Stromal pleiotrophin regulates repopulation behavior of hematopoietic stem cells

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Abstract

Pleiotrophin (Ptn) is strongly expressed by stromal cells which maintain hematopoietic stem cells (HSC). However, in vivo, Ptn deficiency does not alter steady-state hematopoiesis. On the other hand, knockdown of Ptn (PtnKD) in stromal cells increases production of hematopoietic progenitors as well as HSC activity in cocultures, suggesting that Ptn may have a role in HSC activation. Indeed, transplantations of wild-type (WT, Ptn+/+) HSC into Ptn-/- mice show increased donor cell production in serial transplantations and dominant myeloid regeneration caused by Ptn-dependent regulation of HSC repopulation behavior. This regulation of LSK function is associated with increased proliferation, and, on a molecular level, with upregulated expression of cyclin D1 (Ccnd1) and C/EBPa (Cebpα), but reduced of PPARγ (Pparg). The known HSC regulator β-catenin is, however, not altered in the absence of Ptn. In conclusion, our results point to different Ptn-mediated regulatory mechanisms in normal hemostasis and in hematopoietic regeneration and in maintaining the balance of myeloid and lymphoid regeneration. Moreover, our results support the idea that microenvironmental Ptn regulates hematopoietic regeneration through β-catenin-independent regulation of Ccnd1 and Cebpα.
Introduction

All mature blood cells derive from hematopoietic stem cells (HSC). These HSC have been shown to reside mainly in specialized microenvironments, referred to as niches. It is thought that the niche regulates HSC quiescence (dormancy), self-renewal, and differentiation by expression of surface molecules and secretion of soluble factors. Which signals are provided by the niche and how exactly these signals affect HSC, still remains uncertain.1

We have established a number of stromal cell clones from midgestation embryonic sources, of which we identified two cell lines (EL08-1D2 and UG26-1B6) which maintain fetal as well as adult HSC, even though they had no direct contact with the hematopoietic cells (non-contact co-cultures).2,3 In gene expression studies, we observed that - in comparison to a number of non-supporting stromal cell lines - those two cell lines both expressed larger amounts of mRNA corresponding to a number of secreted molecules. We recently described that one of these factors, secreted frizzled-related protein 1, is required for sustained self-renewal of HSC in vivo, and that this was due to extrinsic regulation of HSC by the microenvironment, most likely, through regulating β-catenin (Ctnnb1) and peroxisome proliferator-activated receptor gamma (Pparg), both mediators of the Wnt signalling pathways.4 One other overrepresented factor was the pleiotrophic cytokine Pleiotrophin (Ptn).3 Pleiotrophin was also found to be overexpressed by other HSC supportive cells, like human brain endothelial cells5,6 or the stromal cell line AFT024,7 suggesting that high expression of Ptn may be a common feature among HSC-supportive stromal cells.

Pleiotrophin, due to its pleiotrophic activities, is known under many alternative names, including heparin-binding growth-associated molecule (HB-GAM) and osteoblast-
stimulating factor (OSF). Ptn is a highly conserved 17 kDa cytokine,\(^8\) which, together with Midkine, forms a small family of low molecular weight factors.\(^9\) Several receptors are known to bind Ptn as a ligand: receptor protein tyrosine phosphatase \(\beta\zeta\) (\(Rptpz1\)),\(^10\) nucleolin,\(^11\) and N-syndecan.\(^12\) It was recently shown that Rptpz1 is expressed on bone marrow-derived lineage-negative Ly6a\(^+\) Kit\(^+\) (LSK) cells.\(^6\) Binding of Ptn to RPTP \(\beta\zeta\) inactivates the phosphatase domain through dimerization of the receptor. This leads to an increasing phosphorylation status of the numerous targets of RPTP \(\beta\zeta\), including \(\beta\)-catenin, ALK, \(\beta\)-adducin, CD81, c-Fyn and others.\(^10,13\) The effects of Ptn on proliferation and differentiation appear to converge in \(\beta\)-catenin- and its downstream factor Dlk1.\(^10,14\) Interestingly, Dlk1 has previously been identified to be overrepresented in the Ptn overexpressing HSC-maintaining cell line AFT024, and to promote cobblestone area formation by HSC-derived progeny.\(^15\) Since it was shown that Rptpz1 is expressed on bone marrow-derived LSK cells,\(^6\) these pathways may well be relevant in HSC regulation.

