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Eltrombopag, a thrombopoietin receptor agonist, enhances human umbilical cord blood hematopoietic stem/primitive progenitor cell expansion and promotes multi-lineage hematopoiesis

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Abstract

Umbilical cord blood (UCB) transplantation has emerged as promising therapy, but is challenged by scarcity of stem cells. Eltrombopag is a non-peptide, thrombopoietin (TPO) receptor agonist, which selectively activates *c-Mpl* in humans and chimpanzees. We investigated eltrombopag's effects on human UCB hematopoietic stem cell (HSC) and hematopoietic progenitor cell (HPC) expansion, and its effects on hematopoiesis in vivo. Eltrombopag selectively augmented the expansion of human CD45+, CD34+, and CD41+ cells in bone marrow compartment without effects on mouse bone marrow cells in the NOD/SCID mice xenotransplant model. Consequently, eltrombopag increased peripheral human platelets and white blood cells. We further examined effects in the STAT and AKT signaling pathways in serum-free cultures. Eltrombopag expanded human CD34+CD38−, CD34+, and CD41+ cells. Both eltrombopag and recombinant human TPO (rhTPO) induced phosphorylation of STAT5 of CD34+CD41−, CD34−CD41+, and CD34−CD41− cells. rhTPO preferentially induced pSTAT3, pAKT, and more pSTAT5 in CD34−C41+ cells, while eltrombopag had no effects on pSTAT3. In conclusion, eltrombopag enhanced expansion of HSCs/HPCs of human UCB in vivo and in vitro, and promoted multi-lineage hematopoiesis through the expansion of bone marrow HSCs/HPCs of human UCB in vivo. Eltrombopag differed somewhat from rhTPO in the signal transduction pathways by favoring earlier HSC/HPC populations.

Keywords

Eltrombopag; thrombopoietin; *c-Mpl*; CD34+; CD34+CD38−; CD 41+; NOD/SCID xenotransplant; STAT5; STAT3; AKT

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INTRODUCTION

Umbilical cord blood transplantation (UCBT) holds great promise with apparent clinical advantages, but the transplant success rate remains much poorer than peripheral blood stem cell (PBSC) or bone marrow stem cell sources. This is due to the scarcity of stem cells in each unit, thus ex vivo expansion of UBC continues to be an area of active research (Koestenbauer et al., 2009). Nonetheless, human UCB stem cells are useful for patients without matched related or unrelated donors, because an estimated 40%–80% of patients will not be able to find an acceptable adult stem cell donor for stem cell transplantation (Ballen et al., 2007; Barker, et al., 2003; Brunstein et al., 2007; Misawa et al., 2006; Rocha, et al., 2009). Human UCB offers practical advantages as an alternative source of bone marrow stem cell or PBSC, which include (1) the relative ease of procurement (ability to store fully tested and HLA-typed UCB available for immediate use); (2) the absence of risk to the mother and the donors; (3) the reduced likelihood of transmitting infections; (4) immaturity of immune cells, thus reduced risk of GVHD; (5) less stringent criteria for HLA matching for donor-recipient selection (with the potential of finding donors for minority populations); (6) absence of donor attrition (Chao et al., 2004); and (7) availability: UCB banks have been established for related and unrelated UCB transplantation with more than 100,000 units being available based on published data (Barker et al., 2002; Gluckman et al., 2001; Kernan et al., 1993; Rocha et al., 2000). Thus agents that can facilitate human UCB hematopoietic stem cell (HSC) and hematopoietic progenitor cell (HPC) expansion are highly desirable.

The cytokine thrombopoietin (TPO) plays a crucial role in thrombopoiesis, in the regulation of HSCs, and in the proliferation of primitive HPCs. Both murine and human HSCs are highly enriched in cells expressing the TPO receptor *c-Mpl* (Solar et al., 1998; Yoshihara et al., 2007). Administration of TPO to myelosuppressed animals not only significantly alleviates thrombocytopenia, but also accelerates multiple lineage recovery (Akahori et al., 1996; Farese et al., 1996; Grossmann et al., 1996a; Grossmann et al., 1996b; Kaushansky et al., 1996; Neelis et al., 1997), promotes the reconstitution of multiple-lineage immature progenitors/precursors in bone marrow and spleen (Farese et al., 1996; Grossmann et al., 1996a; Kaushansky et al., 1996; Neelis et al., 1997), and augments the responses to GM-CSF and G-CSF (Farese et al., 1996; Grossmann et al., 1996a; Neelis et al., 1997). In vitro, TPO acts alone and most effectively in synergy with other early cytokines to promote survival and proliferation of HSCs and HPCs, and supports their expansion and differentiation into multiple-lineage colony-forming progenitors (Borge et al., 1997; Kobayashi et al., 1996; Ku et al., 1996; Luens et al., 1998; Ramsfjell et al., 1997; Petzer et al., 1996; Sitnicka et al., 1996; Young et al., 1996). TPO is also known to be critical for the replenishment of HSCs after bone marrow transplantation (Fox et al., 2002; Qian et al., 2007). These observations established the crucial roles of TPO receptor (*c-Mpl)* signaling on not only megakaryocytopoiesis, but also early hematopoiesis including regulation of HSCs.

