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Pleiotrophin regulates the expansion and regeneration of hematopoietic stem cells

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Hematopoietic stem cell (HSC) self-renewal is regulated by both intrinsic and extrinsic signals. Although some of the pathways that regulate HSC self-renewal have been uncovered, it remains largely unknown whether these pathways can be triggered by deliverable growth factors to induce HSC growth or regeneration. Here we show that pleiotrophin, a neurite outgrowth factor with no known function in hematopoiesis, efficiently promotes HSC expansion *in vitro* and HSC regeneration *in vivo*. Treatment of mouse bone marrow HSCs with pleiotrophin caused a marked increase in long-term repopulating HSC numbers in culture, as measured in competitive repopulating assays. Treatment of human cord blood CD34$^+$CDCD38$^-$Lin$^-$ cells with pleiotrophin also substantially increased severe combined immunodeficient (SCID)-repopulating cell counts in culture, compared to input and cytokine-treated cultures. Systemic administration of pleiotrophin to irradiated mice caused a pronounced expansion of bone marrow stem and progenitor cells *in vivo*, indicating that pleiotrophin is a regenerative growth factor for HSCs. Mechanistically, pleiotrophin activated phosphoinositide 3-kinase (PI3K) signaling in HSCs; antagonism of PI3K or Notch signaling inhibited pleiotrophin-mediated expansion of HSCs in culture. We identify the secreted growth factor pleiotrophin as a new regulator of both HSC expansion and regeneration.

HSCs possess the unique capacity to self-renew and give rise to all of the mature cell types within the blood and immune systems[1–3]. HSC self-renewal is regulated by both intrinsic and extrinsic signals[1–9], but the mechanisms involved in the control of this process are incompletely understood. Several growth factors have been identified whose action is associated with mouse HSC self renewal, including Notch ligands[4,5], Wnt3a[6], angiopoietin-like proteins[8] and prostaglandin E_2 (ref. 9). Coculture of HSCs with supportive stromal or endothelial cells[10,11] or the enforced expression of the transcription factors homeobox protein B4 or homeobox protein A9 (refs. 1,12) can also cause robust expansions of HSCs in culture. However, strategies that require cell co-culture or genetic modification of HSCs are not readily translatable into the clinic[13]. Moreover, despite advances in the understanding of the biology of HSC self-renewal and differentiation, it has not yet been possible to induce HSC expansion or regeneration in a manner that can be translated to clinical practice. Here we describe pleiotrophin, an 18-kDa heparin-binding growth factor that is mitogenic for neurons[14–16] and has angiogenic and proto-oncogene activity[17–19] as a potent, secreted regulator of HSC expansion and regeneration.

RESULTS

Treatment with pleiotrophin induces HSC proliferation

We have previously shown that human endothelial cells isolated from adults support the expansion of human HSCs in culture[20,21]. In contrast to co-culture studies with stromal cells[22], which have a demonstrated requirement for cell-to-cell contact for HSC maintenance, we have shown that primary human brain endothelial cells (HUBECs) produce a soluble activity capable of inducing a tenfold expansion of human HSCs *ex vivo*[11,20]. To identify the HUBEC-secreted proteins responsible for this HSC-amplifying activity, we performed genome-wide expression analysis of HUBECs as compared to nonbrain human endothelial cells that lack HSC-supportive activity (**Fig. 1a**). We identified 13 genes that were more than fivefold overexpressed in HUBECs and that were predicted to produce secreted gene products (**Supplementary Table 1**). We found that the expression of pleiotrophin, a heparin-binding growth factor with no known role in hematopoiesis[14–19], was 25-fold higher in HUBECs versus nonbrain endothelial cells (**Fig. 1b**). Quantitative RT-PCR confirmed a >100-fold higher pleiotrophin expression in HUBECs, and analysis of HUBEC-conditioned medium by ELISA showed a higher concentration of pleiotrophin compared to nonbrain endothelial cell–conditioned medium (6.9 ± 0.3 pg ml^{-1} compared to 0.02 ± 0.01 pg ml^{-1}, **Fig. 1b**).

We next examined whether bone marrow progenitor cells express one or more of the pleiotrophin receptors, receptor protein tyrosine phosphatase-β/ζ (RPTP-β/ζ), syndecan or anaplastic lymphoma kinase[14–16]. Both bone marrow mononuclear cells and bone marrow c-Kit$^+$Sca-1$^+$Lin$^-$ (KSL) progenitor cells expressed RPTP-β/ζ ($n = 3$ mice, mean 87.0% ± 8.8% and 89% of cells stain positive, respectively; **Fig. 1c**), whereas neither population expressed syndecan or anaplastic

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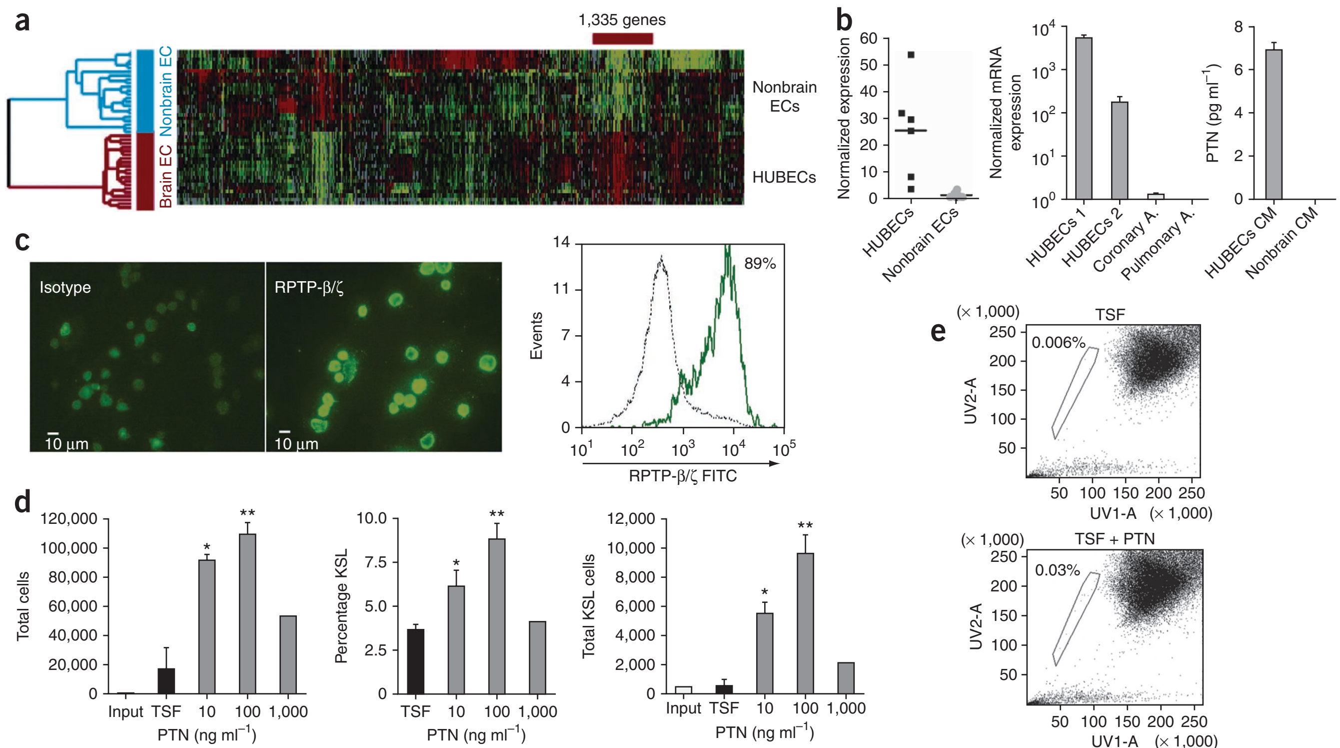

