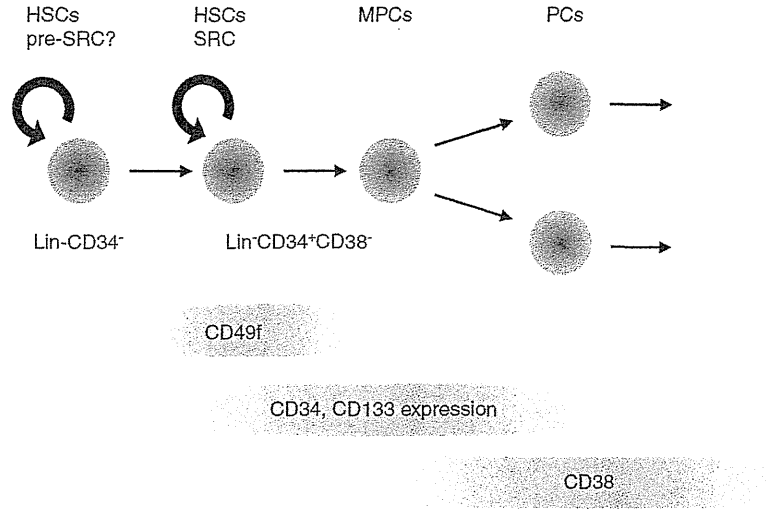

Figure 1. Expression profile of cellular markers on HSCs/PCs.
HSCs: Hematopoietic stem cells; MPCs: Multipotent hematopoietic progenitor cells; PCs: Hematopoietic progenitor cells; SRCs: SCID-repopulating cells.

Of note, Notta *et al.* identified CD49f as a specific marker for HSCs and succeeded in purifying human HSCs at single-cell resolution in the $Lin^-CD34^+CD38^-CD90^+Rho^{lo}$ $CD45RA^-CD49f^+$ fraction [34].

Elucidation of the hierarchy of HSCs/PCs and their cellular markers (Figure 1) is essential for the precise and efficient isolation of human HSCs/PCs, a step necessary for subsequent *ex vivo* expansion.

3. Regulators of HSC self-renewal

To formulate an effective and implementable strategy to establish *ex vivo* culture conditions that assure the expansion of lifelong reconstituting cells, or HSCs, comprehensive understanding of the factors that control HSC self-renewal and their mechanism of action is essential (Figure 2).

3.1 Intrinsic regulators

Although many advances have been made in identifying the intracellular signaling pathways that govern the proliferation, differentiation, senescence and cell death of HSCs, recent findings revealed the importance of transcriptional regulation of HSC self-renewal. The blood transcription factors such as GATA2, GFI1, TEL, JUNB, SOX17, PU.1 are reported to be necessary for mouse HSC self-renewal and summarized in a review by Zon [35]. Evi1, a transcription factor of the SET/PR domain protein family, has also been found to be essential for HSC maintenance and self-renewal in mice, and its expression marks cells with long-term multilineage repopulating activity [36]. Overexpression and direct delivery of the gene product of *SALL4*, a gene encoding a zinc-finger transcription factor originally identified as a critical regulator of embryonic stem cell (ESC) function, has been shown to enhance human HSC expansion [37]. The authors previously demonstrated that STAT5, a member of the family of signal transducers and activators of transcription, has a key function in regulating HSC self-renewal. Constitutive activation of Stat5 in mouse HSCs led to the prominent expansion of multipotent progenitors and promoted HSC self-renewal *ex vivo* [38]. As for human $CD34^+$ cells, intermediate levels of STAT5 activation are sufficient to induce HSC proliferation, and likely through the STAT5-mediated regulation of the expression levels of growth-promoting oncogenes, such as *PIM1* and *MAF* [39]. These studies suggest that manipulation of STAT5 activity may be of use in expanding HSCs in culture.

Homeobox B4 (HOXB4) is a member of transcription factors that share the common homeodomain and one of the most examined and important regulators of HSC self-renewal. Forced expression of *HoxB4* in mouse HSCs induces a drastic expansion of long-term reconstituting HSCs *ex vivo* [40]. This is also true in human HSCs. High-level ectopic expression of *HOXB4* in CB $CD34^+$ cells confers a significant expansion of HSCs, although it impairs myeloerythroid differentiation and significantly reduces B-cell output [41]. Human HSC expansion can also be achieved by direct delivery of the HOXB4 protein [42].

Recent studies have also demonstrated that epigenetic regulation of HSCs is important for HSC self-renewal and lineage restriction. Among epigenetic regulators, polycomb group (PcG) proteins have been the best characterized in the maintenance of HSCs. PcG proteins maintain the self-renewal capacity of HSCs by repressing tumor suppressor genes and keep differentiation programs poised for activation in HSCs by repressing a cohort of hematopoietic developmental regulator genes via bivalent histone domains [43-46]. Overexpression of

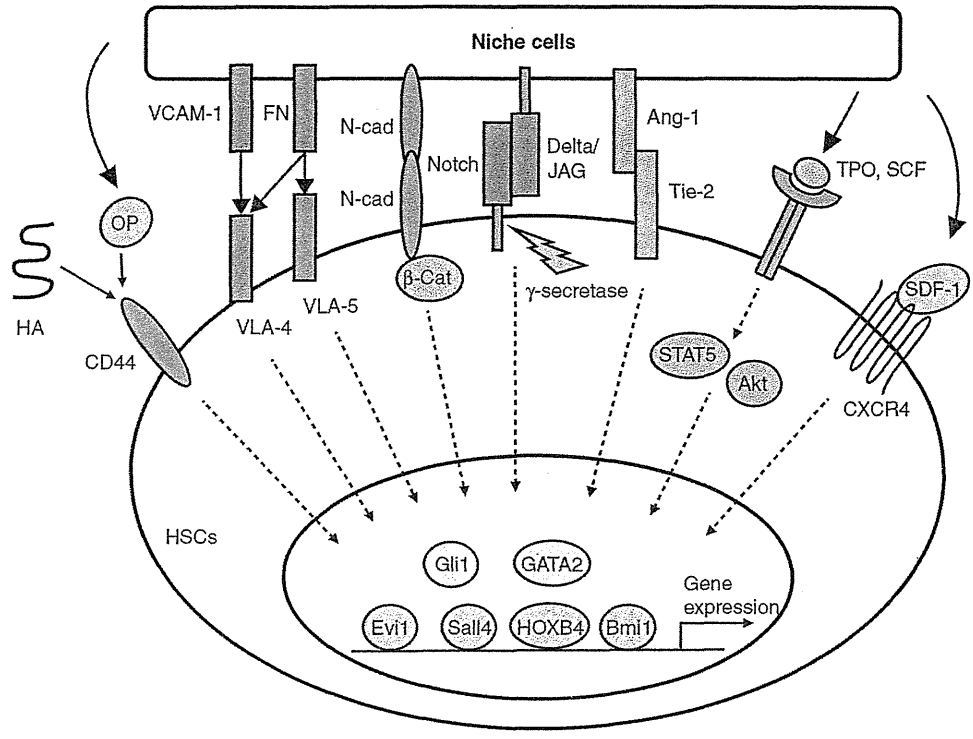

Figure 2. Intrinsic and extrinsic regulators of HSCs.
Ang-1: Angiopoietin-1; CXCR4: C-X-C chemokine receptor type 4; FN: Fibronectin; HA: Hyaluronic acid; HSCs: Hematopoietic stem cells; JAG: Jagged; N-cad: N-cadherin; OP: Osteopontin; SCF: Stem cell factor; SDF-1: Stromal cell-derived factor 1; SNO cells: Spindle-shaped N-cadherin$^+$CD45$^-$ osteoblasts; TPO: Thrombopoietin; VCAM-1: Vascular cell adhesion molecule-1; VLA-4 and -5: Very late antigen-4 and -5.

the PcG genes *Bmi1* and *Ezh2* augments the self-renewal capacity of mouse HSCs. Recent studies also showed a similar result from overexpression of *BMI1* in human CB CD34$^+$ cells [47]. Of interest, HoxB4 and Bmi1 function in a complementary, but independent, fashion in HSC self-renewal [44,48]. Overexpression of Fbxl10/Kdm2b, a demethylase specific to the histone H3 mono/di-methylated at lysine 36, also augments HSC function [49]. The research field on epigenetic regulation of HSCs is rapidly growing and attracting much attention.

3.2 Extrinsic regulators

HSCs/PCs reside in a special microenvironment called the niche. The niche is a specialized anatomic location composed of supporting cells and extracellular matrices that serve as essential surroundings for maintaining HSCs/PCs in the BM [50,51]. It has been reported that HSCs interact with osteoblasts lining the endosteal surface of the bone, termed spindle-shaped N-cadherin$^+$CD45$^-$ osteoblastic (SNO) cells, which form the endosteal niche [52]. HSCs are also reported to associate with sinusoidal endothelial cells lining blood vessel, which form vascular niche [16]. The relationship between the endosteal and vascular niches has not been fully elucidated, but a recent report has suggested that these niches are not mutually exclusive in regulating HSC maintenance and mobilization [53]. Other potential niche cells include marrow stromal cells, adipocytes, perivascular cells, CXCL12-abundant reticular (CAR) cells, non-myelinating Schwann cells ensheathing autonomic nerve, macrophages, megakaryocytes and so on [54-56]. Although there is the unresolved question of how these complicated niche cells regulate HSCs within the BM, the niche emanates a variety of signals to HSCs/PCs in the form of cytokines, chemokines, growth factors, hormones, cell adhesion proteins and extracellular matrices.