Ptn is known to play important roles in proliferation and differentiation in various cell types. It was shown that Ptn is mitogenic for fibroblasts, epithelial and endothelial cells.\(^16\) Ptn leads to an increased expansion and differentiation of human osteoprogenitor cells.\(^17\) A similar effect was found in murine osteoprogenitors, but only with low concentrations of Ptn, higher concentrations showed no effect.\(^18\) In human embryonic stem cells, the addition of Ptn induces increased clonal growth, without differentiation.\(^19\) In neural stem cells, however, Ptn lead to an decreased proliferation and an increase of differentiation of these cells.\(^20\) Recently, it was demonstrated that Ptn also affects cellular proliferation of HSC. Although Ptn by itself does not affect proliferation, it enhances proliferation of HSC stimulated in vitro using a 3 growth factor cocktail. Interestingly, Ptn-enhanced proliferation, does not lead to
HSC loss, but also increases absolute HSC number, suggesting Ptn increases HSC self-renewal *in vitro*.⁶

Thus, Ptn can have opposing effects, probably dependent on the Ptn dose and the expression of its different receptors. In this paper, we studied the effect of HSC *in vivo*. In particular, we were interested in how the absence of Ptn in microenvironmental stromal cells would affect early hematopoiesis and we here report a differential role for Ptn in the myeloid and lymphoid engraftment response of hematopoietic stem cells.
Materials and methods

Mice. Ptn deficient (Ptn⁻/⁻) mice²¹ were backcrossed to (129S2 x C57BL/6.J)F1 (129B6) mouse strains. For experiments, C57BL/6. B6.SJL-Ptproᵃ Pep3ᵇ/BoyJ (Ly5.1) were used as wild-type (WT) donor cells, whereas littermates of (129S2xC57BL/6.J) F2 background (129.B6 Ptn⁺/⁺, Ptn⁻/⁻) were used as recipients. In transplantations of 129.B6 Ptn⁺/⁺ or Ptn⁻/⁻ donor cells, WT (129S2xLy5.1)F1 (129.Ly5.1; Ly5.1 x Ly5.2) mice were used as recipients.

Stromal cells and cell lines. Primary stromal cells were prepared from aorta-gonads-mesonephros regions of genotyped Ptn⁺/⁺ and Ptn⁻/⁻ embryo (E11.5) as previously described.² The stromal cell lines EL08-1D2, UG26-1B6, UG15-1B7, EL28-1B3, and AM30-3F4 were cultured as described previously.²,³ Lentiviral shRNAmir in pLKO.1 vector (OpenBiosystems) was used for stable knockdown of Ptn (PtnKO) in the stromal cell lines as described previously.⁴ As a control (pLKO.1) stromal cells transformed with empty vector were used. Infected cells were selected by 5 µg/ml puromycin in the medium for 3 days after infection or thawing.

Flow cytometry. Surface markers were stained using antibodies from eBioscience (Natutec, Heidelberg Germany), except for CD150-PE, which was obtained from BioLegend (Biozol, Eching, Germany). For intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm Buffer (BD Biosciences) and stained with anti-Ccnd1, 92G2 (Cell Signaling). FACS analyses were performed on a Coulter EPICS
XL (Beckman Coulter) or CyAn ADP Lx P8 (Coulter-Cytomation). Data was analysed with FlowJo software (TreeStar Inc). Sorting of cell populations was done using a BD FACS Aria II lu (Becton Dickinson).