Figure 1 Pleiotrophin is overexpressed by HUBECs, and treatment with pleiotrophin induces the expansion of phenotypic HSCs in culture. (**a**) Unsupervised hierarchical cluster analysis of 1,335 genes upregulated in HUBECs (the region of the heat map indicated by the red bar) compared to nonbrain endothelial cells (ECs) (red, increased expression; green, decreased expression). (**b**) Left, scatter plot of pleiotrophin gene expression determined by microarray analysis in HUBECs versus nonbrain ECs (mean 25.1 ± 7.4 versus 1.0 ± 0.3, n = 6–8 samples per group, P = 0.001). Horizontal lines represent mean pleiotrophin expression in each group. Middle, pleiotrophin expression determined by quantitative RT-PCR in HUBECs versus coronary and pulmonary artery ECs (means ± s.e.m., n = 2 or 3 samples per group, HUBECs 1 versus coronary, P = 0.004; HUBECs 1 versus pulmonary artery (A.), P = 0.004). Right, pleiotrophin concentrations determined by ELISA of HUBEC-conditioned medium (CM) compared to nonbrain EC–conditioned medium (means ± s.e.m., n = 3 samples per group, *P = 0.04). 1 and 2 refer to two different primary HUBEC lines. (**c**) Left, a representative high-power field microscopic image of RPTP-β/ζ staining in mouse bone marrow mononuclear cells versus isotype control (n = 3 mice). Right, flow cytometric analysis of RPTP-β/ζ expression on mouse bone marrow KSL cells (n = 1). (**d**) The total number of bone marrow cells, the percentage of KSL cells and the number of KSL cells after 7-d culture of highly purified $CD34^-$KSL cells (97.3% ± 2.1%) with cytokines (TSF) with or without pleiotrophin (PTN). Left, data are means ± s.d., n = 3 experiments per group, *P = 0.01, **P = 0.006 versus TSF. Middle, data are means ± s.d., n = 3, *P = 0.04, **P = 0.004 versus TSF. Right, data are means ± s.d., n = 3 experiments per group, *P = 0.005, **P = 0.006 versus TSF. All comparisons were one-tailed t tests. (**e**) Bone marrow $CD34^-$KSL cells were placed in culture with TSF alone or TSF plus pleiotrophin for 7 d and representative FACS plots show the presence of side population cells in the progeny of TSF alone and TSF plus pleiotrophin cultures (n = 2 experiments; percentage of cells that efflux the Hoechst 33342 dye are shown in the gates; UV1-A represents Hoechst red and UV2-A represents Hoechst blue staining).

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lymphoma kinase (data not shown). We next isolated bone marrow $CD34^-$KSL cells from C57BL/6 mice by FACS; $CD34^-$KSL cells are highly enriched for HSCs[3,23]. We placed the cells in liquid suspension culture with 20 ng ml^{-1} thrombopoietin, 125 ng ml^{-1} stem cell factor (SCF) and 50 ng ml^{-1} Flt-3 ligand (TSF, these culture conditions support the proliferation of HSCs) without pleiotrophin or with 10, 100 or 1,000 ng ml^{-1} pleiotrophin for 7 d. We observed a dose-dependent increase in the number of total cells, the percentage of KSL cells and the number of total KSL cells in response to the addition of 10–100 ng ml^{-1} of pleiotrophin (**Fig. 1d**). In this experiment, the addition of 100 ng ml^{-1} pleiotrophin caused a 6.4-fold increase in total cell counts and a 17.7-fold increase in total KSL cell counts compared to TSF alone (P = 0.006 and P = 0.006, respectively, **Fig. 1d**). Similarly, cell cultures with TSF plus pleiotrophin contained twofold more 'side population' cells, which are highly enriched for primitive HSCs[24], compared to cell cultures with TSF alone (mean 0.02% versus 0.01%, n = 4, P = 0.03, **Fig. 1e**). A 14-d replating study confirmed that pleiotrophin induced the expansion of $CD34^-$KSL cells; at day 14, cell cultures with TSF plus pleiotrophin contained 3.2-fold more $CD34^-$KSL cells compared to input (day 0) bone marrow $CD34^-$KSL cells, whereas cell cultures with TSF alone contained a 1.6-fold increase in $CD34^-$KSL cells compared to day 0 (data not shown). Notably, treatment of bone marrow $CD34^-$KSL cells with 10-1,000 ng ml^{-1} pleiotrophin alone yielded no viable cells at day 7 of culture (n = 3 experiments), indicating that pleiotrophin cannot act alone as a survival factor for bone marrow and that other cytokines are required to maintain HSCs in culture.

Treatment with pleiotrophin induces the expansion of LT-HSCs

To determine whether treatment with pleiotrophin could induce functional HSC expansion in culture, we performed competitive repopulating unit (CRU) assays with limiting dilutions of donor CD45.1$^+$ bone marrow $CD34^-$KSL cells transplanted into lethally irradiated CD45.2$^+$ C57BL/6 mice. We collected peripheral blood from recipient mice at 4, 12 and 24 weeks after transplant to assess the engraftment of donor CD45.1$^+$ cells. At 12 weeks, mice that had been transplanted with the progeny of $CD34^-$KSL cells cultured with TSF plus pleiotrophin showed more than tenfold higher total CD45.1$^+$ cell engraftment (means ± s.d., P = 0.006) and significantly higher B lymphoid

($P = 0.003$), myeloid ($P = 0.03$) and T cell engraftment ($P = 0.006$) at 12 weeks compared to mice transplanted with the same dose of day 0 bone marrow CD34$^-$KSL cells or their progeny after culture with TSF alone ($P = 0.007$, $P = 0.004$, $P = 0.04$ and $P = 0.007$, respectively; one tailed *t* test, **Fig. 2a**,**b**). These data indicate that pleiotrophin promotes the expansion of HSCs in culture. Poisson statistical analysis ($n = 75$ mice) showed that the 12-week CRU frequency within day 0 bone marrow CD34$^-$KSL cells was 1 in 39 cells (95% confidence interval: 1 in 21 to 1 in 70, **Fig. 2c** and **Supplementary Table 2**). The CRU frequency within the progeny of CD34$^-$KSL cells after culture with TSF was reduced to 1 in 58 cells (95% confidence interval: 1 in 31 to 1 in 108). Conversely, the CRU frequency within the progeny of CD34$^-$KSL cells cultured with TSF and pleiotrophin was 1 in 10 cells (95% confidence interval: 1 in 5 to 1 in 20). Therefore, the addition of pleiotrophin induced a fourfold increase in HSC frequency compared to input and a sixfold increase compared to the progeny of cells treated with TSF alone. Mice transplanted with CD34$^-$KSL cells treated with TSF and pleiotrophin also showed increased numbers of CD45.1$^+$ cells in the peripheral blood at all time points through 24 weeks compared to mice transplanted with day 0 CD34$^-$KSL cells or their progeny after culture with TSF alone (**Fig. 2d**). This correlated with an increased CRU frequency in the pleiotrophin-treated CD34$^-$KSL cells compared to day 0 CD34$^-$KSL cells at all time points. At 4 weeks, the short-term CRU frequency was 6.4-fold higher in the progeny of CD34$^-$KSL cells cultured with TSF and pleiotrophin compared to input CD34$^-$KSL cells (1 in 5 cells (95% confidence interval: 1 in 2 to 1 in 10) versus 1 in 32 cells (95% confidence interval: 1 in 18 to 1 in 57)). At 24 weeks, the CRU frequency was fourfold increased in the pleiotrophin-treated progeny compared to day 0 CD34$^-$KSL cells (1 in 13 (95% confidence interval: 1 in 6 to 1 in 30) versus 1 in 52 (95% confidence interval:1 in 25 to 1 in 106)).