SCF signaling via its receptor, c-Kit, has been well investigated in regulating both proliferation and survival of HSCs/PCs [57]. Recent studies have revealed that SCF produced by endothelial and perivascular cells has a crucial function in the maintenance of HSCs [58]. SNO cells also secrete a variety of cytokines and chemokines that contribute to the maintenance and migration of HSCs. TPO and its receptor MPL (myeloproliferative leukemia virus oncogene) have been reported to enhance the quiescence of long-term HSCs in the endosteal niche [59,60]. Angiopoietin-1 (Ang-1) secreted from SNO cells binds to Tie-2, a cell surface receptor on HSCs, and the interaction of Ang-1 and Tie-2 has been reported to enhance the quiescence of HSCs by activating β1-integrin and N-cadherin [61]. Osteopontin, a matrix

Table 1. Clinical trials using *ex vivo* expanded CB.

Type of expansion	Number of patients	Additive factors	Culture period (day)	Expansion of $CD34^+$ cells (fold)	Days to neutrophil recovery	Survival (%) (duration of follow-up)	Ref.
Liquid culture	37	SCF, TPO, G-CSF	10	4	28	35 (30 months)	[81]
	10	SCF, TPO, FL, G-CSF, IL-6, TEPA	21	6	30	60 (180 days)	[84-87]
Co-culture with stromal cells	32	SCF, TPO, FL, G-CSF with stromal cells	14	14	15	40 (1 year)	[90,91]
Continuous perfusion culture in bioreactors	27	PIXY321, FL, EPO	12	0.5	22	39 (41 months)	[94]

EPO: Erythropoietin; FL: Fms-like tyrosine kinase 3 ligand; G-CSF: Granulocyte colony-stimulating factor; PIXY321: Granulocyte-macrophage colony-stimulating factor/IL-3 fusion protein; SCF: Stem cell factor; TEPA: Tetraethylenepentamine; TPO: Thrombopoietin.

glycoprotein that is produced by osteoblasts has been reported to be a negative regulator of HSC proliferation in the endosteal niche and limits the size of the stem cell pool [62,63]. Although many reports proposed an important role of the Notch signaling in self-renewal of HSCs [64,65], its role in HSCs is still controversial [66,67]. Stromal cell-derived factor 1 (SDF-1; also called CXCL12), a chemokine expressed by endothelial cells, SNO cells and stromal cells, is a well-characterized factor regulating HSC mobilization and homing. The signaling of SDF-1 and its receptor CXCR4 (C-X-C chemokine receptor type 4) also has a function in cell survival and maintenance of HSCs [68,69].

Accumulating data have indicated that adhesion molecules also play an important role in the interaction of HSCs with the niche through cell–cell interactions, and include N-cadherin/β-catenin, VCAM-1/VLA-4, fibronectin/VLA-4 and -5 and CD44/hyaluronic acid. Although hematopoiesis is normal in N-cadherin-deficient mouse, HSCs that express a dominant negative form of N-cadherin have reduced engraftment potential in BM [52,70,71]. VLA-4 (very late antigen 4, integrin $\alpha4\beta1$) is expressed on the surface of HSCs and mediates the retention of HSCs within the BM through interactions with VCAM-1 (vascular cell adhesion molecule 1), which is expressed on endothelial cells and osteoblasts. The expression of VCAM-1 correlates with the capacity of HSCs to home to the BM [54]. Fibronectin, one of the major extracellular components of the niche, is recognized by VLA-4 and -5 (integrin $\alpha5\beta1$) and this interaction supports the proliferation of human HSCs/PCs *in vitro* and *in vivo* [72]. CD44 glycoprotein and its ligand, hyaluronic acid, are essential for homing of human $CD34^+$ HSCs/PCs to the BM of NOD/SCID mice [73]. CD44 is also the receptor for osteopontin and the interaction of the two molecules may have various effects on HSCs/PCs including regulation of cell migration and proliferation.

4. Attempts to expand HSCs/PCs

The ultimate goals of *ex vivo* expansion of CB HSCs/PCs are to increase the number of PCs, thus permitting rapid recovery of immune cells and platelets, and to augment the number of stem cells in order to increase the chances of life-long hematopoietic reconstitution after transplantation. To accomplish these goals, there are several requirements to be met for *ex vivo* expansion of HSCs/PCs: i) net expansion of HSCs and PCs without loss of stem cell capacity via excess differentiations and senescence, ii) no abnormalities in cell cycling and no risk of carcinogenesis, iii) a balanced cell population in expanded cells, iv) optimized amount of cell number and time for transplantation and v) precise selection of HSCs/PCs with the highest regenerative potential. In this section, the authors will focus on the past and current approaches for HSC/PC expansion.

4.1 Early approaches

Cumulative efforts to develop techniques for *ex vivo* expansion of CB HSCs/PCs have been underway for the past two decades. There are currently three basic methods: liquid culture with cytokines, co-culture with stromal cells and continuous perfusion culture in bioreactors (Table 1).

4.1.1 Liquid culture with cytokines

CB cells ($CD133^+$ or $CD34^+$ cells) were usually cultured in liquid with combinations of cytokines, growth factors and other additives by using various plates, flasks, bags or containers as culture vessels. Cytokines have been extensively used in *ex vivo* culture of HSCs/PCs because of their important role in regulating cell fate of HSCs/PCs. Although the optimal combination of cytokines has yet to be precisely defined, common cytokines used in *ex vivo* HSC/PC expansion include SCF, TPO, FL, IL-3, IL-6, granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GM-CSF) and erythropoietin (EPO) [74]. The most commonly used combination of cytokines is a mixture of SCF [75], TPO [76,77] and FL [78]. This may be associated with the finding that SCF and TPO function to regulate proliferation of HSCs/PCs in the niche as described before. FL has been shown to enhance the adhesion to endothelial cells via modulating VLA-4 and -5

expression on PCs [79]. SCF has also been reported to improve SCID-repopulating activity of CB HSCs/PCs through the induction of matrix metalloproteinases [80].

Shpall *et al.* were the first to show the efficacy of *ex vivo* expansion of CB CD34$^+$ cells in a clinical trial [81]. They used a combination of SCF, TPO and G-CSF to expand a portion of a single CB unit where the non-expanded fraction was infused on day 0 and the remaining fraction was infused on day 10 of expansion. The resulting cultures increased the total cell number 56-fold and the CD34$^+$ cell number 4-fold and resulted in no toxicities to patients, although the time to neutrophil recovery was not significantly reduced. Based on this trial, a further randomized study of double CBT with and without *ex vivo* expansion is now in progress.

Additional efforts to improve liquid culture system have established further optimized culture conditions that employ serum free medium [82,83]. Furthermore, Peled *et al.* developed a culture system using tetraethylenepentamine (TEPA), a copper chelator, based on the studies that cellular coppers are associated with proliferation and differentiation of HSCs/PCs [84,85]. A Phase I/II trial was conducted by de Lima *et al.* and evaluated the safety and potential efficacy of cultured cells with TEPA and cytokines (SCF, TPO, FL and IL-6) [86]. This expansion of HSCs/PCs resulted in a 56-fold increase of total cell number and a 6-fold increase of the number of CD34$^+$ cells. While the cultured cells in this trial attained engraftment in human and showed safety, there was no significant improvement in neutrophil and platelet recovery, similar to previous reports. Additional studies will be required to demonstrate the efficacy of culture systems with TEPA.

4.1.2 Co-culture with stromal cells

As described above, HSCs/PCs are surrounded by niche cells in the BM microenvironment that provide the molecular signals necessary for self-renewal, proliferation and differentiation of HSCs/PCs. Co-culture of HSCs/PCs with stromal cells in the presence of growth factors *ex vivo* has been developed as a culture strategy with the intention of mimicking the interaction between HSCs/PCs and the niche cells. Stromal cells are supposed to provide positive effect on HSCs/PCs; not only through physical support and the signal transduction of cell–cell interaction but also by the secretion of soluble regulatory factors. Among niche cells, mesenchymal stem cells (MSCs), which are a component of the BM niche and can differentiate into osteoblasts, chondrocytes and adipocytes, have been reported to promote engraftment of CB CD34$^+$ cells when co-administered, and also possess the ability to modulate the immune system [87-89]. Using CB cells expanded for 14 days on related donor MSCs with cytokines (SCF, TPO, FL and G-CSF) combined with an unexpanded CB unit, de Lima *et al.* conducted clinical trials, which showed a 14-fold expansion of CD34$^+$ cells in cultured cells with no toxicities and rapid engraftment of platelets and neutrophils [90,91].

4.1.3 Continuous perfusion culture in bioreactors

Researchers in this field usually use conventional culture flasks and gas-permeable blood bags for *ex vivo* expansion of HSCs/PCs. By contrast, automated bioreactors with continuous perfusion device that supply stable oxygen concentration, pH and culture medium have been designed to control and monitor the culture condition of HSCs/PCs [92,93]. Jaroscak *et al.* reported in the first bioreactor-based clinical trial that CB cells expanded by using Aastrom Replicell bioreactor technology (Aastrom Biosciences, Inc., Ann Arbor, MI, USA) for 12 days could engraft in human BM safely [94]. However, no difference in the time to myeloid cell and platelet engraftment was observed. Further clinical trials are needed to evaluate the value of the bioreactors in HSC/PC expansion.