**Short-term colony assay.** The number of colony forming cells was determined using culture in growth factor-supplemented methylcellulose medium as described by the manufacturer (MethoCult GF M3434, Stemcell Technologies, Bonn, Germany). 2.5·10⁴ BM cells or, after culturing on stroma, 1000 Lin⁻ (see below) input equivalent cells were seeded per 3 cm dish.

**Stromal cell co-cultures.** For all co-cultures, lineage depleted bone marrow cells (Lin⁻) were co-cultured with confluent and irradiated stromal cells (30 Gy for cells lines and 15 Gy for primary cells). Lin⁻ cells were negatively selected from flushed bone marrow (Lineage depletion kit, Miltenyi Biotec, Bergisch Gladbach, Germany). 5000 Lin⁻ cells were plated on stromal cells in a 3 cm dish. For long-term co-cultures, cells were cultured in long-term culture medium (M5300, StemCell Technologies). Each week, half of the supernatant was replaced with fresh medium.

**In vivo engraftment.** In all experiments, recipient mice were lethally irradiated with a 9 Gy (KD2 Mevatron, Siemens, Munich, Germany). Three types of experiments were performed. Firstly, bone marrow cells from Ptn⁺/⁺ and Ptn⁻/⁻ mice were serially transplanted into tail vein of WT (129.Ly5.1) recipients. Alternatively, WT Ly5.1 were transplanted into either Ptn⁻/⁻ or their Ptn⁺/⁺ littermates. 1° transplants were injected
with 2·10⁵, 2° with 1·10⁶, 3°- with 2·10⁶ and finally 4° with 5·10⁶ total bone marrow cells isolated from previous recipient respectively. In the second set of experiments, Lin- cells from the BM of Ly5.1 mice were were firstly co-cultured with pLKO.1 and PtnKD stroma for 3 weeks. The input equivalent of 2500 co-cultured Lin- cells were transplanted into WT B6.Ly5.2 recipients. For secondary transplants, 5·10⁶ cells bone marrow cells were transplanted into the recipients with same background. In the third set of experiments, Lin-Kit+ cells were sorted from primary Ptn+/+ and Ptn-/- recipients of 2x10⁵ WT (Ly5.1) bone marrow cells, 16 weeks after initial transplantation. The equivalent of 1000 Lin-Kit+Sca1+ (LSK) cells was then transplanted into lethally irradiated secondary B6.Ly5.2 recipients, together with 2x10⁵ recipient-type competitor cells.

In the first 5 weeks after transplantation, all recipients received 1 mg/ml neomycin sulfate (Sigma) and 500 units/ml Polymyxin B (Sigma) with the drinking water. Peripheral blood was analysed 5 and 10 weeks after transplantation by flow cytometry. 16 weeks after transplantation mice were sacrifice, bone marrow, spleen and peripheral blood were analysed by flow cytometry. BM cells were also used as donor cells for further transplantations. Donor and recipient cells were identified due to differential expression of Cd45 (Ly5 ) gene (Ly5.1 and Ly5.2 alleles). Mice were counted positive with at least 1% myeloid and 1% lymphoid donor engraftment.

**High-resolution tracking of cell division.** Lin- cells were depleted from the WT bone marrow, labeled with 5/6-Carboxyfluorescein succinimidyl ester (CFSE, Invitrogen) and cultured overnight in long-term culture medium (M5300, Stem Cell Technologies). The next day, cells were stained with antibodies and a tight peak of
CFSE+ LSK and MMP cells was sorted to facilitate detection of single divisions, as described earlier. Co-cultures were set up with MMP cells and karyomax (Invitrogen) to enable detection of undivided cells. LSK cells were then co-cultured on primary WT or Ptn+/- stroma for three days and analyzed for the presence of Lin-, Kit+ and Ly6a+ cells in combination with different cell divisions.

**Real time PCR.** cDNA was generated using Dynabeads mRNA DIRECT micro kit (Invitrogen) and Quantitect RT kit (Qiagen). For the real time PCR reaction Power SYBR Green PCR Master Mix (Applied Biosystems) was used, samples were run in an Applied Biosystems 7900HT. All primers used in this study are shown in supplementary table S1.