To confirm that pleiotrophin caused an amplification of long-term-HSCs (LT-HSCs) with serial repopulating capacity, we performed secondary transplants. CD45.2$^+$ mice transplanted with bone marrow collected at 24 weeks from the primary recipients of pleiotrophin plus TSF–treated CD34$^-$KSL cells showed more than a tenfold higher CD45.1$^+$ cell engraftment at 12 weeks after transplantation compared to mice transplanted with bone marrow from primary mice in the day 0 CD34$^-$KSL cell group or in the TSF alone group ($P = 0.003$ and $P = 0.02$, respectively; **Fig. 2e**). Moreover, mice transplanted with bone marrow from primary mice that had been transplanted with pleiotrophin plus TSF–treated CD34$^-$KSL cells showed normal multilineage differentiation at 12 weeks (**Fig. 2e**,**f**). These data indicate that treatment with pleiotrophin induces a substantial expansion of long-term repopulating HSCs in culture, and that this amplification does not alter their multilineage differentiation potential.

Given that altered homing capacity of HSCs can affect HSC frequency estimates in competitive repopulation assays, we compared the homing efficiency of mouse bone marrow progenitor cells with the progeny of bone marrow progenitor cells cultured with TSF or TSF plus pleiotrophin. We irradiated C57BL/6 mice with 950 cGy total body irradiation (TBI) and transplanted them with CD45.1$^+$ Sca-1$^+$Lin$^-$ bone marrow cells or their progeny after culture with TSF or TSF plus pleiotrophin, as previously described[25]. At 24 h after transplantation, we observed no significant differences in the degree of donor CD45.1$^+$ cell engraftment between mice transplanted with day 0 bone marrow cells or with the progeny of TSF-alone or TSF plus pleiotrophin cultures (**Fig. 2g**). These results indicate that augmented homing capacity does not contribute to the increased HSC frequency estimate in pleiotrophin plus TSF–treated cultures compared to day 0 bone marrow cells or TSF alone-treated progeny.

Because pleiotrophin treatment increased the numbers of both short-term and long-term HSCs in culture, we sought to determine whether transplantation of pleiotrophin-treated bone marrow HSCs could accelerate neutrophil or platelet recovery in lethally irradiated mice. We irradiated C57BL/6 mice with 950 cGy and then transplanted them with a limiting dose (100 cells) of bone marrow CD34$^-$KSL cells or their progeny after culture with TSF or TSF plus pleiotrophin. All mice (13 of 13) transplanted with day 0 bone marrow CD34$^-$KSL cells had severe, persistent neutropenia and thrombocytopenia and died by day 18 (**Supplementary Fig. 1**). Conversely, mice transplanted with either the progeny of bone marrow CD34$^-$KSL cells treated with TSF alone or TSF plus pleiotrophin survived through day 25 (**Supplementary Fig. 1**). Notably, mice transplanted with the progeny of TSF plus pleiotrophin cultures showed higher neutrophil counts at days 21 and 25 and higher platelet counts at day 21 compared to mice transplanted with the progeny of TSF-alone cultures (**Supplementary Fig. 1**). These data suggest that treatment with pleiotrophin produces a graft capable of accelerating neutrophil and platelet recovery compared to input CD34$^-$KSL cells or treatment with TSF alone.

Because we identified pleiotrophin from a gene expression analysis of HUBECs, we also tested whether addition of a neutralizing pleiotrophin-specific antibody to HUBEC cultures could block HUBEC-mediated expansion of LT-HSCs[11,20,21]. Competitive repopulating assays confirmed that the progeny of noncontact cocultures of CD34-KSL cells with HUBECs contained more than a threefold higher number of LT-HSCs compared to day 0 bone marrow CD34$^-$KSL cells, and the addition of the pleiotrophin-specific antibody completely abolished this expansion (**Supplementary Fig. 2**).

Treatment with pleiotrophin induces human HSC expansion

In order to determine if pleiotrophin is a growth factor for human HSCs, we sorted human cord blood CD34$^+$CD38$^-$Lin$^-$ cells, which are enriched for HSCs[20], and placed them in culture with TSF alone or TSF plus 100 or 500 ng ml^{-1} pleiotrophin for 7 d. The increase in total cell numbers was not significantly different between the TSF alone and TSF plus pleiotrophin groups, but the frequency of CD34$^+$CD38$^-$Lin$^-$ cells was significantly higher in cultures treated with TSF plus pleiotrophin versus TSF alone (**Fig. 3a**). Similarly, treatment of cord blood CD34$^+$CD38$^-$Lin$^-$ cells with TSF plus pleiotrophin for 7 d resulted in a fourfold increase in total colony-forming cell (CFC) content compared to TSF alone (**Fig. 3b**). Taken together, these data suggest that treatment with pleiotrophin induces the expansion of cord blood progenitor cells in culture.

To determine whether pleiotrophin treatment causes an increase in human HSCs in culture, we transplanted nonobese diabetic (NOD)-SCID mice with limiting doses of human cord blood CD34$^+$CD38$^-$Lin$^-$ cells or their progeny after culture with TSF or TSF plus pleiotrophin. First, we observed that mice transplanted with the progeny of cord blood CD34$^+$CD38$^-$Lin$^-$ cells cultured with TSF plus pleiotrophin had threefold higher human CD45$^+$ cell engraftment at 4 weeks in the peripheral blood compared to mice transplanted with day 0 CD34$^+$CD38$^-$Lin$^-$ cells or with the progeny of cells cultured with TSF alone (**Fig. 3c**). These results indicate that treatment of human cord blood HSCs with pleiotrophin produces a graft capable of accelerated engraftment in transplanted mice. At 8 weeks after transplant using a dose of 500 cells, only one of nine mice (11%) transplanted with day 0 CD34$^+$CD38$^-$lin$^-$ cells showed human CD45$^+$ cell engraftment ($\geq$0.1% human CD45$^+$ cells) in the bone