4.2 Recent approaches employing protein factors

The attempts described above have indicated that methods using cytokines, stromal cells and bioreactors are safe and result in a moderate expansion of HSCs/PCs. However, the clinical trials utilizing these methods have not clearly demonstrated sufficient expansion of HSCs/PCs to overcome the delay of neutrophil and platelet engraftment in BM. To overcome this barrier in HSC/PC expansion, strategies that target signal transduction pathways involved in self-renewal of HSCs have been exploited by a variety of technologies.

To find a solution for the problem of differentiation and loss of stemness in the process of HSC/PC expansion, several protein factors have been thoroughly screened to enhance HSC self-renewal. The combination of Ang-like (ANGPTL)-5, insulin growth factor-binding protein (IGFBP)-2, SCF, TPO and fibroblast growth factor (FGF)-1 induced a 20-fold expansion of SRCs [95]. The key components, ANGPTL-5 and IGFBP-2, supposedly activate signal transduction pathway that cannot be activated by other cytokines which results in a synergistic effect on HSC proliferation but not differentiation. Pleiotrophin, a growth factor with high affinity for heparin, has recently been reported to expand human SRCs [96]. In addition, administration of pleiotrophin to myeloablative mice enhanced the regeneration of BM HSCs/PCs *in vivo*. This suggests that pleiotrophin has pharmaceutical potential for hematopoietic recovery in patients after myeloablation.

Among protein factors which can be used to expand HSCs/PCs *ex vivo*, Notch ligands are particularly remarkable. The Notch pathway has been well investigated and shown to function as a master regulator of cell fate in developmental processes. Although the physiological function of Notch pathway in the maintenance of HSCs in the niche is controversial as mentioned above, recent findings suggest that activation of the Notch pathway maintains HSCs/PCs in an undifferentiated state and enhances HSC self-renewal *in vitro* [97,98]. On the basis of these results, Delaney *et al.* demonstrated that CB CD34$^+$ HSCs/PCs expanded with immobilized Delta-1, the Notch ligand, engraft better than unmanipulated cells in a clinical setting [99]. The most important result in this

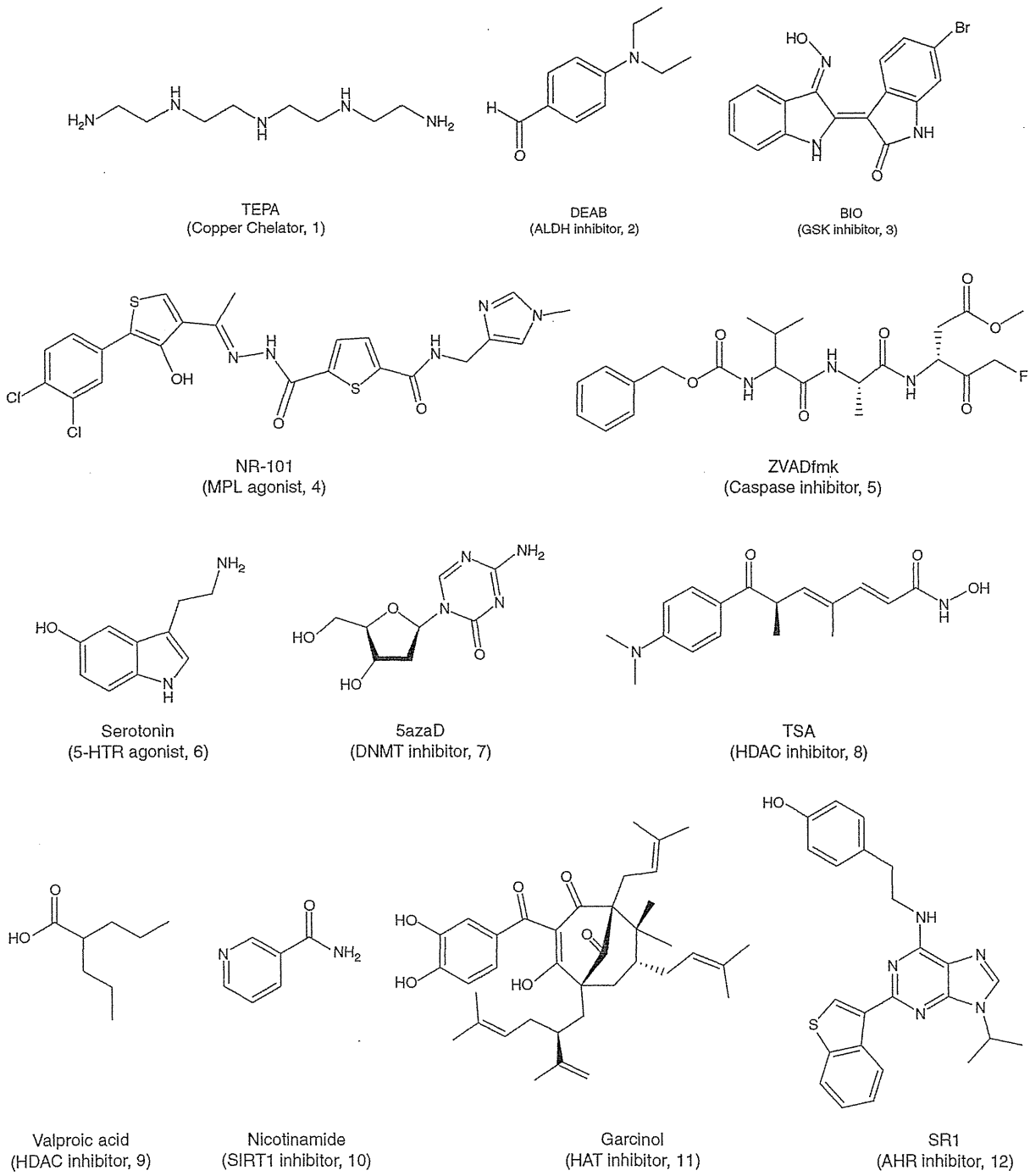

Figure 3. Chemical structures of the compounds that expand HSCs/PCs.

5azaD: 5-aza-2′-deoxycytidine; 5-HTR: 5-hydroxytryptamine receptor; AHR: Aryl hydrocarbon receptor; ALDH: Aldehyde dehydrogenase; BIO: 6-bromoindirubin 3′-oxime; DEBA: Diethylaminobenzaldehyde; DNMT: DNA methyltransferase; GSK-3β: Glycogen synthase kinase-3β; HAT: Histone acetyltransferase; HDAC: Histone deacetylase; MPL: Myeloproliferative leukemia virus oncogene; SIRT1: Sirtuin 1; SR1: StemRegenin 1; TEPA: Tetraethylenpentamin; TSA: Trichostatin A.

study is that the time to neutrophil recovery is substantially shortened with Delta-1 treatment. Although further studies should be continued for optimal combination of cytokines and these protein factors, target-oriented research will offer promising approaches for *ex vivo* expansion of HSCs/PCs.

4.3 Novel approaches by using small-molecule compounds

SMCs are also attractive tools for HSC/PC expansion (**Figure 3**). While SMCs, which comprise natural and chemically synthesized products, have been used in molecular biology and pharmaceutical therapy for many years, their application to stem cell technologies has recently come into wide use [100]. As a representative of SMCs controlling HSC proliferation, TEPA (1) enhances the *ex vivo* expansion of HSCs/PCs and is under evaluation in a clinical trial as noted above. Besides TEPA, inhibition of aldehyde dehydrogenase, which is highly active in HSCs, with diethylaminobenzaldehyde (DEAB, 2) has been reported to result in a 3.4-fold expansion of SRCs [101]. DEAB is thought to suppress the differentiation of HSCs through retinoid signaling. 6-Bromoindirubin 3′-oxime (BIO, 3), a chemical inhibitor of glycogen synthase kinase-3β (GSK-3β), has been shown to promote the engraftment of cultured $CD34^+$ cells in NOD/SCID mouse when added to the culture with SCF, TPO and FL for 24 h [102]. GSK-3β serves as a key inhibitory factor in the Wnt signaling that is suggested to be necessary for self-renewal of HSCs [103,104]. In this way, specific manipulation of the Wnt pathway by SMCs can be used to modulate HSCs/PCs. This was a successful example of the application of SMCs in order to modulate specific signaling pathways critical to hematopoietic cells. These findings encouraged us to screen for compounds that activate the TPO/MPL pathway, a pathway which has an essential function in the maintenance of HSCs. The authors identified NR-101 (4), a small-molecule MPL agonist, as a superior stimulator of HSC/PC expansion in comparison with TPO [105]. SRCs were increased 2.9-fold during a 7-day culture with NR-101 compared with freshly isolated $CD34^+$ cells, and 2.3-fold compared with TPO. Of note, NR-101 persistently activated STAT5 but not STAT3. Furthermore, NR-101 induced a long-term accumulation of hypoxia-inducible factor-1α (HIF-1α) protein and enhanced activation of its downstream target genes. The authors' findings demonstrated that SMCs could artificially modulate signaling pathways in HSCs. This establishes that the approach of using SMCs that modulate the signal transduction in stem cells is also a viable strategy for HSC/PC expansion. Several approaches to develop small molecule mimics of cytokines or growth factors are in progress [106]. In addition, a novel role of apoptotic protease or caspase inhibitors, zVADfmk (5) and zLLYfmk has been reported to promote the *ex vivo* expansion of HSCs/PCs [107]. The reduced level of apoptosis by these compounds in $CD34^+$ cells culture increases the number of $CD34^+CD38^-$ cells and enhances the engraftment of expanded cells. Serotonin (6), a monoamine neurotransmitter that activates 5-hydroxytryptamine (5-HT) receptors, has also been reported to increase the number of SRCs during culture of $CD34^+$ cells due to its anti-apoptotic effects [108].