While TPO holds promise in thrombopoiesis and in promoting stem cell proliferation and differentiation, phase II-III clinical trials of rhTPO and its shorter polyethylene glycol-conjugated form, PEG-rHuMGDF yielded mixed results. This was due to the fact that both were associated with autoantibodies that cross-reacted with and neutralized endogenous TPO, ultimately leading to low platelet counts. Clinical trials of these agents were discontinued in the United States several years ago (Basser et al., 2002; Li et al., 2001). Since then, a peptide mimetic (romiplostim), and a few non-peptide, small molecule *c-Mpl* agonists have been developed as alternatives to recombinant TPO (Erickson-Miller et al., 2009; Fukushima-Shintani et al., 2009; Inagaki et al., 2004; Nakamura et al., 2006; Nogami et al., 2008). Among these, eltrombopag (SB-497115) is an oral thrombopoietic receptor agonist, which interacts with the transmembrane domain of the *c-Mpl*. The latter confers

unique species-specificity of eltrombopag, which binds to *c-Mpl* of humans and chimpanzees only (Erickson-Miller et al., 2005; Erickson-Miller et al., 2009). Eltrombopag selectively activates the *c-Mpl* signaling pathway, stimulates the growth of TPO-dependent cell lines, promotes isolated human CD34+ cells to become megakaryocytes, and increases platelet count dose-dependently [Erickson-Miller et al., 2005; Erickson-Miller et al., 2009; Jenkins et al., 2007). The treatment of chronic Idiopathic Thrombocytopenic Purpura (ITP) or chronic hepatitis C patients with eltrombopag effectively increased platelet number and reduces thrombocytopenia-related complications (Bussel et al., 2007; Bussel et al., 2009; McHutchison et al., 2007).

Eltrombopag is only capable of activating parts of the *c-Mpl* signaling pathways, but whether eltrombopag retains the function of TPO in enhancing stem cells and early progenitor cells remains largely unknown (Erhardt et al., 2009; Will et al., 2009). As eltrombopag is a *c-Mpl* agonist, we hypothesize that eltrombopag can expand HSCs and HPCs of UCB, thus can enhance multi-lineage hematopoiesis. Given the species specificity of eltrombopag to only humans and chimpanzees, our investigation utilized an in vitro serum-free culture system, and an in vivo NOD/SCID xenotransplant model. Here we present results of our investigation of eltrombopag in promoting multi-lineage hematopoiesis through the expansion of bone marrow HSCs and HPCs of human UCB in vivo using the NOD/SCID human bone marrow xenotransplant model. We further examined eltrombopag effects on the expansion of human UCB CD34+CD38− cells in serum-free cultures, and compared the differential effects between eltrombopag and TPO on the HSCs/HPCs and the intracellular phosphorylation of STAT5, STAT3, and AKT pathways.

RESULTS

Eltrombopag promoted multi-lineage hematopoiesis of engrafted human UCB CD34+ cells in NOD/SCID mice

We examined effects of eltrombopag on hematopoiesis of human UCB CD34+ cells transplanted in sublethally irradiated NOD/SCID mice. The mice were gavaged with eltrombopag daily (50mg/kg/day) vs. vehicle for 28 days, starting one-day post-human UCB CD34+ cell transplantation. Day-zero was defined as the day of human UCB transplantation. Eltrombopag treatment significantly promoted thrombopoiesis (Figure 1A), leading to a 3.6 fold increase in circulating human platelets between weeks 4 and 5 when compared with vehicle treated mice. In addition, eltrombopag treatment significantly increased the circulating human white blood cells (WBCs) at week 5 (Figure 1C). Note that eltrombopag had no effect on murine thrombopoiesis (Figure 1B), nor murine WBC counts (Figure 1D). As the progenitors for thrombopoiesis and for WBCs are from different HPC lineages, our observation supported that eltrombopag promoted multiple lineage proliferation and differentiation of human UCB CD34+ cells. By week 9, which was 5 weeks after cessation of eltrombopag treatment, the number of human platelets as well as WBCs in the drug-treated NON/SCID mice was still higher than those in the vehicle-treated mice, although the differences were not statistically significant due to variations among mice in the same treatment group.

Eltrombopag enhanced expansion of HSCs/HPCs of human UCB CD34+ cells in the bone marrow of NOD/SCID mice

To assess if the increase of peripheral human platelets and WBCs is a result of eltrombopag effect on human HSCs and HPCs in the bone marrow compartment of NOD/SCID mice, we examined the cell surface markers of cells extracted from the bone marrow of NOD/SCID mice. We found that bone marrow human CD45+ (marker for WBC lineage), CD34+ (marker for HSC/HPC) and CD41+ (marker for thrombocyte lineage) cells were augmented

by eltrombopag treatment. Eltrombopag-treated mice had 1.6 and 2 fold more bone marrow CD45+ on week 3 ($p<0.01$) and week 4 ($p<0.01$), respectively (Figure 2A). By week 9, which was 5 weeks after cessation of eltrombopag treatment, the number of human CD45+ bone marrow cells was still significantly higher (70%) than that in the control mice. For HSC/HPC, there was 70% increase of bone marrow CD34+ cells in the eltrombopag-treated group as early as week 1 ($p<0.05$) (Figure 2C), with further increase to more than 150% on week 2 ($p<0.01$) and week 4 ($p<0.05$). For thrombopoiesis, the administration of eltrombopag induced a 1.8 fold increase in human CD41+ bone marrow cells by the end of 4-week treatment ($p<0.01$). There were also more human CD34+ and CD41+ bone marrow cells in the mice treated with eltrombopag at week 9, though the differences were not statistically significant. Again, eltrombopag did not affect the proliferation of mouse CD45+ bone marrow cells (Figure 2B).