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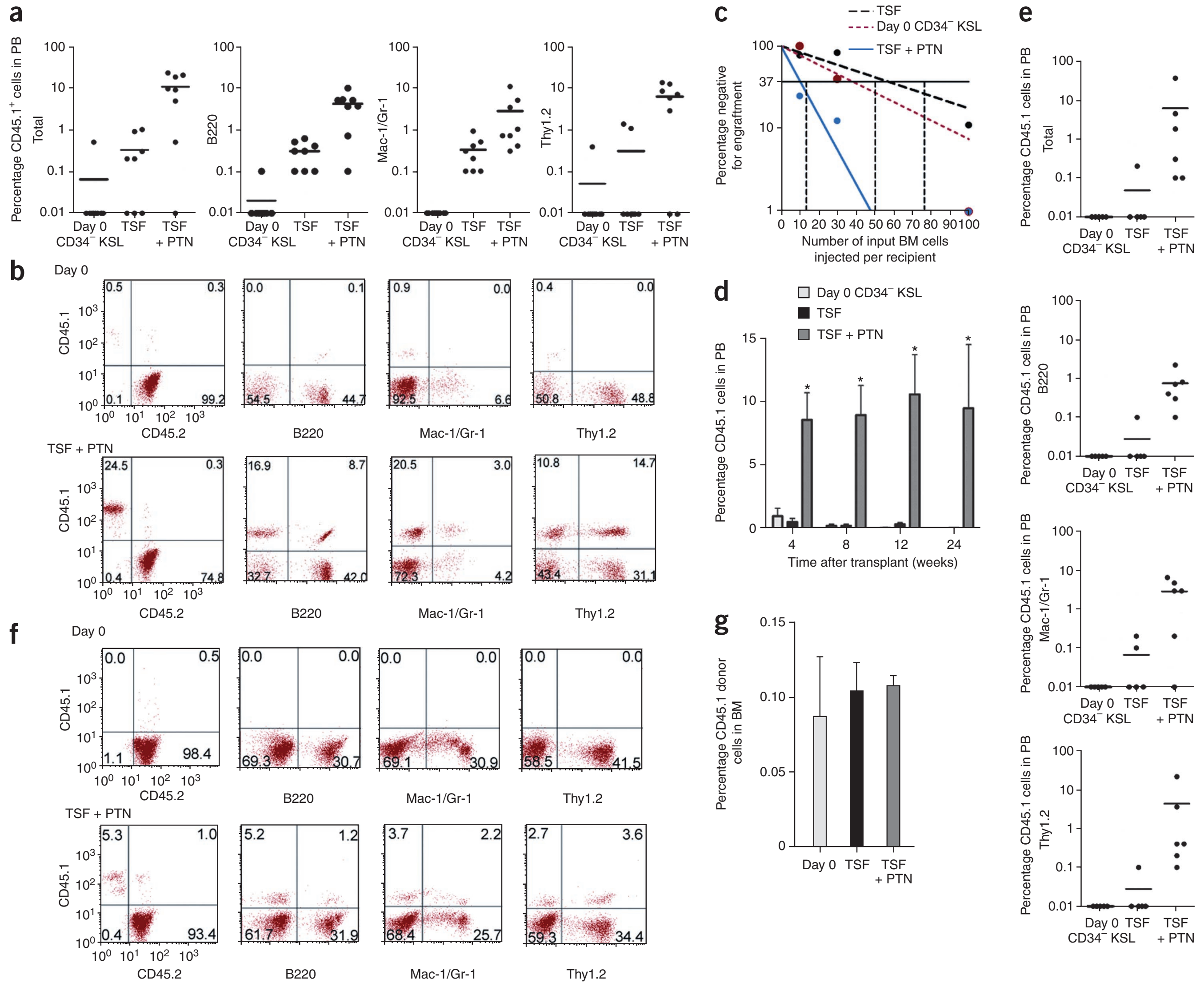

Figure 2 Treatment with pleiotrophin induces the expansion of mouse short- and long-term HSCs. (a) Scatter plots showing the percentages of total CD45.1+ donor cells and donor-derived B220+ (B lymphoid), Mac-1+/Gr-1+ (myeloid) and Thy1.2+ (T cell) populations in the peripheral blood (PB) of mice transplanted with 10 bone marrow CD34−KSL cells or their progeny after culture (n = 8–10 mice per group; means ± s.d). Horizontal lines represent the mean engraftment levels for each group. PB, peripheral blood. (b) Representative flow cytometric analysis of peripheral blood donor-derived (CD45.1+) multilineage engraftment at 12 weeks after transplantation in mice transplanted with ten bone marrow CD34−KSL cells versus mice transplanted with the progeny of ten bone marrow CD34−KSL cells after culture with TSF and 100 ng ml^{-1} pleiotrophin. The percentage of cells in each quadrant is indicated (representative of analysis of eight to ten mice per group). (c) Poisson statistical analysis after limiting-dilution analysis; plots were obtained to allow estimation of CRU content within each condition (n = 8–10 mice transplanted at each dose per condition; n = 75 mice total). The plot shows the percentage of recipient mice containing less than 1% CD45.1+ cells in the peripheral blood at 12 weeks after transplantation versus the number of cells injected per mouse. BM, bone marrow. The horizontal line indicates the point at which 37% of the transplanted mice are nonengrafted in each group; the CRU frequency is estimated at the point where 37% of the mice are nonengrafted, by conventional Poisson statistical methods and limiting-dilution analysis; the vertical dashed lines are meant to highlight the various CRU frequencies in each condition. (d) The number of CD45.1+ donor-derived cells in the peripheral blood of mice transplanted with day 0 CD34−KSL cells or with pleiotrophin plus TSF-treated or TSF-alone–treated CD34−KSL cells at 4, 8, 12 and 24 weeks after transplantation (means ± s.e.m., n = 6–10 per group, *P = 0.006, *P = 0.002, *P = 0.006, P = 0.05; ^P = 0.005, ^P = 0.002, ^P = 0.007, P = 0.05). (e) Donor-derived CD45.1+ cell engraftment 12 weeks after transplantation in secondary recipient mice transplanted with bone marrow isolated from primary mice transplanted with day 0 CD34− KSL cells or with TSF plus pleiotrophin or TSF-alone treated CD34−KSL cells (means ± s.e.m., n = 5 or 6 per group, P = 0.003 and 0.02 for TSF plus pleiotrophin group versus day 0 CD34−KSL group and TSF alone group, respectively; Mann-Whitney test). Horizontal bars represent mean levels of CD45.1+ cell engraftment in the peripheral blood. (f) Representative FACS analysis of CD45.1+ cell engraftment and B220+, Mac-1+/Gr-1+ and Thy 1.2+ engraftment at 12 weeks after transplantation in secondary recipient mice transplanted with bone marrow from primary mice transplanted with day 0 CD34−KSL cells or with TSF plus pleiotrophin treated CD34−KSL cells (representative of n = 5 or 6 mice per group). (g) The mean levels of donor CD45.1+ cells in the bone marrow (BM) of CD45.2+ recipient mice at 24 h after transplantation of CD45.1+ bone marrow Sca-1+Lin− cells (4 × 10^4) or their progeny after 7 d of culture with TSF or TSF plus pleiotrophin (n = 3–5 per group, means ± s.d.).

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marrow at 8 weeks, whereas 6 of 13 mice (46%) transplanted with the progeny of CD34+CD38−Lin− cells cultured with TSF showed human CD45+ cell engraftment (**Fig. 3d**). Conversely, 13 of 13 mice (100%) transplanted with the progeny of CD34+CD38−Lin− cells cultured with TSF plus pleiotrophin showed human hematopoietic cell engraftment at 8 weeks after transplant (**Fig. 3d**). At 8 weeks after transplant of a dose of 2,500 cells, four of seven mice (57%) transplanted with day 0 CD34+CD38−Lin− cells and 8 of 11 mice (72%) transplanted with the progeny of TSF-alone cultures showed human CD45+ cell engraftment. Conversely, 12 of 12 mice (100%) transplanted with the progeny of TSF plus pleiotrophin cultures showed human CD45+ cell engraftment (**Fig. 3d**). Mice transplanted with the progeny of TSF plus pleiotrophin cultures also showed normal multilineage differentiation *in vivo* (**Fig. 3e**), confirming that treatment with pleiotrophin does not alter the normal differentiation program of human HSCs.