The attempt to expand HSCs/PCs by modulating epigenetic mechanism with SMCs has also yielded positive results. Sequential treatment with 5-aza-2′-deoxycytidine (5azaD, 7), a DNA methyltransferase inhibitor, followed by Trichostatin A (8), a histone deacetylase (HDAC) inhibitor, during *ex vivo* culture of $CD34^+CD90^+$ cell up-regulated the self-renewal of marrow repopulating cells [109,110]. Valproic acid (9), a HDAC inhibitor, also has the capacity to enhance the cytokine-induced expansion of human HSCs [111]. Very recently, nicotinamide (10) has been reported to delay differentiation and increase engraftment capacity of CB $CD34^+$ cells through its inhibitory activity against sirtuin 1 (SIRT1), a nicotinamide adenine dinucleotide (NAD)-dependent HDAC [112]. On the other hand, the authors have shown that garcinol (11), a histone acetyltransferase (HAT) inhibitor, also expands the numbers of $CD34^+CD38^-$ cells and SRCs, even though its capacity to expand HSCs/PCs is correlated with its inhibitory effect on HATs [113]. It is controversial whether the level of histone acetylation in HSCs directly affects their proliferation and self-renewal, but epigenetic regulators could be valuable targets of SMCs in *ex vivo* culture of HSCs/PCs.

One of the most encouraging findings in recent chemical approaches is the discovery of StemRegenin 1 (SR1, 12), a SMC that promotes the self-renewal of human HSCs through its antagonizing effect on the aryl hydrocarbon receptor (AHR) [114]. Boitano *et al.* reported that $CD34^+$ cells treated with SR1 in the presence of SCF, TPO, FL and IL-6 for 2 weeks contained > 10-fold of short- and long-term SRCs as compared with uncultured cells or cells cultured with cytokines alone. The positive effect of SR1 on HSC/PC expansion was also confirmed in an automated culture system employing a controlled fed-batch media approach [115]. SR1 has revealed a physiological role for AHR in controlling stemness and also has suggested that signaling molecules downstream of AHR may be promising targets for modulation by SMCs in *ex vivo* HSC/PC expansion. **Table 2** summarizes the characteristics of HSC/PC expansion by SMCs compared with expansion mediated by protein factors alone.

4.4 Generation of HSCs/PCs from pluripotent stem cells

Human ESCs and induced pluripotent stem cells (iPSCs) have a potential to serve an alternative source for HSCs/PCs. Of particular interest are iPSCs which, when derived from a patient's cells, could potentially be used as a source of autologous HSCs/PCs [116]. Long-term reconstituting HSCs have been obtained by forced expression of the transcription factor genes *Hoxb4* or *Lhx2* in HPCs derived from mouse ESCs [117,118]. By contrast, HSCs/PCs derived from human ESCs have only a limited potential for long-term engraftment. HPCs induced by co-culture of human ESCs with stromal cells from aorta-gonad-mesonephros (AGM)

Table 2. Representative new approaches for *ex vivo* expansion of HSCs/PCs.

Means	Representative example	Mechanism	Effects	Advantages	Disadvantages
Supplementation of soluble or immobilized protein factors to medium	Delta-1	Notch signaling activation	16-Fold increase in SRC frequency and decrease in time to neutrophil recovery in human BM	Remarkable expansion of $CD34^+$ cells and effectiveness in clinical trial	Time- and cost-consuming procedure to immobilize Delta-1 on culture plates
Overexpression of transcription factors that regulate HSC self-renewal	HOXB4	Activation of the signal in HSC self-renewal	> 50-Fold increase in engraftment of cultured cells	Easy to manipulate specific signals in HSCs	Risk of carcinogenesis caused by virus vector
Supplementation of SMCs to medium	SR1	Antagonization of AHR	> 10-Fold increase in short- and long-term SRCs	Cost-effective and stable production of active compound	Unexpected side effects from off-target binding

AHR: Aryl hydrocarbon receptor; BM: Bone marrow; HOXB4: Homeobox B4; HSCs: Hematopoietic stem cells; SMCs: Small-molecule compounds; SRCs: SCID-repopulating cells.

region have shown the best engraftment in the primary recipient mice, establishing chimerism of human $CD45^+$ cells up to 16% in the PB. However, they exhibited poor engraftment in the secondary recipient mice [119]. Indeed, HSCs derived from human pluripotent stem cells have a limited engraftment potential compared with human adult HSCs, such as human CB $CD34^+$ cells. Furthermore, forced expression of *HOXB4* has little effect on HPCs from human ESCs. HSCs develop through multiple steps by changing niches from the yolk sac and/or AMG (autometallography) to the fetal liver and eventually to the BM. It is possible that the authors are missing some critical developmental steps in their culture system for induction of long-term reconstituting HSCs from human ESCs. Precise understanding of hematopoietic development including the differences between human and mouse hematopoiesis might be fundamental to overcome the current problems in generation of true HSCs from human ESC/iPSCs.

5. Conclusions

Although CB has several advantages in transplantation therapy for patients with high-risk hematological malignancies and other blood diseases, mortality due to infection, which is most often caused by delayed recovery of neutrophils after transplantation, has yet to be improved for CB transplantation. Nevertheless, recent studies have revealed specific cell surface markers, which aid in purifying HSCs/PCs, and factors that control the behavior of HSCs *in vivo* and *in vitro*. Moreover, promising approaches to overcome the failings of CB transplantation through *ex vivo* expansion of HSCs/PCs have been developed utilizing factors that control HSCs. Notch ligand (Delta-1) and AHR antagonists (SR1) have appeared as leading-edge tools to establish better methods for *ex vivo* expansion of HSCs/PCs. Use of SMCs such as SR1 is becoming more and more common in this field. With these evolving technologies to expand HSCs/PCs, the applications in transplantation and cell-based therapies of CB cells will continue to grow.

6. Expert opinion

Although the challenges that exist in the expansion of human HSCs/PCs *ex vivo* have not been completely overcome in a clinical setting, further advances in this area will be accomplished by combinatorial application of methods to modulate the signaling pathways governing the fate of HSCs/PCs, which are referred to above. This means that it is necessary to keep trying to elucidate the pathways fundamentally supporting the proliferation, self-renewal, maintenance and survival of HSCs/PCs and to assess the suitability of these pathways for practical manipulation in *ex vivo* expansion. For example, recent finding revealed that PcG proteins are indispensable for the self-renewal capacity of HSCs. Bmi1, a member of PcG proteins, has been thoroughly examined and demonstrated to play a key role in regulating HSC self-renewal. To maximize and apply this accumulated evidence to clinically useful expansion of HSCs, renewed efforts to clarify the mechanism of Bmi1 signaling including its downstream targets and associated molecules will be required. In addition, evaluation systems that can precisely predict *in vivo* behavior of HSCs/PCs are useful in validating whether net and credible self-renewal of HSCs occurs in the process of *ex vivo* culture. However, the xenotransplantation system using immunodeficient mice still has some limitations, including inefficient engraftment of human HSCs/PCs and short life span of the host mice. Further improvement in the xenotransplantation system will be important in making preclinical assessments.

The strategies of how to regulate these key pathways in HSC self-renewal are also important for establishing robust and efficient approaches for HSC/PC expansion. Conventional methods to expand HSCs/PCs have depended mainly on

cytokines and protein factors. However, extensive investigations of the HSC niche have revealed a variety of extrinsic factors that control HSC behaviors and an important role of the oxygen concentrations in the maintenance of HSC quiescence [120,121]. A device that keeps HSCs in a hypoxic condition, thereby inducing a high level of HIF-1α expression, would accomplish optimal HSC expansion. Furthermore, a novel technology using nanofibers that mimic the *in vivo* niche has a potential to improve the expansion of HSCs/PCs [122].

Recent studies noted above have demonstrated promising capability of SMCs to serve as the manipulators of fate, state and function of HSCs via targeting of stem cell-specific signaling pathways and/or proteins. The merits of using SMCs for this purpose are summarized as follows.

1) A great number of screening sources: millions of SMCs are now available from compound library suppliers, which provide a variety of chemically diverse structures that can be used to identify useful compounds.
2) Highly advanced techniques from drug discovery research can be of use in the identification and optimization of the desired chemical compounds; for example, systems for high-throughput screening, combinatorial chemistry, structure-based drug design and chemical biology.
3) Cost-effective and stable production of desired SMCs for clinical use.
4) Easy to combine a variety of SMCs that regulate different pathways, in varying proportions.
5) Opportunity to develop conventional therapeutics that stimulate regeneration of HSCs/PCs *in vivo*; the SMCs expanding HSCs/PCs *ex vivo* have potential to use for the treatment of hematocytopenia such as aplastic anemia, pure red cell aplasia and anemia after HSCT therapy.

By contrast, SMCs may cause unexpected side effects when used for HSC/PC expansion. Detailed evaluation of the safety of SMCs, especially the confirmation of no carcinogenicity, is an essential step in the preclinical stages of any new potential therapy. Furthermore, while TPO can be substituted by chemically synthesized TPO mimetics, elimination of all conventional cytokines (e.g., SCF and FL) may be challenging because small-molecule agonists for membrane-bound receptors are hard to find [105,106]. Replacement of all cytokines with SMCs and the accomplishment of complete synthetic medium for HSCs/PCs will require more time.