Eltrombopag enhanced expansion of human CD34+CD38− cells in vitro

It is known that human HSCs are highly enriched in the CD34+CD38− fraction, and the measure of CD34+CD38− is a close estimate of HSCs (Bhatia et al., 1997; Larochelle et al., 1996). To further expand on our observation that eltrombopag promoted expansion of bone marrow HSC/HPCs in the NOD/SCID xenotransplant model, we conducted assessment of eltrombopag's effect on the expansion of HSCs/HPCs using a well-defined, serum-free culture system for UCB. Eltrombopag effects on promoting proliferation vs. differentiation of human UCB were compared with the rhTPO effects in vitro. Human UCB CD34+ cells were cultured in serum-free medium supplemented with rhSCF and rhFL plus either rhTPO or eltrombopag for 7 days. Human CD34+CD38− cells (more primitive cell population) were measured along with all cells expressing CD34+ and cells expressing CD41+, the marker for megakaryocytes and other HPCs committed to thrombopoiesis. Scatter plots of cell marker phenotypes were analyzed by flow cytometry, and are shown in Figure 3A. The cell number (bars) and percentage (squares) are presented in Figure 3B. Compared to the control culture (SF) of rhSCF and rhFL only, the addition of 3μg/ml eltrombopag significantly increased CD34+ cells by 26%, CD34+CD38− cells by 42%, and CD41+ cells by 400%. In comparison, the cultures containing 10ng/ml rhTPO (SFT10) had significantly increased CD34+ cells by 130%, CD34+CD38− cells by 160%, CD41+ cells by 900%. The increase in rhTPO concentration from 10ng/ml to 50ng/ml did not lead to more expansion, supporting that the effect of rhTPO was saturated at 10ng/ml. The "percentages" (squares) of CD34+, CD34+CD38− and CD41+ cells were almost the same in the cultures with 10ng/ml rhTPO vs. 3μg/ml eltrombopag. Thus, our data support that rhTPO supported greater cell proliferation than eltrombopag, while the extent of cell differentiation induction, maintaining primitive hematopoietic progenitors, and promotion of megakaryopoiesis are almost the same between eltrombopag and rhTPO. We also noted that the increase of eltrombopag from 3μg/ml to 6μg/ml inhibited proliferation in all cell populations and almost eliminated CD41+ cells, showing the potential toxicity of eltrombopag at higher concentrations.

Eltrombopag induced phosphorylation of STAT5, but not STAT3 and AKT

To gain insight into potential differences between eltrombopag and rhTPO in the activation of intracellular signaling pathway related to the activation of TPO receptor *c-Mpl*, we analyzed the induction of phosphorylation of STAT5, STAT3, and AKT pathways after treatment with rhTPO vs. eltrombopag (Figure 4). The analysis was flow-cytometry-based assay, which offered the advantage of analyzing phosphorylation of the target proteins at the single cell level. Three cell types: CD34+CD41−, CD34−CD41+, and CD34−CD41− (Figure 4A) were gated to measure the levels of pSTAT5, pSTAT3, and pAKT in these cells after treatment by either eltrombopag or rhTPO. We first analyzed the TPO-R levels of these three cell types. Our culture condition contained rhSC and rhFL to enrich TPO-R (*c-Mpl*)

expression for the investigation of intracellular signal pathways, and we achieved an average of 97% ± 2% of cells expressing TPO-R. We found that expression levels of TPO-R were not different in these three different cell populations at all time points for the untreated control, the eltrombopag treated group, and the rhTPO treated group (Figure 4B, last row of panels). After gating the cells, less than 0.1% of extreme outliers of fluorescence intensity were gated away and the mean of fluorescence intensity were calculated (Figure 4B). Both rhTPO and eltrombopag significantly induced phosphorylation of STAT5 in CD34+CD41−, CD34−CD41+, and CD34−CD41−cells when compared with untreated control. Eltrombopag and rhTPO induced STAT5 phosphorylation to the same extent in CD34+CD41− and CD34−CD41− cell types, while rhTPO induced significantly more phosphorylation of STAT5 in CD34−CD41+ cells than eltrombopag at all time points ($p<0.05$, two-sided paired t-test). In contrast to rhTPO, the level of eltrombopag induced phosphorylation of CD34+CD41−, CD34+CD41+, and CD34−CD41− cells were comparable among all three cell-types (Figure 4, first row of panels).