PI3k and Notch pathways mediate the response to pleiotrophin

To determine a potential mechanism through which pleiotrophin mediates HSC expansion, we examined whether pleiotrophin treatment alters signaling pathways known to be affected by RPTP-β/ζ[14–16,26]. Canonical pleiotrophin signaling occurs via binding and inactivation of RPTP-β/ζ[14], which facilitates the tyrosine phosphorylation of several intracellular substrates, including Akt and β-catenin[27,28]. As pleiotrophin has been shown to mediate mitogenic effects outside the hematopoietic system via activation of the PI3K-Akt pathway[27], we tested whether this pathway is involved in pleiotrophin-induced HSC amplification. We treated mouse bone marrow CD34−KSL cells with TSF with or without 100 ng ml^{-1} pleiotrophin in the presence or absence of 10 μM LY294002, a PI3K inhibitor[26,27]. The addition of LY294002 to TSF plus pleiotrophin caused an 88% decrease in total cell expansion and a 92% decrease in KSL cell expansion compared to cultures treated TSF plus pleiotrophin without

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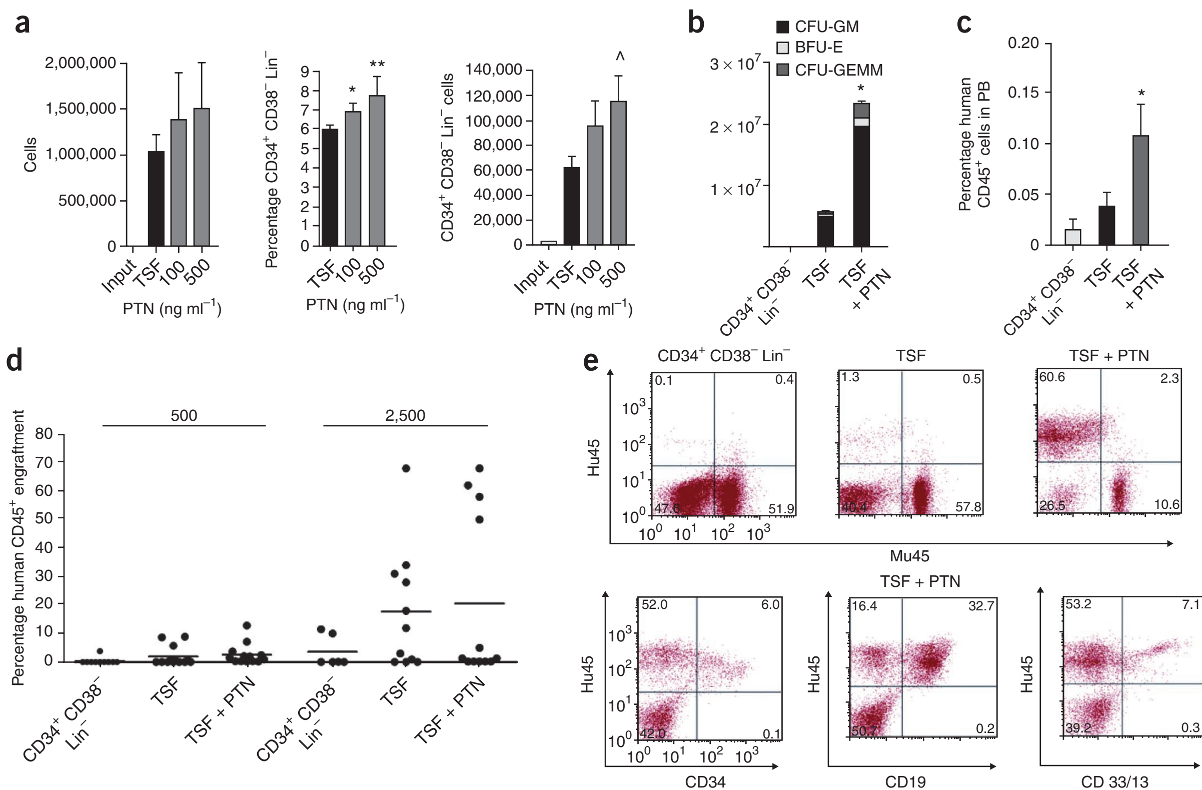

Figure 3 Treatment with pleiotrophin induces the expansion of human HSCs. (**a**) Total number of cells, percentage of CD34+CD38−Lin− cells and number of CD34+CD38−Lin− cells after culture of highly purified human cord blood CD34+CD38−Lin− cells (98.6% ± 0.4%; input, day 0, 3×10^3 cells per culture) with pleiotrophin (either 100 or 500 ng^{-1} ml) plus TSF or TSF alone (means ± s.d., $n = 3$ per group, *$P = 0.02$ versus TSF, **$P = 0.04$ versus TSF, ^$P = 0.04$ versus TSF). (**b**) The number of CFCs obtained from 1×10^3 day 0 cord blood CD34+CD38−Lin− cells or after culturing of these cells with pleiotrophin plus TSF or TSF alone (means ± s.d., $n = 3$ per group). *$P = 0.0004$ and 0.01 versus day 0 and TSF group, respectively. CFU-GM, colony-forming unit–granulocyte-monocyte; BFU-E, burst-forming unit–erythroid; CFU-GEMM, colony-forming unit–granulocyte, erythrocyte, monocyte and megakaryocyte. (**c**) Mean levels of human CD45+ cell engraftment in the peripheral blood of NOD-SCID mice at 4 weeks in mice transplanted with cord blood CD34+CD38−Lin− cells (1,000 cells) or their progeny after 7 d of culture with TSF or TSF plus pleiotrophin (means ± s.d., $n = 10–13$ per group; *$P = 0.04$ and 0.05 versus day 0 CD34+CD38−Lin− and TSF groups, respectively). (**d**) A scatter plot showing the levels of human CD45+ cell engraftment in the bone marrow of NOD-SCID mice 8 weeks after transplantation with 500 or 2,500 cord blood CD34+CD38−Lin− cells or their progeny after culture with TSF or TSF plus pleiotrophin ($n = 7–13$ mice per group). Horizontal bars represent the mean levels of human CD45+ cell engraftment in the bone marrow in each group. 500 cell dose: CD34+CD38−Lin− group = 0.3% ± 0.3% human CD45+ cells, TSF = 1.9% ± 0.9% human CD45+ cells, TSF plus pleiotrophin = 2.6% ± 1.0 human CD45+ cells; 2,500 cell dose: CD34+CD38−Lin− group = 3.1% ± 1.9% human CD45+ cells, TSF = 17% ± 6.4% human CD45+ cells, TSF plus pleiotrophin = 21% ± 8.0% human CD45+ cells. (**e**) FACS plots of representative human CD45+ hematopoietic cell engraftment (top) and multilineage differentiation (bottom) in NOD-SCID mice at 8 weeks after transplantation with 2,500 cord blood (CB) CD34+CD38−Lin− cells or their progeny after culture with TSF or TSF plus pleiotrophin (representative of 7–13 mice per group).