Taken together, elucidation of the cell signaling pathways implicated in proliferation, self-renewal, maintenance and survival of HSCs/PCs combined with the development of SMCs that target these signaling pathways can establish efficient, cost-effective, riskless and robust methods for *ex vivo* expansion of HSCs/PCs. With these efforts, improved HSC transplantation and gene therapy can be accomplished in the near future. By that time, SMCs may be applicable not only in HSC therapy but also in the *ex vivo* manipulation of other stem cells, including MSCs, neural stem cells and pluripotent stem cells. SMCs that target critical developmental steps of human HSCs could be a viable tool to generate long-term econstituting HSCs from pluripotent stem cells, which have not been established by using protein factors.

Acknowledgements

The authors would like to thank G Wendt for critical reading of the manuscript.

Declaration of interest

The authors state no conflict of interest and have received no payment for the preparation of this manuscript. This work was supported in part by a Grant-in-aid for Core Research for Evolutional Science and Technology (CREST) from the Japan Science and Technology Corp. (JST).

Bibliography

Papers of special note have been highlighted as either of interest (•) or of considerable interest (••) to readers.

1. Becker AJ, Mc CE, Till JE. Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature 1963;197:452-4
2. Wu AM, Till JE, Siminovitch L, McCulloch EA. Cytological evidence for a relationship between normal hemotopoietic colony-forming cells and cells of the lymphoid system. J Exp Med 1968;127:455-64
3. Morrison SJ, Uchida N, Weissman IL. The biology of hematopoietic stem cells. Annu Rev Cell Dev Biol 1995;11:35-71
4. Weissman IL. Stem cells: units of development, units of regeneration, and units in evolution. Cell 2000;100:157-68
5. Thomas ED, Lochte HL Jr, Lu WC, Ferrebee JW. Intravenous infusion of bone marrow in patients receiving radiation and chemotherapy. N Engl J Med 1957;257:491-6
6. Gluckman E, Broxmeyer HA, Auerbach AD, et al. Hematopoietic reconstitution in a patient with Fanconi's anemia by means of umbilical-cord blood from an HLA-identical sibling. N Engl J Med 1989;321:1174-8
7. Appelbaum FR. Hematopoietic-cell transplantation at 50. N Engl J Med 2007;357:1472-5
8. Yazaki M, Atsuta Y, Kato K, et al. Incidence and risk factors of early bacterial infections after unrelated cord blood transplantation. Biol Blood Marrow Transplant 2009;15:439-46
9. Delaney C, Gutman JA, Appelbaum FR. Cord blood transplantation for haematological malignancies: conditioning regimens, double cord transplant and infectious complications. Br J Haematol 2009;147:207-16
10. Escalon MP, Komanduri KV. Cord blood transplantation: evolving strategies to improve engraftment and immune reconstitution. Curr Opin Oncol 2010;22:122-9
11. Sauvageau G, Iscove NN, Humphries RK. In vitro and in vivo expansion of hematopoietic stem cells. Oncogene 2004;23:7223-32
12. Hofmeister CC, Zhang J, Knight KL, et al. Ex vivo expansion of umbilical cord blood stem cells for transplantation: growing knowledge from the hematopoietic niche. Bone Marrow Transplant 2007; 39:11-23
13. Tung SS, Parmar S, Robinson SN, et al. Ex vivo expansion of umbilical cord blood for transplantation. Best Pract Res Clin Haematol 2010;23:245-57
14. Osawa M, Hanada K, Hamada H, Nakauchi H. Long-term lymphohematopoietic reconstitution by a single CD34-low/negative hematopoietic stem cell. Science 1996;273:242-5
• **The first report on the purification of mouse HSCs to a single cell level.**
15. Kiel MJ, Yilmaz OH, Iwashita T, et al. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 2005;121:1109-21
16. Kim I, He S, Yilmaz OH, et al. Enhanced purification of fetal liver hematopoietic stem cells using SLAM family receptors. Blood 2006;108:737-44
17. Baum CM, Weissman IL, Tsukamoto AS, et al. Isolation of a candidate human hematopoietic stem-cell population. Proc Natl Acad Sci USA 1992;89:2804-8
18. McCune JM, Namikawa R, Kaneshima H, et al. The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. Science 1988;241:1632-9
19. Ito M, Hiramatsu H, Kobayashi K, et al. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. Blood 2002;100:3175-82
20. Shultz LD, Lyons BL, Burzenski LM, et al. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J Immunol 2005;174:6477-89
21. Wang JC, Doedens M, Dick JE. Primitive human hematopoietic cells are enriched in cord blood compared with adult bone marrow or mobilized peripheral blood as measured by the quantitative in vivo SCID-repopulating cell assay. Blood 1997;89:3919-24
• **The first report on the SCID-repopulating assay that can evaluate human HSCs.**
22. Rocha V, Labopin M, Sanz G, et al. Transplants of umbilical-cord blood or bone marrow from unrelated donors in adults with acute leukemia. N Engl J Med 2004;351:2276-85
23. Krause DS, Fackler MJ, Civin CI, May WS. CD34: structure, biology, and clinical utility. Blood 1996;87:1-13
24. Vogel W, Scheding S, Kanz L, Brugger W. Clinical applications of CD34(+) peripheral blood progenitor cells (PBPC). Stem Cells 2000;18:87-92
25. Nakamura Y, Ando K, Chargui J, et al. Ex vivo generation of CD34(+) cells from CD34(-) hematopoietic cells. Blood 1999;94:4053-9
26. Wang J, Kimura T, Asada R, et al. SCID-repopulating cell activity of human cord blood-derived CD34- cells assured by intra-bone marrow injection. Blood 2003;101:2924-31
27. Yahata T, Ando K, Sato T, et al. A highly sensitive strategy for SCID-repopulating cell assay by direct injection of primitive human hematopoietic cells into NOD/SCID mice bone marrow. Blood 2003;101:2905-13
28. Bhatia M, Wang JC, Kapp U, et al. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci USA 1997;94:5320-5
29. Craig W, Kay R, Cutler RL, Lansdorp PM. Expression of Thy-1 on human hematopoietic progenitor cells. J Exp Med 1993;177:1331-42
30. Yin AH, Miraglia S, Zanjani ED, et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. Blood 1997;90:5002-12
31. Majeti R, Park CY, Weissman IL. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell Stem Cell 2007;1:635-45
32. Bertoncello I, Williams B. Hematopoietic stem cell characterization by Hoechst 33342 and rhodamine 123 staining. Methods Mol Biol 2004;263:181-200

33. McKenzie JL, Takenaka K, Gan OI, et al. Low rhodamine 123 retention identifies long-term human hematopoietic stem cells within the Lin-CD34+CD38- population. Blood 2007;109:543-5

34. Notta F, Doulatov S, Laurenti E, et al. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science 2011;333:218-21

•• **The first report on the purification of human HSCs to a single cell level.**

35. Zon LI. Intrinsic and extrinsic control of haematopoietic stem-cell self-renewal. Nature 2008;453:306-13

•• **An extensive review on the regulators that control HSC self-renewal.**

36. Kataoka K, Sato T, Yoshimi A, et al. Evi1 is essential for hematopoietic stem cell self-renewal, and its expression marks hematopoietic cells with long-term multilineage repopulating activity. J Exp Med 2011;208:2403-16

37. Aguila JR, Liao W, Yang J, et al. SALL4 is a robust stimulator for the expansion of hematopoietic stem cells. Blood 2011;118:576-85

38. Kato Y, Iwama A, Tadokoro Y, et al. Selective activation of STAT5 unveils its role in stem cell self-renewal in normal and leukemic hematopoiesis. J Exp Med 2005;202:169-79

39. Wierenga AT, Vellenga E, Schuringa JJ. Maximal STAT5-induced proliferation and self-renewal at intermediate STAT5 activity levels. Mol Cell Biol 2008;28:6668-80

40. Antonchuk J, Sauvageau G, Humphries RK. HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. Cell 2002;109:39-45

41. Schiedlmeier B, Klump H, Will E, et al. High-level ectopic HOXB4 expression confers a profound in vivo competitive growth advantage on human cord blood CD34+ cells, but impairs lymphomyeloid differentiation. Blood 2003;101:1759-68

• **The first report on the *ex vivo* expansion of human HSCs using HOXB4, an intrinsic regulator of HSC self-renewal.**

42. Amsellem S, Pflumio F, Bardinet D, et al. Ex vivo expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein. Nat Med 2003;9:1423-7

43. Park IK, Qian D, Kiel M, et al. Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature 2003;423:302-5

44. Iwama A, Oguro H, Negishi M, et al. Enhanced self-renewal of hematopoietic stem cells mediated by the polycomb gene product Bmi-1. Immunity 2004;21:843-51

• **An important study demonstrating the role of the polycomb protein Bmi1 in the regulation of HSC self-renewal.**

45. Oguro H, Iwama A, Morita H, et al. Differential impact of Ink4a and Arf on hematopoietic stem cells and their bone marrow microenvironment in Bmi1-deficient mice. J Exp Med 2006;203:2247-53

46. Oguro H, Yuan J, Ichikawa H, et al. Poised lineage specification in multipotential hematopoietic stem and progenitor cells by the polycomb protein Bmi1. Cell Stem Cell 2010;6:279-86