For STAT3 and AKT pathways, we observed that phosphorylation of STAT3 and AKT were not increased by eltrombopag in these three cell types, while rhTPO did increase phosphorylation of STAT3 and AKT in CD34−CD41+ cells, but not in CD34+CD41− and CD34−CD41− cells. The cell-type dependant phosphorylation of STAT5, STAT3, and AKT induced by rhTPO was not caused by any difference in the level of TPO-R expression. Eltrombopag differed from rhTPO in the lack of inducing phosphorylation of pSTAT5 in “more committed progenitor cells”. Such observations highlighted the target cell-type differences of eltrombopag vs. rhTPO, and the potent effects of TPO on megakaryopoiesis.

DISCUSSION

In the stem cell transplantation setting, reconstitution of bone marrow and the subsequent recovery of peripheral blood cells are dependent on the expansion and differentiation of the HSCs and HPCs of donor cells. In this report, we investigated eltrombopag effect on human UCB CD34+ cells in the NOD/SCID mice xenotransplants after receiving human UCB CD34+ cells. We found that eltrombopag treatment accelerated expansion of human CD34+ progenitors, human CD45+ and CD41+ cells in the bone marrow, and promoted the production of circulating human platelets and WBCs without any influence on mouse hematopoietic cells (Figures 1 and 2). By week 9, which was 5 weeks after the cessation of eltrombopag feeding, the differences between the eltrombopag-treated mice and the control mice persisted. The multiple lineage and long-term effect of eltrombopag strongly suggested that it acted on “earlier hematopoiesis” at the HSC/HPC levels in addition to its anticipated effect on promoting megakaryocpoiesis, which is a more committed later stage.

To further examine eltrombopag’s effect on early hematopoiesis through HSCs/HPCs, we tested the ability of eltrombopag on the expansion of human UCB CD34+ cells in the presence of SCF and FL in the serum-free media (Figure 3). Eltrombopag significantly expanded CD34+CD38− HSCs/HPCs, CD34+ immature progenitors and CD41+ cells. In comparison with the effects of 3μg/ml eltrombopag, 10ng/ml rhTPO did not affect the percentage of CD34+CD38−, CD34+, and CD41+ cells but did markedly increase the expansion, supporting the fact that eltrombopag is as effective as TPO in regulating the balance of proliferation and differentiation among CD34+CD38−, CD34+, and CD41+ cells. However, eltrombopag was not as potent as TPO in promoting proliferation. Our finding was consistent with a recent report showing that eltrombopag was able to expand CD34+CD38− cells in the presence of SCF in the serum-free culture (Nishino et al., 2009), suggesting that it at least partly retained the function of TPO on early hematopoiesis.

The differential findings between eltrombopag and TPO suggested the possibility that separate intracellular signaling pathways might have taken place in mediating proliferation vs. differentiation of HSC/HPCs mediated by *c-Mpl.* To look for the potential mechanisms behind the functional difference between TPO and eltrombopag, we conducted a cytometry-based method to analyze the phosphorylation of STAT3, STAT5 and AKT in the signaling pathway of TPO receptor (*c-Mpl)*, in three different cell types: CD34+CD41− HSC/ HPC, CD34−CD41+ megakaryocytes, and CD34−CD41− cells. We found that the level of phosphorylation of STAT5 induced by eltrombopag was similar in all three cell-populations and were comparable to the level stimulated by TPO in CD34+CD41− and CD34−CD41− cells, but TPO stimulated higher levels of STAT5 phosphorylation in CD34−CD41+ cells. This was the cell population that was more committed to thrombopoiesis than the other two cell type populations. We also observed that TPO activated STAT3 and AKT in CD34−CD41+ cell, while eltrombopag did not cause the phosphorylation of STAT3 and AKT pathways in CD34−CD41+ cells. Because eltrombopag promoted CD41+ differentiation as effectively as TPO in our in vivo model, our finding suggested that STAT3 and AKT signaling pathway might not be important for the differentiation of CD34+ cells to CD41+ cells. It is known that the binding of TPO to *c-Mpl* activates MAPK p42/p44, AKT, and STAT proteins in normal human CD34+ cells, megakaryocytes, and platelets (Majka et al., 2000; Majka et al., 2002), while eltrombopag activates MAPK p42/p44 and STAT5 in N2C-TPO cells, and STAT5 in human megakaryocytes (Erickson-Miller et al., 2009). In human platelets, eltrombopag induces STAT1, STAT3, and STAT5 activation, but spares AKT phosphorylation (Erhardt et al., 2009). In bone marrow cells from patients with acute myeloid leukemia and myelodysplastic syndrome, eltrombopag activates STAT5 but not STAT3 (Will et al., 2009). Combining our findings with the published reports support the observation that the activation of the downstream signaling pathway by eltrombopag differs from TPO, despite the fact both exert their functions through the TPO-R (*c-Mpl)* binding.