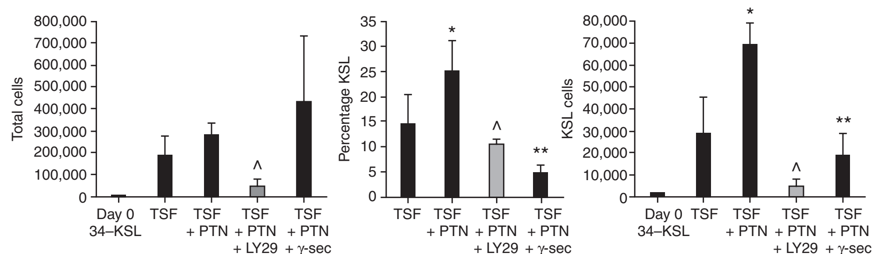

Figure 4 Pleiotrophin mediates bone marrow progenitor cell expansion via activation of PI3K and Notch signaling. Depicted are the total number of cells, percentage of KSL cells and number of KSL cells in untreated bone marrow CD34⁻KSL cells (5×10^2) or after treatment of these cells with TSF plus pleiotrophin or TSF alone. TSF plus pleiotrophin treatment was with or without addition of LY294002 (LY29), a PI3K inhibitor, or 30 μM γ-secretase inhibitor (γ-sec), a Notch signaling inhibitor. Data are means ± s.d., $n = 3$ per group. * $P = 0.04$ and 0.02 for comparison of TSF plus pleiotrophin versus TSF alone; ^$P = 0.002$, 0.03 and 0.002 for comparison of TSF plus pleiotrophin and LY29 versus TSF plus pleiotrophin; ** $P = 0.01$ and $P = 0.002$ for TSF plus pleiotrophin plus γ-secretase inhibitor versus TSF plus pleiotrophin.

LY294002 ($P = 0.002$ and $P = 0.002$, respectively; **Fig. 4**). These data suggest that PI3K signaling contributes to pleiotrophin-induced bone marrow stem/progenitor cell expansion. Because treatment with pleiotrophin induced a 2.4-fold increase ($P = 0.04$, data not shown) in the expression of hairy and enhancer of split-1 (HES-1), a mediator of Notch signaling[29], we also examined the effect of pleiotrophin treatment of bone marrow CD34-KSL cells with and without a γ-secretase inhibitor, which inhibits Notch signaling[30]. Treatment of bone marrow CD34⁻KSL bone marrow CD34⁻KSL cells with a γ-secretase inhibitor reduced the expansion of KSL cells by 72% in response to pleiotrophin, suggesting that pleiotrophin mediates effects on bone marrow stem and progenitor cell expansion via Notch signaling (**Fig. 4**). Given that HES-1 has been shown to induce PI3K-Akt signaling in leukemogenesis[31], it is plausible that treatment of CD34⁻KSL cells with pleiotrophin induced HES-1 expression, which induced PI3K-Akt signaling in HSCs (**Fig. 4**). Moreover, pleiotrophin treatment of bone marrow CD34⁻KSL cells downmodulated PTEN expression, a negative regulator of PI3K-Akt signaling[32] (data not shown). Of note, bone marrow CD34⁻KSL cells treated with pleiotrophin showed no increase in the activated (nonphosphorylated) form of β-catenin (data not shown), which is a downstream target of RPTP-β/ζ and a positive regulator of HSC self-renewal[7]. Furthermore, we observed no difference in the ability of pleiotrophin to amplify KSL cells in cultures of bone marrow KSL cells isolated from mice bearing a deletion of β-catenin compared to cells isolated from wild-type littermate control mice (**Supplementary Fig. 3**). Taken together, these data suggest that activation of the PI3K and Notch signaling pathways contributes to pleiotrophin-induced HSC expansion.

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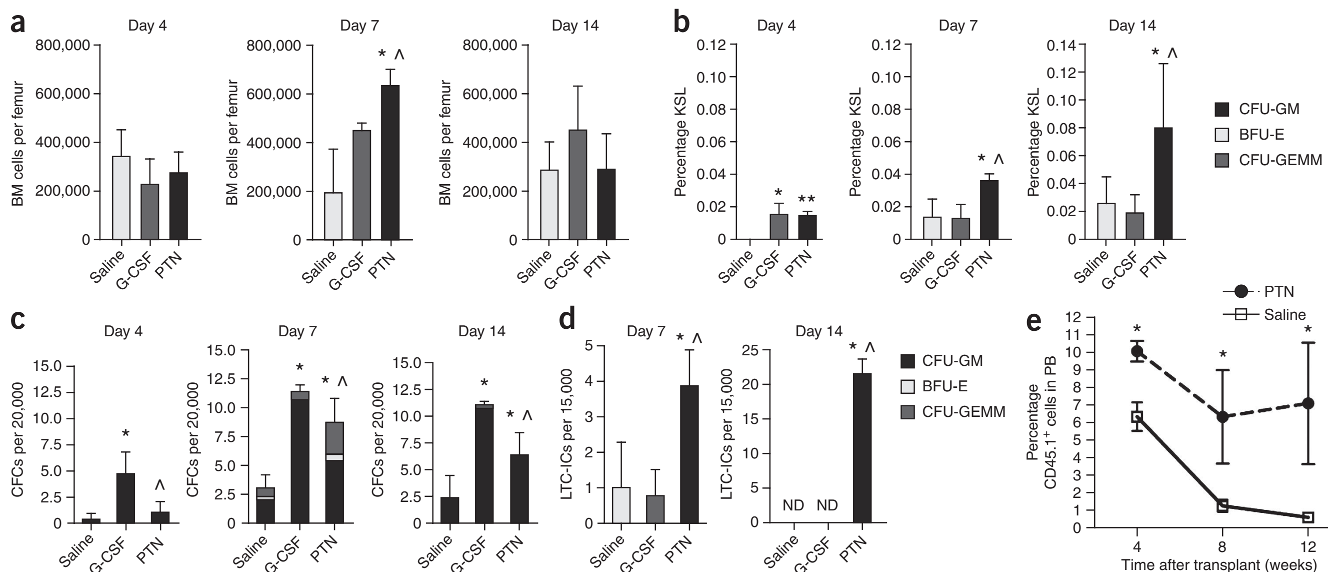

Figure 5 Pleiotrophin induces bone marrow stem and progenitor cell regeneration *in vivo*. Bone marrow stem and progenitor cell content and function are shown for mice irradiated with 700 cGy total body irradiation and subsequently treated intraperitoneally for 7 d with pleiotrophin, G-CSF or saline (days 4, 7 and 14 indicate the time after irradiation). (**a**) Total number of bone marrow cells (means ± s.d., $n = 10$, *$P = 0.02$ versus saline, ^$P = 0.04$ versus G-CSF). (**b**) Percentage KSL cells (means ± s.d., $n = 5$ per group; day 4, *$P = 0.04$ and **$P = 0.003$ versus saline; day 7, *$P = 0.03$ and ^$P = 0.004$ versus saline- and G-CSF groups, respectively; day 14, *$P = 0.04$ and ^$P = 0.02$ versus saline- and G-CSF-treated groups, respectively). (**c**) Bone marrow CFCs ($n = 3$ per group, means ± s.d.; *$P = 0.03$, 0.004 and 0.006 for comparison between G-CSF and saline groups at days 4, 7 and 14, respectively; *$P = 0.004$ and 0.04 for comparison between pleiotrophin and saline groups at days 7 and 14, respectively; ^$P = 0.04$, 0.04 and 0.02 for comparison between pleiotrophin and G-CSF groups at days 4, 7 and 14, respectively. (**d**) Bone marrow LTC-ICs ($n = 4$–6 per group, means ± s.d.; *$P = 0.02$ and $P = 0.02$ versus saline group at days 7 and 14, respectively; ^$P = 0.03$ and $P = 0.02$ versus G-CSF group at days 7 and 14, respectively). (**e**) Competitive repopulation assays, performed with 5×10^5 CD45.1⁺ bone marrow cells collected at day 7 from mice that had been irradiated with 700 cGy and subsequently treated with pleiotrophin or saline for 7 days. Bone marrow was collected at weeks 4, 8 and 12 after transplantation, and donor CD45.1⁺ cell engraftment was measured in the peripheral blood of recipient CD45.2⁺ mice ($n = 9$ per group, means ± s.d. *$P = 0.001$, 0.04 and 0.04 at weeks 4, 8 and 12, respectively).