47. Rizo A, Olthof S, Han L, et al. Repression of BMI1 in normal and leukemic human CD34(+) cells impairs self-renewal and induces apoptosis. Blood 2009;114:1498-505

48. Faubert A, Chagraoui J, Mayotte N, et al. Complementary and independent function for Hoxb4 and Bmi1 in HSC activity. Cold Spring Harb Symp Quant Biol 2008;73:555-64

49. Konuma T, Nakamura S, Miyagi S, et al. Forced expression of the histone demethylase Fbxl10 maintains self-renewing hematopoietic stem cells. Exp Hematol 2011;39:697-709

50. Adams GB, Scadden DT. The hematopoietic stem cell in its place. Nat Immunol 2006;7:333-7

51. Morrison SJ, Spradling AC. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. Cell 2008;132:598-611

52. Zhang J, Niu C, Ye L, et al. Identification of the haematopoietic stem cell niche and control of the niche size. Nature 2003;425:836-41

53. Ellis SL, Grassinger J, Jones A, et al. The relationship between bone, hemopoietic stem cells, and vasculature. Blood 2011;118:1516-24

54. Lo Celso C, Scadden DT. The haematopoietic stem cell niche at a glance. J Cell Sci 2011;124:3529-35

• **A comprehensible and brief review on the HSC niche.**

55. Nagasawa T, Omatsu Y, Sugiyama T. Control of hematopoietic stem cells by the bone marrow stromal niche: the role of reticular cells. Trends Immunol 2011;32:315-20

56. Yamazaki S, Ema H, Karlsson G, et al. Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone marrow niche. Cell 2011;147:1146-58

57. Broudy VC. Stem cell factor and hematopoiesis. Blood 1997;15:1345-64

58. Ding L, Saunders TL, Enikolopov G, Morrison SJ. Endothelial and perivascular cells maintain haematopoietic stem cells. Nature 2012;481:457-62

59. Yoshihara H, Arai F, Hosokawa K, et al. Thrombopoietin/MPL signaling regulates hematopoietic stem cell quiescence and interaction with the osteoblastic niche. Cell Stem Cell 2007;13:685-97

60. Qian H, Buza-Vidas N, Hyland CD, et al. Critical role of thrombopoietin in maintaining adult quiescent hematopoietic stem cells. Cell Stem Cell 2007;13:671-84

61. Arai F, Hirao A, Ohmura M, et al. Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 2004;118:149-61

62. Stier S, Ko Y, Forkert R, et al. Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. J Exp Med 2005;201:1781-91

63. Nilsson SK, Johnston HM, Whitty GA, et al. Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood 2005;106:1232-9

64. Calvi LM, Adams GB, Weibrecht KW, et al. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 2003;425:841-6

65. Varnum-Finney B, Xu L, Brashem-Stein C, et al. Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. Nat Med 2000;6:1278-81

66. Mancini SJ, Mantei N, Dumortier A, et al. Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. Blood 2005;105:2340-2

67. Maillard I, Koch U, Dumortier A, et al. Canonical notch signaling is dispensable for the maintenance of adult hematopoietic stem cells. Cell Stem Cell 2008;2:356-66

68. Lapidot T, Dar A, Kollet O. How do stem cells find their way home? Blood 2005;106:1901-10

69. Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12-CXCR4 chemokine signaling in bone marrow stromal cell niches. Immunity 2006;25:977-88

70. Kiel MJ, Acar M, Radice GL, Morrison SJ. Hematopoietic stem cells do not depend on N-cadherin to regulate their maintenance. Cell Stem Cell 2009;4:170-9

71. Hosokawa K, Arai F, Yoshihara H, et al. Cadherin-based adhesion is a potential target for niche manipulation to protect hematopoietic stem cells in adult bone marrow. Cell Stem Cell 2010;6:194-8

72. Yokota T, Oritani K, Mitsui H, et al. Growth-supporting activities of fibronectin on hematopoietic stem/progenitor cells in vitro and in vivo: structural requirement for fibronectin activities of CS1 and cell-binding domains. Blood 1998;91:3263-72

73. Avigdor A, Goichberg P, Shivtiel S, et al. CD44 and hyaluronic acid cooperate with SDF-1 in the trafficking of human CD34+ stem/progenitor cells to bone marrow. Blood 2004;103:2981-9

74. Hai-Jiang W, Xin-Na D, Hui-Jun D. Expansion of hematopoietic stem/progenitor cells. Am J Hematol 2008;83:922-6

75. Abboud MR, Xu F, Payne A, Laver J. Effects of recombinant human Steel factor (c-kit ligand) on early cord blood hematopoietic precursors. Exp Hematol 1994;22:388-92

76. Ohmizono Y, Sakabe H, Kimura T, et al. Thrombopoietin augments ex vivo expansion of human cord blood-derived hematopoietic progenitors in combination with stem cell factor and flt3 ligand. Leukemia 1997;11:524-30

77. Piacibello W, Sanavio F, Garetto L, et al. The role of c-Mpl ligands in the expansion of cord blood hematopoietic progenitors. Stem Cells 1998;16:243-8

78. McKenna HJ, de Vries P, Brasel K, et al. Effect of flt3 ligand on the ex vivo expansion of human CD34+ hematopoietic progenitor cells. Blood 1995;86:3413-20

79. Solanilla A, Grosset C, Duchez P, et al. Flt3-ligand induces adhesion of haematopoietic progenitor cells via a very late antigen (VLA)-4- and VLA-5-dependent mechanism. Br J Haematol 2003;120:782-6

80. Zheng Y, Sun A, Han ZC. Stem cell factor improves SCID-repopulating activity of human umbilical cord blood-derived hematopoietic stem/progenitor cells in xenotransplanted NOD/SCID mouse model. Bone Marrow Transplant 2005;35:137-42

81. Shpall EJ, Quinones R, Giller R, et al. Transplantation of ex vivo expanded cord blood. Biol Blood Marrow Transplant 2002;8:368-76

82. Yao CL, Chu IM, Hsieh TB, Hwang SM. A systematic strategy to optimize ex vivo expansion medium for human hematopoietic stem cells derived from umbilical cord blood mononuclear cells. Exp Hematol 2004;32:720-7

83. Yao CL, Feng YH, Lin XZ, et al. Characterization of serum-free ex vivo-expanded hematopoietic stem cells derived from human umbilical cord blood CD133(+) cells. Stem Cells Dev 2006;15:70-8

84. Peled T, Landau E, Mandel J, et al. Linear polyamine copper chelator tetraethylenepentamine augments long-term ex vivo expansion of cord blood-derived CD34+ cells and increases their engraftment potential in NOD/SCID mice. Exp Hematol 2004;32:547-55

85. Peled T, Glukhman E, Hasson N, et al. Chelatable cellular copper modulates differentiation and self-renewal of cord blood-derived hematopoietic progenitor cells. Exp Hematol 2005;33:1092-100

86. de Lima M, McMannis J, Gee A, et al. Transplantation of ex vivo expanded cord blood cells using the copper chelator tetraethylenepentamine: a phase I/II clinical trial. Bone Marrow Transplant 2008;41:771-8

• **An important report on the effect of TEPA on expansion of human HSCs/PCs in clinical trial.**

87. in 't Anker PS, Noort WA, Kruisselbrink AB, et al. Nonexpanded primary lung and bone marrow-derived mesenchymal cells promote the engraftment of umbilical cord blood-derived CD34(+) cells in NOD/SCID mice. Exp Hematol 2003;31:881-9

88. Le Blanc K, Frassoni F, Ball L, et al. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 2008;371:1579-86

89. Bernardo ME, Ball LM, Cometa AM, et al. The therapeutic potential of mesenchymal stem cell transplantation as a treatment for multiple sclerosis: consensus report of the International MSCT Study Group. Bone Marrow Transplant 2011;46:200-7

90. Kelly SS, Sola CB, de Lima M, Shpall E. Ex vivo expansion of cord blood. Bone Marrow Transplant 2009;44:673-81

91. de Lima M, Robinson S, McMannis J, et al. Mesenchymal stem cell based cord blood expansion leads to rapid engraftment of platelets and neutrophils. Blood 2010;116:abstract 362

92. Palsson BO, Paek SH, Schwartz RM, et al. Expansion of human bone marrow progenitor cells in a high cell density continuous perfusion system. Biotechnology 1993;11:368-72

93. Liu Y, Liu T, Fan X, et al. Ex vivo expansion of hematopoietic stem cells derived from umbilical cord blood in rotating wall vessel. J Biotechnol 2006;124:592-601

94. Jaroscak J, Goltry K, Smith A, et al. Augmentation of umbilical cord blood (UCB) transplantation with ex vivo-expanded UCB cells: results of a phase I trial using the AastromReplicell System. Blood 2003;101:5061-7

95. Zhang CC, Kaba M, Iizuka S, et al. Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation. Blood 2008;111:3415-23

96. Himburg HA, Muramoto GG, Daher P, et al. Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells. Nat Med 2010;16:475-82

97. Stier S, Cheng T, Dombkowski D, et al. Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over

myeloid lineage outcome. Blood 2002;99:2369-78

98. Suzuki T, Yokoyama Y, Kumano K, et al. Highly efficient ex vivo expansion of human hematopoietic stem cells using Delta1-Fc chimeric protein. Stem Cells 2006;24:2456-65

99. Delaney C, Heimfeld S, Brashem-Stein C, et al. Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution. Nat Med 2010;16:232-6

•• **The first report on the notch-mediated expansion of human HSCs/PCs in clinical trial.**