To our knowledge, our manuscript is the first of its kind in finding that the thrombopoietic agent, ie, eltrombopag, has the potential to expand UCB CD34+ “in vivo”. The implication is significant in that despite much work has been done on “ex vivo” UCB CD34+ cell expansion, the current success rate of UCB stem cell transplantation remains poor when compared with PBSC transplantation or bone marrow stem cell transplantation. If eltrombopag can improve UCB transplant directly in vivo, which will need to be tested in future clinical trials, it potentially can either negate the need of “ex vivo” UCB stem cell expansion procedures, or may work synergistically with “ex vivo” expansion of UCB CD34+ cells to enhance UCB transplant success rate.

CONCLUSION

Our in vivo data demonstrated that eltrombopag was effective in accelerating the expansion of human UCB CD34+ HSCs/HPCs and in promoting multi-lineage hematopoiesis in the animal human bone marrow xenotransplant model. The in vitro data further supported eltrombopag effects on the proliferation of CD34+CD38− primitive progenitors in the serum-free culture. In vitro studies also delineated the effects of eltrombopag vs. TPO on different cell populations in the induction of intracellular signaling pathways. Our data suggests that eltrombopag at least partly retains the function of TPO on early hematopoiesis, and appears to have fewer effects on intracellular signaling in the more committed (CD41+) progenitor cell population. Our finding has the potential in clinical applications to improve the success rate of UCB transplantation by enhancing engraftment of human UCB for stem cell transplantation.

MATERIALS AND METHODS

NOD/SCID mouse for human cord blood xenotransplant

Due to species specificity of eltrombopag in humans and chimpanzees, the in vivo study was conducted using NOD/SCID mice transplanted with human UCB. All animal experimentation protocols were approved by the University Committee on Animal Resources at the University of Rochester. NOD/SCID mice (Jackson Lab) were maintained under specific pathogen-free conditions, and were provided with sterile water containing Sulfatrim. Nine to 10 week old mice were sublethally irradiated with 3.5 Gy total body irradiation (TBI) using a Cs^{137} source to partially ablate mouse bone marrow cells. One day after TBI, cryopreserved human UCB CD34+ cells (Stemcells) were thawed and washed with IMDM supplemented with 10% heat-inactivated FBS. Human CD34+ cells at $1x10^5$ were intravenously inoculated into the mice through the tail vein. The mice received daily gavage of eltrombopag for 28 days at the dose of 50mg/kg in sterile water or vehicle (sterile water), starting one-day after UCB transplantation. The dose 50mg/kg/day was chosen based on the renal toxicity information of mice in the dose range between 25mg–75mg/kg/day (Eltrombopag Investigator Brochure, 2012). Mice were sacrificed at 1, 2, 3, 4, 5, and 9 weeks from the starting day of eltrombopag treatment. Whole blood was drawn by cardiac puncture for cell counts. Bone marrow cells were extracted by flushing femurs and tibia. Complete blood counts and bone marrow mononuclear cell counts were obtained using Heska HemaTure Hematology Analyzer and FACSCalibur flow cytometer.

Analysis of human UCB engraftment in NOD/SCID mice

To detect human cells in murine peripheral blood, multicolor flow-cytometry analysis was performed using FACSCalibur flow cytometer (BD Biosciences). Human platelets were differentially detected by staining whole blood with anti-human CD41a-PE and anti-mouse CD61-FITC antibodies. CountBright Absolute Counting Beads (Molecular Probe) were added to samples as internal counting control to obtain the number of platelets. Human WBCs were examined by staining whole blood with anti-human-CD45-FITC and anti-mouse CD45-PE antibodies. The RBCs were then depleted by FACS lysing solution (BD PharMingen). The Draq5 (Biostatus) was added at the final concentration of 2μM and incubated at room temperature for 5 minutes to stain the mononuclear cells before the cells were subjected to analysis with FACSCalibur. The percentage of human CD45+ WBCs in total WBCs was measured by the cytometry. The number of human CD45+ WBCs was calculated by multiplying the percentage of human CD45+ WBCs in total WBCs with the number of total WBCs measured by the Heska HemaTure Hematology Analyzer. The bone marrow cells were stained either with anti-human CD45-FITC and anti-mouse CD45-PE antibodies or anti-human CD34-FITC and anti-human CD41a-PE antibodies. Draq5 was used to stain MNCs and excluded RBCs. The number of subpopulation was calculated by multiplying the percentage of the population in total MNCs measured by flow cytometry with the number of bone marrow total MNCs. The antibodies were purchased from BD or eBioscience.

Human UCB CD34+ cell in serum-free culture

The human UCB CD34+ cells with more than 90% purity (Stemcells) were plated at 5×10^4 cells/ml in HPGM serum-free media (Lonza) and cultured for 7 days at 37°C in humidified air with 5% CO_2. rhTPO or eltrombopag at different concentrations was added with or without 25ng/ml recombinant human SCF and 50ng/ml recombinant human FL. On the fourth day, the culture was diluted 1:1 with fresh medium containing the cytokines. The cells were harvested on the seventh day. The viable cells were distinguished by Trypan blue and counted using a hemacytometer (Hausser Scientific, Horsham, PA). Cells were stained with anti-human CD41, anti-human CD34 and anti-human CD38 before analyses using

FACSCanto II. Propidium iodide (Sigma) was added before flow sample running to exclude the dead cells.