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Pleiotrophin induces bone marrow HSC regeneration *in vivo*

We next tested whether pleiotrophin administration could augment bone marrow HSC regeneration *in vivo* after myelosuppression. For these experiments, we irradiated mice with 700 cGy TBI, which causes a 96% decrease in bone marrow HSC content[23], and then treated the mice with 2 μg pleiotrophin, 2 μg granulocyte colony–stimulating factor (G-CSF) or saline intraperitoneally daily for 7 d. Pleiotrophin administration caused a significant increase in the number of total bone marrow cells at day 7 compared to G-CSF- or saline-treated mice (**Fig. 5a**; $P = 0.02$ and $P = 0.04$, respectively). Pleiotrophin treatment also caused a threefold increase in the number of bone marrow KSL cells at day 7 and a fourfold and 4.3-fold increase in the number of bone marrow KSL cells at day 14 compared to mice treated with saline or G-CSF, respectively (**Fig. 5b**). Both G-CSF- and pleiotrophin-treated mice showed significantly higher bone marrow CFC content after irradiation than mice treated with saline, and G-CSF-treated mice had the highest bone marrow CFC content overall over time (**Fig. 5c**). Notably, mice treated with pleiotrophin had an increase in the number of bone marrow long-term culture–initiating cells (LTC-ICs) by fourfold and >20-fold at days 7 and 14 after irradiation, respectively, compared to saline-treated and G-CSF-treated mice (**Fig. 5d**). Taken together, these results indicate that systemic treatment with pleiotrophin causes the selective regeneration of phenotypic and functional bone marrow stem/progenitor cells *in vivo* after myelosuppressive injury. As confirmation of the effect of pleiotrophin administration on bone marrow HSC regeneration *in vivo*, we also measured bone marrow HSC content via competitive repopulation assays using 5×10^5 bone marrow cells from irradiated, pleiotrophin-treated mice versus irradiated, saline-treated mice. Mice transplanted with bone marrow cells from pleiotrophin-treated mice showed significantly higher multilineage engraftment of donor $CD45.1^+$ cells at 4, 8 and 12 weeks after transplant compared to mice transplanted with saline-treated bone marrow cells (**Fig. 5e** and **Supplementary Fig. 4**). These results confirm that systemic treatment with pleiotrophin induces the regeneration of short- and long-term HSCs *in vivo* after irradiation.

Of note, systemic administration of pleiotrophin to mice irradiated with lower-dose TBI (300 cGy) showed no consistent differences in the numbers of total bone marrow cells, bone marrow KSL cells or CFCs as compared to G-CSF-treated mice (**Supplementary Fig. 5**). However, nonirradiated mice treated with pleiotrophin for 7 d showed a significant expansion in the number of bone marrow KSL cells ($P = 0.009$) compared to saline-treated controls (**Supplementary Fig. 5**). These data suggest that systemic pleiotrophin administration can induce bone marrow stem/progenitor cell expansion in normal mice, an idea that will be tested in future studies.

DISCUSSION

Our results demonstrate that pleiotrophin is a secreted growth factor for HSCs, and the addition of pleiotrophin is sufficient to promote a substantial expansion of mouse LT-HSCs in culture, as shown using primary and secondary competitive repopulation assays. Furthermore, we show that treatment of human cord blood HSCs with pleiotrophin plus cytokines increases the short-term and long-term SCID-repopulating capacity of these cells compared to treatment with the cytokines alone. Pleiotrophin, therefore, has potential therapeutic application for an area of unmet clinical need: the production of cord blood grafts capable of accelerated engraftment in adult transplant candidates who lack a histocompatible adult donor. In addition, we show that systemic administration of pleiotrophin causes a substantial increase in the regeneration of both short- and long-term repopulating bone marrow HSCs *in vivo* after total body irradiation. Therefore, pleiotrophin regulates not only mouse and human HSC expansion *in vitro* but also HSC regeneration *in vivo*, a process that is largely uncharacterized.

Because bone marrow HSCs express RPTP-β/ζ and our *in vitro* studies showed a direct effect of pleiotrophin on HSCs, we propose that pleiotrophin acts directly on bone marrow HSCs to induce bone marrow HSC regeneration *in vivo*. However, it will be crucial to examine the effects of pleiotrophin administration on the bone marrow microenvironment. Pleiotrophin has been shown to have angiogenic activity[17,18] and we and others have shown that bone marrow vascular endothelial cells can regulate hematopoietic reconstitution after injury[21,23,33]. Therefore, it is plausible that pleiotrophin might contribute indirectly to bone marrow HSC regeneration *in vivo* by promoting recovery of the bone marrow vascular niche. Given that little is known about the extrinsic or microenvironmental signals that regulate bone marrow HSC regeneration *in vivo*[34], the demonstration that pleiotrophin induces bone marrow HSC regeneration *in vivo* provides a basis to begin to understand this process. Furthermore, as a soluble growth factor capable of inducing bone marrow HSC regeneration *in vivo*, pleiotrophin has unique translational potential compared to previously described pathways shown to regulate HSC expansion *in vitro*[6,10,12].

As a possible mechanism of action, we showed that inhibition of PI3K signaling blocks pleiotrophin-induced expansion of bone marrow KSL cells in culture. Pleiotrophin also induced the expression of HES-1, a mediator of Notch signaling and a positive regulator of PI3K signaling[29,31], suggesting the possibility that pleiotrophin promotes HSC amplification via activation of Notch signaling. Consistent with this hypothesis, we found that inhibition of Notch signaling with a γ-secretase inhibitor also blocked pleiotrophin-induced bone marrow KSL cell expansion. In future studies, we will test the role of HES-1 and Notch signaling in mediating pleiotrophin effects on HSC expansion using genetic strategies in mice. It has recently been reported that deletion of the gene encoding PTEN, a negative regulator of PI3K-Akt signaling, was associated with exhaustion of 12-week CRU in mice[35]; in addition, deletion of Foxo3a, a transcription factor that negatively regulates HSC cycling and is inhibited by Akt, has been associated with depletion of LT-HSCs in mice[36]. Therefore, it will be useful to determine whether pleiotrophin-mediated expansion of HSCs is dependent upon PI3K-Akt signaling or whether pleiotrophin-mediated HSC expansion is independently caused by activation of alternative HSC regulatory pathways (for example, Notch signaling).