**Immunocytofluorescence Staining.** Single cell staining assays were performed as modified from our previously described protocol. In brief, 500 cells were spotted on poly-L-lysine coated slides. Spotted cells were fixed with 4% PFA and blocked with 10% FCS and 0,1 % Triton-X in PBS. Cells were stained with anti-Ctnnb1 (L54E2 Alexa Fluor 488-conjugated Mouse antibody, Cell Signaling), rabbit anti-Ccnd1 (92G2, Cell Signaling) or anti-Cebpa (2295, Cell Signaling). As a secondary antibody we used anti-rabbit, Alexa Fluor 488-conjugated antibody (4412 (Cell Signaling)). All stains were counterstained with DAPI (4,6-diamino-2-phenylindole, dihydrochloride) (Invitrogen). A Leica DM RBE fluorescent microscope (Leica, Wetzlar, Germany) was used for detection. Fluorescence intensities of stained cells were quantified in total pixels using ImageJ (NIH, Bethesda, USA). Each stain included a negative Ig control, the detected pixels of which were deducted from the total pictures as background.
Nuclear and cytoplasmic regions were differentially defined based on the boundaries of the DAPI staining, and the ratio of total pixels within the nuclear region and the cytoplasm was determined as described previously.\textsuperscript{4}

**Western blotting.** For Ptn expression analysis, 15 µl of supernatant from confluent cultures (10 ml of total medium) were denatured with 3µl of 5X Lämi-buffer and loaded on 18% reducing polyacrylamide gels. After blotting the proteins on PVDF membranes (Millipore), 2% horse serum in PBST was used for blocking and dilution of anti-Ptn antibody (ab14025, Abcam). Primary antibody was detected using an anti-rabbit horseradish peroxidase-conjugated secondary antibody (Pierce, St Augustin, Germany) and developed using Super Signal chemo luminescent substrates (Pierce).

**Statistics.** For statistical analysis, unpaired Welsh-corrected and paired Student’s t-tests were used where appropriate (InStat, Graphpad Software). Stem cell frequencies in co-cultures were estimated from single cell doses using the L-Calc software (StemCell Technologies).
Results

Loss of Ptn alters hematopoiesis in vitro. We previously demonstrated that pleiotrophin is overrepresented in the cell lines EL08-1D2 and UG26-1B6 which support maintenance of HSC in culture. Here, we confirmed this finding using real time PCR (Figure 1a). To study the importance of Ptn for stem cell maintenance, we stably knocked down expression of Ptn (Ptn\textsuperscript{KD}) in both cell lines (Figure1b, 1c). Co-cultures of wild-type Lin- cells on Ptn\textsuperscript{KD} stromal cells displayed increased production of committed progenitors (Figure 1d) compared to the control (pLKO.1) cells. To ascertain that a soluble factor was responsible for this increase in colony formation, we also performed co-cultures in which Lin- cells and UG26-1B6 were separated by a membrane. In these non-contact co-cultures we found a similar increase in colony-forming cells (CFC) as in the contact co-cultures (Figure 1d). These results indicate that the knockdown of Ptn affects CFC production in an extrinsic manner. To rule out intrinsic effects of the Ptn loss, we used Lin- cells from Ptn\textsuperscript{+/+} and Ptn\textsuperscript{-/-} mice\textsuperscript{21} for co-cultures on pLKO.1 and Ptn\textsuperscript{KD} UG26-1B6 cells. Here, we observed a same effect independently from the genotype of co-cultured Lin- cells. (Figure 1e).