Phosphorylation STAT3, STAT5 and AKT signaling pathway

We applied modified flow cytometric phospho-protein analysis (Larochelle et al., 1996) to measure the phosphorylation status of STAT3, STAT5 and AKT in the *c-Mpl* signal pathway of different cell types. Human UCB CD34+ cells were cultured in the serum-free media with 25ng/ml rhSCF, 50ng/ml rhFL and 10ng/ml rhTPO for 7 to 8 days. The cells were then washed with fresh medium twice and 7 to 8 hours serum starvation was allowed in the HPGM serum-free medium. Cells were then treated with 100ng/ml rhTPO or 3μg/ml eltrombopag for 10 to 60 minutes at 37°C. At the end of treatment, the cells were immediately fixed by adding 1:1 (vol:vol) BD cytofix fixation buffer (BD Bioscience) and incubated at 37°C for 10 minutes. Cells were then washed and permeabilized with BD phosphoflow Perm Buffer III for 30 minutes on ice. The cells were washed again and stained with anti-phospho-STAT3 (pY705), anti-phospho-STAT5 (pY694), anti-phospho-AKT (pS473), anti-human CD41, anti-human CD34, and anti-human TPO-receptor (*c-Mpl)* (allophycocyanin (APC)-conjugated mouse monoclonal anti-human Thrombopoietin R antibody, R&D Systems Inc., Minneapolis, MN). The stained cells were analyzed using FACSCanto II flow cytometer (BD Biosciences).

Statistical analysis

Data are presented as the mean ± standard error of the mean. The difference between two groups of data in the experiments of xenotransplantated NOD/SCID mice was analyzed by nonparametric two-tail Mann-Whitney U Test, as data were not normally distributed. In all other cases, two groups of data were compared by two-sided paired t-tests.

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Abbreviations

FBS	fetal bovine serum
FL	flt-3 ligand
G (M)-CSF	granulocyte(macrophage) colony-stimulating factor
GVHD	graft versus host disease
HLA	human leukocyte antigen
HPC	hematopoietic progenitor cell
HSC	hematopoietic stem cell
ITP	idiopathic thrombocytopenic purpura

MNCs	mononuclear cells
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency
PEG-rHuMGDF	pegylated recombinant megakaryocyte growth and development factor
PBSC	peripheral blood stem cells
RBC	red blood cells
rhTPO	recombinant human thrombopoietin
UCB(T)	umbilical cord blood (transplantation)
TPO	thrombopoietin
TPO-R	thrombopoietin receptor
WBC	white blood cells

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Highlights

- Eltrombopag expanded HSC/HPC of human cord blood in bone marrow of NOD/SCID mice.
- Eltrombopag increased human platelets and WBCs in NOD/SCID mouse xenotransplants
- Both eltrombopag and TPO expanded human cord blood HSC/HPC in serum-free cultures.
- Both eltrombopag and TPO activated STAT 5 pathway of HSC/HPC of human cord blood.
- In contrast to TPO, eltrombopag lacked activation of STAT3 and AKT of HSC/HPC.

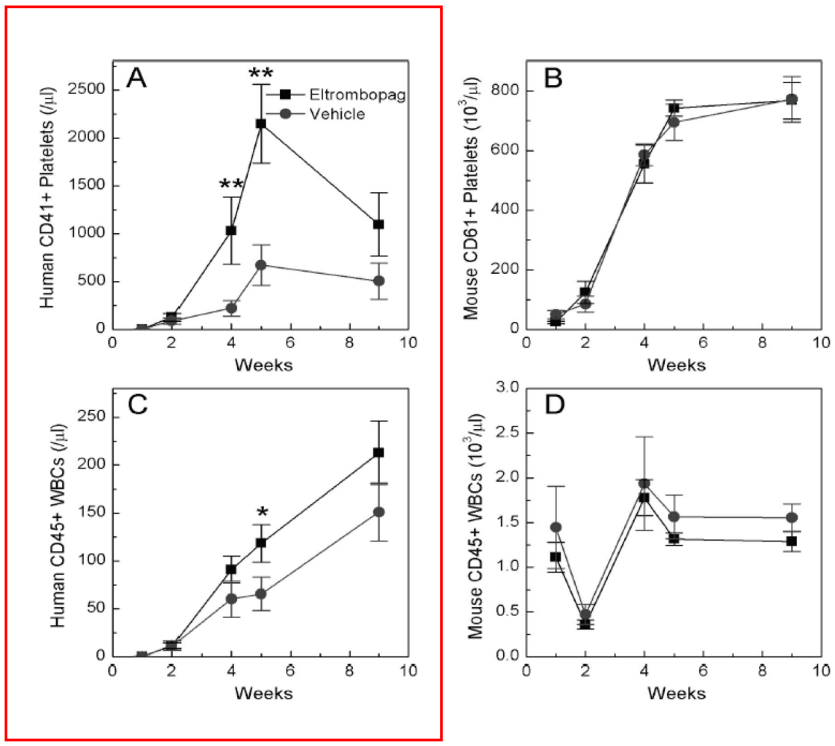

Figure 1.