Much progress has been made in understanding the intrinsic and extrinsic pathways that regulate HSC self-renewal and differentiation[1–3,13,37]. However, the successful development of soluble growth factors or cytokines capable of inducing human HSC expansion *ex vivo* or HSC regeneration *in vivo* has remained an elusive goal[13,37]. Here we show that the soluble growth factor pleiotrophin acts on both mouse and human HSCs and can induce LT-HSC expansion *ex vivo* and HSC regeneration *in vivo*. Pleiotrophin therefore has potential clinical utility for expanding human HSCs *ex vivo* and for accelerating hematopoietic recovery *in vivo* in patients after myelotoxic chemo- or radiotherapy.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Accession codes. Microarray data have been deposited in the Gene Expression Omnibus Database with accession code GSE20243.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS
H.A.H. designed and performed experiments, analyzed data and wrote the paper; G.G.M., P.D., S.K.M., J.L.R., P.D., A.B.S. and W.E.L. performed experiments; J.-T.C. guided the microarray analysis; T.R. and N.J.C. analyzed data and wrote the paper; J.P.C. designed the experiments, analyzed the data and wrote the paper.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Endothelial cell cultures and microarray analysis. We cultured individual primary human endothelial cells derived from uterine, umbilical, iliac, dermal, coronary and pulmonary arteries (Lonza) according to the manufacturer's guidelines. We generated primary HUBECs and cultured them in complete endothelial cell culture medium (M199, Invitrogen; endothelial growth supplement, Sigma; FBS, Hyclone; heparin, Sigma; L-glutamine, Invitrogen), as previously described[11,20]. We cultured the cells from cadaveric explants of human vessel segments from the anterior and posterior cerebral arteries; human donors consented for cadaveric studies, which were approved by the Duke University Institutional Review Board. We amplified RNA from $n = 6$ sources of HUBECs and $n = 8$ sources of nonbrain endothelial cells to a human oligonucleotide spotted microarray (Operon). We analyzed the microarray data with an unsupervised hierarchical cluster analysis[38], and we screened the gene list for annotated soluble proteins. We processed and hybridized samples to Operon Human Arrays (Operon) as previously described[38].

Isolation of bone marrow hematopoietic stem cells and *in vitro* cultures. We performed all studies with mice under a protocol approved by the Duke University Animal Care and Use Committee. We isolated purified bone marrow CD34$^-$KSL cells from C57BL/6 and B6.SJL mice (Jackson Laboratory) by flow cytometric cell sorting as previously described[6,23]. We supplemented liquid suspension cultures of bone marrow CD34$^-$KSL cells with Iscove's modified Dulbecco's medium plus 10% FBS, 1% penicillin-streptomycin, 20 ng ml^{-1} thrombopoietin, 125 ng ml^{-1} stem cell factor and 50 ng ml^{-1} Flt-3 ligand (TSF) with or without recombinant (human) pleiotrophin (R&D Systems). We set up noncontact HUBEC cultures using 0.4-μm transwell inserts (Corning) and supplemented them with TSF medium with or without goat antibody to pleiotrophin (AF-252-PB, R&D Systems) or isotype control antibody (AB-108-C, R&D Systems). We analyzed for surface marker expression of c-Kit, Sca-1 and lineage markers as previously described[21,23].

Mouse competitive repopulating unit assays. We isolated bone marrow CD34$^-$KSL cells from CD45.1$^+$ Bl6.SJL mice for injection into recipient mice and injected them without culturing or cultured them with TSF alone, TSF plus pleiotrophin, TSF plus HUBECs plus goat IgG, or TSF plus HUBECs plus goat pleiotrophin-specific antibody. C57BL/6 mice (CD45.2$^+$) recipient mice were irradiated recipient with 950 cGy TBI and were injected via the tail vein with limiting doses of bone marrow CD34$^-$KSL cells or their progeny after culture. We used 1×10^5 host bone marrow mononuclear cells from C57BL/6 mice as competitor cells. We measured multilineage hematologic reconstitution in the peripheral blood by flow cytometry over time after transplantation as previously described[6,23]. We considered the mice to be engrafted if the donor CD45.1$^+$ cells were present at ≥1% in the peripheral blood[11,20,39]. We made CRU estimates with L-Calc software (Stem Cell Technologies) as previously described[6,21,39].

We performed secondary competitive transplant assays with whole bone marrow collected from primary CD45.2$^+$ mice at 24 weeks after transplantation with either CD45.1$^+$ bone marrow CD34$^-$KSL cells or the progeny of CD34$^-$KSL cells after culture with TSF alone or TSF plus pleiotrophin. By volume, 75% of the primary bone marrow cells were transplanted into recipient mice along with 1×10^5 host competitor bone marrow cells from the C57BL/6 mice. We irradiated secondary recipient CD45.2$^+$ C57BL/6 mice with 950 cGy TBI and analyzed peripheral blood of donor cell engraftments at 12 weeks after transplantation in secondary mice.

Human cord blood hematopoietic cell cultures and nonobese diabetic–severe combined immunodeficient transplantation assays. We isolated human CD34$^+$CD38$^-$Lin$^-$ cells from cord blood units obtained from the Carolinas Cord Blood Bank. We cultured 5×10^3 cord blood CD34$^+$CD38$^-$Lin$^-$ cells with TSF in Iscove's modified Dulbecco's medium containing 10% FBS and 1% penicillin-streptomycin. We added recombinant pleiotrophin at 100–500 ng ml^{-1}. We performed phenotypic analyses and 14-d CFC progenitor cell counts (number of colonies per 1×10^3 cells plated × fold expansion) as previously described[11]. We transplanted limiting doses (0.5×10^3–2.5×10^3) of day 0 cord blood CD34$^+$CD38$^-$Lin$^-$ cells or their progeny after 7 d of culture with TSF alone or TSF plus 500 ng ml^{-1} pleiotrophin into NOD-SCID mice conditioned with 300 cGy TBI. We considered the mice as positively engrafted if we detected ≥ 0.1% human CD45$^+$ cells in the bone marrow at 8 weeks after transplant, as previously described[40]. We measured multilineage human cell repopulation at 8 weeks, as previously described[11,20].

Systemic administration of pleiotrophin. We gave adult B6.SJL mice a single fraction of 700 cGy TBI and then treated them with either with PBS (saline), 2 μg GCSF or 2 μg pleiotrophin intraperitoneally daily for 7 d (beginning 4 h after irradiation). At days 4, 7 and 14 after irradiation, we killed the mice and quantified total viable bone marrow cells. We performed flow cytometric analysis to estimate the percentage of bone marrow KSL cells in each femur[21,23]. We performed CFC assays with MethoCult M3434 medium (Stem Cell Technologies) as previously described[21,23]. We performed LTC-IC assays as follows: we plated mouse M2-10B4 (American Type Culture Collection CRL-1972) bone marrow stromal cells in a 24-well dish and irradiated them with 1,500 cGy. We prepared limiting dilutions (45,000, 90,000 and 180,000) of bone marrow mononuclear cells from irradiated mice that had been treated with either pleiotrophin or PBS, added these cells to the stromal cell layers and maintained the layers in MyeloCult M5300 medium (Stem Cell Technologies) with weekly half-medium changes for 4 weeks. We then collected the nonadherent and adherent cells (15,000 cells per dish) and plated them into 3 × 35 mm dishes (MethoCult, StemCell Technologies). After 2 weeks, we counted and scored hematopoietic colonies. We also performed CRU assays as previously described[23] with a limiting dose of bone marrow cells from mice that had been irradiated with 700 cGy TBI and then treated with saline or pleiotrophin for 7 d to compare bone marrow HSC content within these mice.

Statistical analyses. Data are expressed as the means ± s.d. or s.e.m. We analyzed simple pair-wise comparisons with the Student's t test (one-tailed distribution with unequal variance). For the comparison of donor engraftment in the secondary transplant assays, we used a Mann-Whitney test. For competitive repopulating assays, we performed limiting-dilution assays and calculated CRU frequency with the maximum likelihood estimator for the single-hit Poisson model[11].

Additional methods. Detailed methodology is described in the **Supplementary Methods**.

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