100. Ding S, Schultz PG. A role for chemistry in stem cell biology. Nat Biotechnol 2004;22:833-40

101. Chute JP, Muramoto GG, Whitesides J, et al. Inhibition of aldehyde dehydrogenase and retinoid signaling induces the expansion of human hematopoietic stem cells. Proc Natl Acad Sci USA 2006;103:11707-12

102. Ko KH, Holmes T, Palladinetti P, et al. GSK-3beta inhibition promotes engraftment of ex vivo-expanded hematopoietic stem cells and modulates gene expression. Stem Cells 2011;29:108-18

103. Reya T, Duncan AW. Ailles L, et al. role for Wnt signalling in self-renewal of haematopoietic stem cells. Nature 2003;423:409-14

104. Malhotra S, Kincade PW. Wnt-related molecules and signaling pathway equilibrium in hematopoiesis. Cell Stem Cell 2009;4:27-36

105. Nishino T, Miyaji K, Ishiwata N, et al. Ex vivo expansion of human hematopoietic stem cells by a small-molecule agonist of c-MPL. Exp Hematol 2009;37:1364-77

• **The first study demonstrating the substitution of TPO, an important cytokine to expand HSC *ex vivo*, with SMCs.**

106. Tarasova A, Haylock D, Winkler D. Principal signalling complexes in haematopoiesis: structural aspects and mimetic discovery. Cytokine Growth Factor Rev 2011;22:231-53

107. Sangeetha VM, Kale VP, Limaye LS. Expansion of cord blood CD34 cells in presence of zVADfmk and zLLYfmk improved their in vitro functionality and in vivo engraftment in NOD/SCID mouse. PLoS One 2010;5:e12221

108. Yang M, Li K, Ng PC, et al. Promoting effects of serotonin on hematopoiesis: ex vivo expansion of cord blood CD34+ stem/progenitor cells, proliferation of bone marrow stromal cells, and antiapoptosis. Stem Cells 2007;25:1800-6

109. Araki H, Baluchamy S, Yoshinaga K, et al. Cord blood stem cell expansion is permissive to epigenetic regulation and environmental cues. Exp Hematol 2009;37:1084-95

110. Araki H, Yoshinaga K, Boccuni P, et al. Chromatin-modifying agents permit human hematopoietic stem cells to undergo multiple cell divisions while retaining their repopulating potential. Blood 2007;109:3570-8

111. De Felice L, Tatarelli C, Mascolo MG, et al. Histone deacetylase inhibitor valproic acid enhances the cytokine-induced expansion of human hematopoietic stem cells. Cancer Res 2005;65:1505-13

112. Peled T, Shoham H, Aschengrau D, et al. Nicotinamide, a SIRT1 inhibitor, inhibits differentiation and facilitates expansion of hematopoietic progenitor cells with enhanced bone marrow homing and engraftment. Exp Hematol 2012;published online 20 Dec 2012; doi: 10.1016/j.exphm.2011.12.005

113. Nishino T, Wang C, Mochizuki-Kashio M, et al. Ex vivo expansion of human hematopoietic stem cells by garcinol, a potent inhibitor of histone acetyltransferase. PLoS One 2011;6:e24298

114. Boitano AE, Wang J, Romeo R, et al. Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells. Science 2010;329:1345-8

•• **An important study on the *ex vivo* expansion of HSCs by SR1, a SMC that targets AHR.**

115. Csaszar E, Kirouac DC, Yu M, et al. Rapid expansion of human hematopoietic stem cells by automated control of inhibitory feedback signaling. Cell Stem Cell 2012;10:218-29

116. Kaufman DS. Toward clinical therapies using hematopoietic cells derived from human pluripotent stem cells. Blood 2009;114:3513-23

117. Kyba M, Perlingeiro RC, Daley GQ. HoxB4 confers definitive lymphoid-myeloid engraftment potential on embryonic stem cell and yolk sac hematopoietic progenitors. Cell 2002;109:29-37

118. Kitajima K, Minehata K, Sakimura K, et al. In vitro generation of HSC-like cells from murine ESCs/iPSCs by enforced expression of LIM-homeobox transcription factor Lhx2. Blood 2011;117:3748-58

119. Ledran MH, Krassowska A, Armstrong L, et al. Efficient hematopoietic differentiation of human embryonic stem cells on stromal cells derived from hematopoietic niches. Cell Stem Cell 2008;3:85-98

120. Danet GH, Pan Y, Luongo JL, et al. Expansion of human SCID-repopulating cells under hypoxic conditions. J Clin Invest 2003;112:126-35

121. Suda T, Takubo K, Semenza GL. Metabolic regulation of hematopoietic stem cells in the hypoxic niche. Cell Stem Cell 2011;9:298-310

122. Lu J, Aggarwal R, Pompili VJ, Das H. A novel technology for hematopoietic stem cell expansion using combination of nanofiber and growth factors. Recent Pat Nanotechnol 2010;4:125-35

Affiliation

Taito Nishino[1], Mitsujiro Osawa[2] & Atsushi Iwama[†2] MD PhD
[†]Author for correspondence
[1]Nissan Chemical Industries,
Research Planning Department,
3-7-1 Kanda-nishiki-cho, Chiyoda-ku,
Tokyo 101-0054, Japan
[2]Chiba University,
Graduate School of Medicine,
Department of Cellular and Molecular Medicine,
1-8-1 Inohana, Chuo-ku,
Chiba 260-8670, Japan
Tel: +81 43 226 2187; Fax: +81 43 226 2191;
E-mail: aiwama@faculty.chiba-u.jp

OPEN ACCESS Freely available online

PLoS one

Bmi1 Confers Resistance to Oxidative Stress on Hematopoietic Stem Cells

Shunsuke Nakamura[1,4], Motohiko Oshima[1,4], Jin Yuan[1,4], Atsunori Saraya[1,4], Satoru Miyagi[1,4], Takaaki Konuma[1], Satoshi Yamazaki[2,5], Mitsujiro Osawa[1,4], Hiromitsu Nakauchi[2,5], Haruhiko Koseki[3,4], Atsushi Iwama[1,4]*

1 Department of Cellular and Molecular Medicine, Graduate School of Medicine, Chiba University, Chiba, Japan, **2** Division of Stem Cell Therapy, Center for Stem Cell Biology and Regenerative Medicine, Institute of Medical Science, University of Tokyo, Tokyo, Japan, **3** RIKEN Research Center for Allergy and Immunology, Yokohama, Japan, **4** Japan Science and Technology Agency (JST), CREST, Tokyo, Japan, **5** ERATO, Chiyoda-ku, Tokyo, Japan

Abstract

Background: The polycomb-group (PcG) proteins function as general regulators of stem cells. We previously reported that retrovirus-mediated overexpression of *Bmi1*, a gene encoding a core component of polycomb repressive complex (PRC) 1, maintained self-renewing hematopoietic stem cells (HSCs) during long-term culture. However, the effects of overexpression of *Bmi1* on HSCs *in vivo* remained to be precisely addressed.

Methodology/Principal findings: In this study, we generated a mouse line where *Bmi1* can be conditionally overexpressed under the control of the endogenous *Rosa26* promoter in a hematopoietic cell-specific fashion (*Tie2-Cre;R26Stop^{FL}-Bmi1*). Although overexpression of *Bmi1* did not significantly affect steady state hematopoiesis, it promoted expansion of functional HSCs during *ex vivo* culture and efficiently protected HSCs against loss of self-renewal capacity during serial transplantation. Overexpression of *Bmi1* had no effect on DNA damage response triggered by ionizing radiation. In contrast, *Tie2-Cre;R26Stop^{FL}-Bmi1* HSCs under oxidative stress maintained a multipotent state and generally tolerated oxidative stress better than the control. Unexpectedly, overexpression of *Bmi1* had no impact on the level of intracellular reactive oxygen species (ROS).

Conclusions/Significance: Our findings demonstrate that overexpression of *Bmi1* confers resistance to stresses, particularly oxidative stress, onto HSCs. This thereby enhances their regenerative capacity and suggests that Bmi1 is located downstream of ROS signaling and negatively regulated by it.

Citation: Nakamura S, Oshima M, Yuan J, Saraya A, Miyagi S, et al. (2012) Bmi1 Confers Resistance to Oxidative Stress on Hematopoietic Stem Cells. PLoS ONE 7(5): e36209. doi:10.1371/journal.pone.0036209

Editor: Kevin D. Bunting, Emory University, United States of America

Received December 30, 2011; **Accepted** March 28, 2012; **Published** May 11, 2012

Funding: This work was supported in part by MEXT KAKENHI and the Global COE Program (Global Center for Education and Research in Immune System Regulation and Treatment), MEXT, Japan, a grant for Core Research for Evolutional Science and Technology (CREST) from the Japan Science and Technology Corporation (JST), a grant from the Tokyo Biochemical Research Foundation, and a grant from Astellas Foundation for Research on Metabolic Disorders. No additional external funding was received for this study. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: aiwama@faculty.chiba-u.jp

Introduction

Hematopoietic stem cells (HSCs) are defined as primitive cells that are capable of both self-renewal and differentiation into any of the hematopoietic cell lineages. Cell fate decisions of HSCs (self-renewal vs. differentiation) are precisely regulated to maintain their numbers and lifespan. Defects in these processes lead to hematopoietic insufficiencies and to the development of hematopoietic malignancies.