Eltrombopag treatment increased circulating human platelets and human WBCs in peripheral blood of NOD/SCID mice after transplantation. The mice were transplanted with human UCB CD34+ cells. Mice were gavaged with 50mg/kg eltrombopag or vehicle daily for 28 days. (A) Human platelets. (B) Mouse platelets. (C) Human WBCs and (D) Mouse WBCs. Data are expressed as mean ± standard error of the mean (n=8–12). *p<0.05, **p<0.01 between eltrombopag and vehicle two-tail Mann-Whitney U Test.

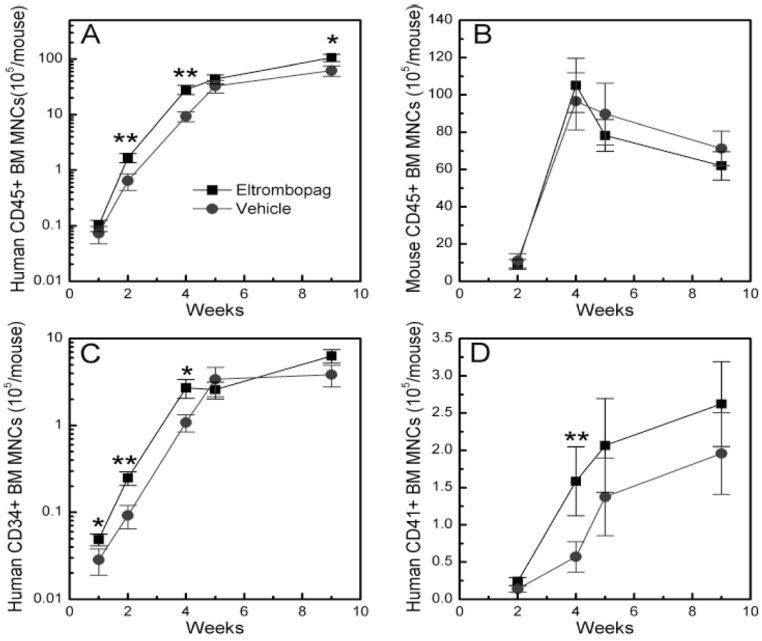

Figure 2.

Eltrombopag promoted rapid establishment of human engraftment in bone marrow of NOD/SCID mice after transplantation. The mice were transplanted with human UCB CD34+ cells. Mice were gavaged with 50mg/kg eltrombopag or vehicle daily for 28 days. (A) Human CD45+ bone marrow MNCs. (B) Mouse CD45+ bone marrow MNCs. (C) Human CD34+ bone marrow MNCs. (D) Human CD41+ bone marrow MNCs. Data are expressed as mean ± standard error of the mean (n=7–12). *p<0.05, **p<0.01 between eltrombopag and vehicle two-tail Mann-Whitney U Test.

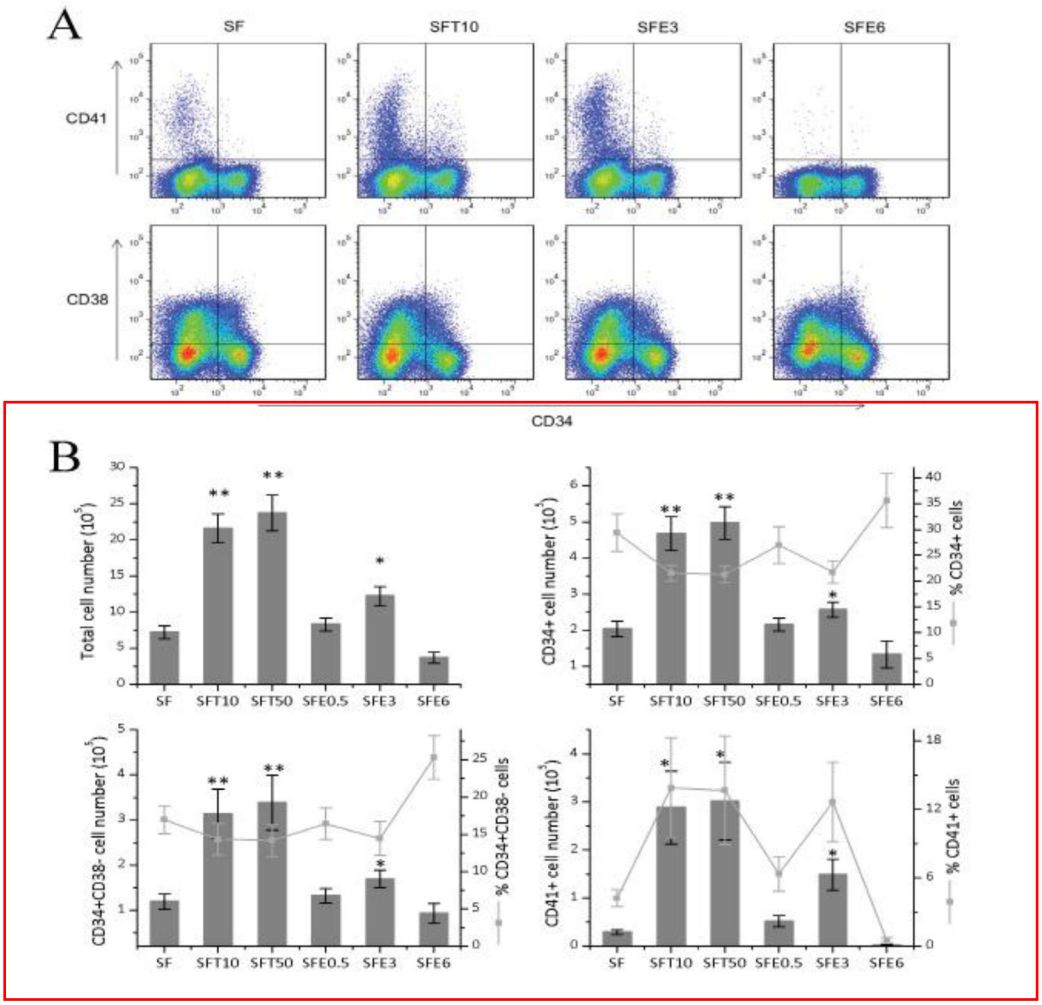

Figure 3.

Expansion of human UCB CD34+ cells in the culture. Human UCB CD34+ cells (> 90% purity) were cultured in the presence of 25ng/ml SCF (S), 50ng/ml FL (F) and 10 or 50ng/ml rhTPO (T10 or T50) or 0.5, 3, 6μg/ml (equivalent to 1.13, 6.78 and 13.6μM, respectively) eltrombopag (E0.5, E3 or E6) for 7 days. The cells were counted and analyzed by flow cytometry. (A) The population distribution after the 7-day culture by flow cytometry. (B) The numbers of total viable cell, CD34+, CD34+CD38− and CD41+ cells are presented in bar graph, while their percentages are shown as squares. 1×10^5 CD34+ human UCB cells were seeded in the beginning of the culture. Data are showed as mean ± standard error of the mean, n=3~5. Compared with the control culture (SF), *p<0.05, **p<0.01 by two-sided paired t-test.

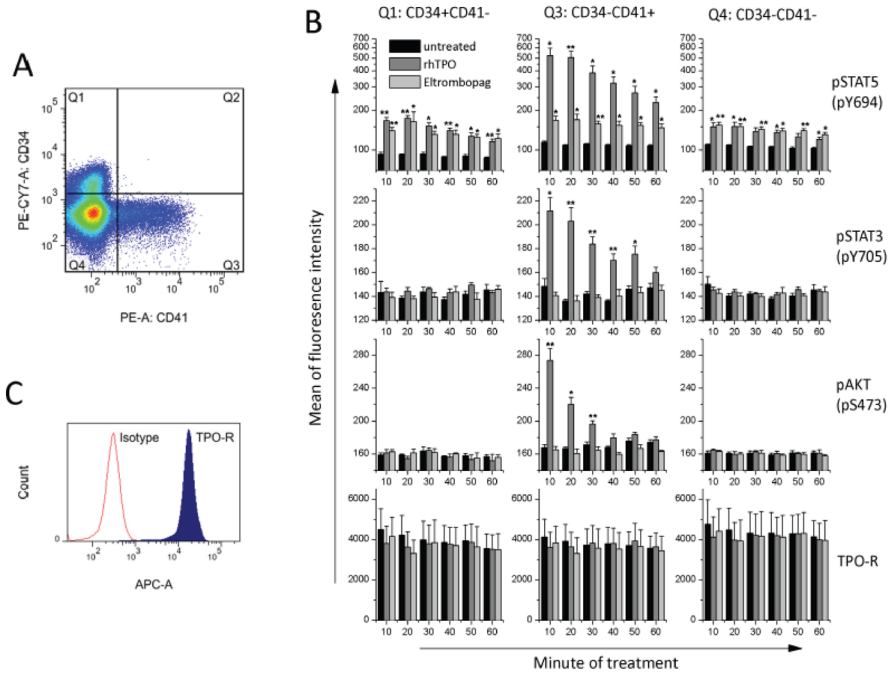

Figure 4.

Phosphorylation of STAT3, STAT5 after treatment with rhTPO or eltrombopag. Human UCB CD34+ cells were expanded in serum-free media with 50 ng/ml FL, 25 ng/ml SCF, and 10 ng/ml rhTPO for 7 days. Then the cells were rendered quiescent in serum-free media without cytokines for 7~8 hours. The cells were exposed to 100 ng/ml rhTPO or 3μg/ml eltrombopag for 10 to 60 min at 37°C. These cells were then fixed, permeabilized, stained for surface markers and intracellular antigens, and analyzed by flow cytometry. (A) Cell population gating. (B) Phosphorylation of STAT3, STAT5 and AKT in CD34+CD41−, CD34−CD41+ and CD34−CD41− cells induced by rhTPO or eltrombopag. Data are shown as mean ± standard error of the mean, n=4. Compared with the untreated control, *p<0.05, **p<0.01 by two-sided paired t-test. (C) Almost all cells expressed TPO receptors (TPO-R) after 7-day culture. The close curve shows the cells stained with anti-TPO-R (anti-c-MPL) antibody, while the open curve represents the cells stained with isotype antibody for non-specific and background binding.