The polycomb-group (PcG) proteins play key roles in the initiation and maintenance of gene silencing through histone modifications. PcG proteins belong to two major complexes, Polycomb repressive complex 1 and 2 (PRC1 and PRC2). PRC1 monoubiquitylates histone H2A at lysine 119 and PRC2 trimethylates histone H3 at lysine 27 [1]. Of note, PcG proteins have been implicated in the maintenance of self-renewing stem cells [2–4]. Among PcG proteins, Bmi1, a core component of PRC1, plays an essential role in the maintenance of self-renewal ability of HSCs at least partially by silencing the *Ink4a/Arf* locus [5–8]. Bmi1 also maintains multipotency of HSCs by keeping developmental regulator gene promoters poised for activation [9]. Furthermore, Bmi1 has been implicated in the maintenance of the proliferative capacity of leukemic stem cells [5]. Consistent with these findings, levels of BMI1 expression in the human CD34$^+$ cell fraction have been reported to correlate well with the progression and prognosis of myelodysplastic syndrome and chronic and acute myeloid leukemia [4,10], suggesting a role of BMI1 in leukemic stem cells.

We previously reported that overexpression of *Bmi1* using a retrovirus maintains self-renewal capacity of HSCs and markedly expands multipotent progenitors *ex vivo*, resulting in an enhancement of repopulating capacity of HSCs after culture. Likewise, forced expression of *BMI1* was demonstrated to promote leukemic transformation of human CD34$^+$ cells by *BCR-ABL* [11].

However, the effects of overexpression of *Bmi1* on hematopoiesis remained to be precisely addressed.

In this study, we generated mice overexpressing *Bmi1* in a hematopoietic cell-specific manner. We analyzed the effects of overexpression of *Bmi1* on hematopoiesis under steady state conditions as well as under multiple stresses. Our findings revealed a protective function for Bmi1 in HSCs from stresses, such as ROS, that usually limit the lifespan of HSCs.

Results

Generation of Mice Overexpressing *Bmi1* in Hematopoietic Cells

To generate tissue-specific *Bmi1*-transgenic mice, we knocked a *loxP*-flanked *neo*^{r}-stop cassette followed by Flag-tagged *Bmi1*, an *frt*-flanked *IRES-eGFP* cassette, and a bovine polyadenylation sequence into the *Rosa26* locus (**Figure 1A**). The obtained mice (hereafter referred to as *R26Stop*^{FL}*Bmi1*) were crossed with *Tie2-Cre* mice [12] to drive *Bmi1* expression in a hematopoietic cell-specific manner. Quantitative RT-PCR analysis of bone marrow (BM) Lineage marker$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells confirmed 6-fold overexpression of *Bmi1* in *Tie2-Cre;R26Stop*^{FL}*Bmi1* mice compared to the *Tie2-Cre* control mice (**Figure 1B**). Western blot analysis also verified overexpression of Bmi1 protein in BM c-Kit$^+$ progenitor cells from *Tie2-Cre;R26Stop*^{FL}*Bmi1* mice (**Figure 1C**).

Steady State Hematopoiesis in *Tie2-Cre;R26Stop*^{FL}*Bmi1* Mice

We first investigated the effect of overexpression of *Bmi1* on hematopoiesis in a steady state. Unexpectedly, 10-week-old *Tie2-Cre;R26Stop*^{FL}*Bmi1* mice did not exhibit any significant differences in the numbers of total BM cells, CD34$^-$LSK HSCs, LSK cells, multipotent progenitors (MPPs), common myeloid progenitors (CMPs), granulocyte/macrophage progenitors (GMPs), megakaryocyte/erythroid progenitors (MEPs), or common lymphoid progenitors (CLPs) compared to the *Tie2-Cre* control mice (**Figure 1D** and **Figure S1A**). The number of white blood cells (WBC) in peripheral blood (PB) did not change upon forced expression of *Bmi1*. Only the proportion of PB Gr-1$^+$/Mac-1$^+$ myeloid cells in *Tie2-Cre;R26Stop*^{FL}*Bmi1* mice was significantly higher than in the control mice, although the difference was not drastic (a difference of only about 2%) (**Figure 1D**). Furthermore, *Tie2-Cre;R26Stop*^{FL}*Bmi1* mice did not show any significant differences in the numbers of total spleen cells, LSK cells in the spleen, total thymic cells, or CD4$^+$CD8$^-$, CD4$^-$CD8$^+$, or CD4$^+$CD8$^+$ cells in the thymus compared to the control mice (**Figure S1A**). These findings indicate that overexpression of *Bmi1* does not largely compromise differentiation of HSCs. We further analyzed the cell cycle status of CD34$^-$LSK HSCs by Pyronin Y staining, but again did not detect any changes (**Figure S1B**). These results indicate that overexpression of *Bmi1* only slightly perturbs hematopoiesis under steady state conditions, suggesting that the level of endogenous Bmi1 is sufficient to repress the transcription of its target genes.

Colony-forming Capacity of Hematopoietic Stem and Progenitor Cells Overexpressing *Bmi1*

We next evaluated the proliferative and differentiation capacity of *Tie2-Cre;R26Stop*^{FL}*Bmi1* HSCs *in vitro*. Single CD34$^-$LSK HSCs were clonally sorted into 96-microtiter plates with the medium supplemented with stem cell factor (SCF), thrombopoietin (TPO), interleukin-3 (IL-3), and erythropoietin (EPO) and allowed to form colonies. At day 14 of culture, the colonies were counted and individually collected for morphological examination. Both *Tie2-Cre* control and *Tie2-Cre;R26Stop*^{FL}*Bmi1* HSCs gave rise to comparable numbers of high proliferative potential (HPP) and low proliferative potential (LPP) colonies with a diameter greater than and less than 1 mm, respectively (**Figure 2A**). The morphological analysis of colonies revealed that the number of colony-forming unit (CFU)-neutrophil/macrophage/erythroblast/megakaryocyte (nmEM) was also comparable between the two groups (**Figure 2A**). CFU-nmEM is a major subpopulation among CD34$^-$LSK HSCs and its frequency is well correlated with that of functional HSCs [13]. These findings indicate that overexpression of *Bmi1* in freshly isolated CD34$^-$LSK HSCs does not affect their colony-forming capacity or differentiation *in vitro*.

We previously reported that overexpression of *Bmi1* by retroviral transduction efficiently maintains hematopoietic stem and progenitor cells during long-term culture [7]. We re-evaluated the effect of forced expression of *Bmi1* using *Tie2-Cre;R26-Stop*^{FL}*Bmi1* HSCs. CD34$^-$LSK cells were cultured for 10 days in a serum-free medium supplemented with SCF and TPO, a cytokine combination which supports the proliferation of HSCs and progenitors rather than their differentiation [14]. Although *Tie2-Cre;R26Stop*^{FL}*Bmi1* HSCs did not show any growth advantage over the control (**Figure 2B**), the *Tie2-Cre;R26Stop*^{FL}*Bmi1* HSC culture contained significantly more HPP-colony-forming cells (CFCs) and CFU-nmEM than the control (**Figure 2B**). Correspondingly, flow cytometric analysis revealed more LSK cells in the *Tie2-Cre;R26Stop*^{FL}*Bmi1* HSC culture than in the control culture at day 14 (**Figure 2C**). There was no significant difference in the frequency of apoptotic cells between the control and *Tie2-Cre;R26Stop*^{FL}*Bmi1* HSC cultures (**Figure S2A**). Of note, however, the *Tie2-Cre;R26Stop*^{FL}*Bmi1* HSC culture contained a significantly higher proportion of LSK cells in the G_0/G_1 stage of cell cycle than the control (**Figure S2B**). These findings suggest that overexpression of *Bmi1* slows down cell cycle of immature hematopoietic cells in culture, leading to no growth advantages over the control cells in spite of an increase in immature progenitors in culture. As we reported previously, the *Ink4a/Arf* locus is a critical target of Bmi1 in HSCs [8]. Quantitative RT-PCR confirmed that *p19*^{Arf} was closely repressed in transcription upon *Bmi1* overexpression (**Figure 2D**). These results support our previous finding that HSCs overexpressing *Bmi1* retain their self-renewal capacity better than the control HSCs under the culture stress.

Overexpression of *Bmi1* Enhances Expansion of HSCs *ex vivo* and Protects HSCs During Serial Transplantation

HSCs are exposed to oxidative stress during long-term culture in 20% O_2 [15]. In order to precisely determine the effect of overexpression of *Bmi1* on HSCs during culture, we next determined the frequency of functional HSCs contained in optimized serum-free culture by competitive repopulating unit (CRU) assay. We first transplanted limiting doses of fresh CD34$^-$LSK cells from *Tie2-Cre* and *Tie2-Cre;R26Stop*^{FL}*Bmi1* mice along with 2×10^5 competitor BM cells. The frequency of long-term repopulating HSCs was 1 in 8 among fresh CD34$^-$LSK cells from both *Tie2-Cre* and *Tie2-Cre;R26Stop*^{FL}*Bmi1* mice (**Figure 3**). We then cultured CD34$^-$LSK cells for 10 days in a serum-free medium supplemented with SCF and TPO in 20% O_2. During the 10-day culture period, functional HSCs increased 4-fold (1 out of 2 CD34$^-$LSK cells) in control CD34$^-$LSK cells. Interestingly, overexpression of *Bmi1* established 2-fold better expansion of HSCs than the control during culture *ex vivo* (**Figure 3**). These findings are the first to show that Bmi1 has a role in the expansion of HSCs.