Ptn deficient mice show normal steady-state hematopoiesis. These in vitro results show that in the absence of Ptn, maintenance of hematopoietic cell subsets in culture is altered, resulting in increased CFC production. Thus, we studied hematopoiesis in Ptn\textsuperscript{-/-} mice in more detail. In general, mature hematopoietic populations were unaltered in Ptn\textsuperscript{-/-} animals compared to their Ptn\textsuperscript{+/+} littermates, except for an increase in myeloid cells in the peripheral blood (PB) of Ptn\textsuperscript{-/-} animals (Figure S1). Characterization of early hematopoietic progenitors and stem cells in the Ptn\textsuperscript{-/-} mice indicates that steady-state hematopoiesis in these mice is very similar to
that of Ptn\(^{+/+}\) littermates (Figure S1). To find out whether knockout of Ptn expression in HSC would affect their ability to engraft and regenerate lethally irradiated recipients, we transplanted Ptn\(^{-/-}\) and Ptn\(^{+/+}\) bone marrow cells into WT recipients. In these experiments, we observed that Ptn\(^{-/-}\) HSC show normal myeloid and lymphoid engraftment behavior in primary (1\(^{\circ}\)) as well as secondary (2\(^{\circ}\)) recipients (Figure S2). However, it is notable that 5 weeks after the initial transplantation of Ptn\(^{-/-}\) cells into 2\(^{\circ}\) recipients, myeloid engraftment is slightly lower. Since at 16 weeks after transplantation this altered engraftment pattern was not found anymore, the finding suggests that the loss of the Ptn gene may have “primed” this short-term repopulation behavior of Ptn\(^{-/-}\) cells. It seems unlikely, that this “priming” is the result of an intrinsic expression of Ptn in stem and progenitors, since in real-time PCR analyses of Ptn in LSK and multipotent myeloid progenitors (MMP) cells from Ptn\(^{-/-}\) and their Ptn\(^{+/+}\) littermates, we failed to detect Ptn mRNA (Figure S3).

**Loss of microenvironmental Ptn leads to enhanced stem cell maintenance in vivo.** The results of the co-cultures of Lin- cells with Ptn\(^{KD}\) stromal cells are reminiscent of similar experiments we previously performed studying Sfrp1.\(^4\) Since stromal cell co-cultures model recapitulate a regenerative response, rather than steady-state hematopoiesis, we also investigated hematopoietic regeneration. For this purpose, WT Ly5.1 bone marrow cells were transplanted in lethally irradiated Ptn\(^{-/-}\) mice and Ptn \(^{+/+}\) littermates. These mice were analyzed 16 weeks after transplantation and showed an increased donor cell compartment in bone marrow and peripheral blood compared to Ptn\(^{+/+}\) recipient mice (Figure 2b, and 2c). In addition, we found an increased amount of donor-derived myeloid progenitors (CMP and GMP) in the bone marrow of these 1\(^{\circ}\) recipients (Figure S4b).
To investigate whether increased primary engraftment reflects over-activation (reduced self-renewal) or increased HSC maintenance (increased self-renewal), bone marrow of these 1° mice was used for transplantation into lethally irradiated 2° WT recipient mice. In contrast to our results in primary Sfrp1−/− knockout recipients we published earlier4, analysis of these 2° recipients receiving cells from the primary Ptn−/− environment 16 weeks after injection showed a clear increase in donor cell regeneration in the peripheral blood (Figure 2e, Figure S4d, e, f), indicating that loss of Ptn in the primary environment does not impair HSC self-renewal.

Further serial transplantations demonstrated that the apparent increase in self-renewal in 1° recipients resulted in a long-lasting increase in engraftment, which is even more outspoken in tertiary (3°) transplants. Analysis of 3° recipients suggest a 4-fold increase of donor engraftment in bone marrow (Figure 2f), accompanied by a 10.3 fold increase in Cd34− LSK (Lin− Ly6a+ Kit+) and a 6.8 fold increase in MMP populations (Figure 2j). This effect is still more outspoken in quaternary (4°) recipients, where we found an increase in engraftment levels of both bone marrow (14.3-fold increase) and blood (3.5-fold increase) in donor engraftment (Figure 2h, 2i). The increase of bone marrow engraftment was accompanied by increased of the numbers of CD34− LSK (26.3-fold increase), and MMP (27.6-fold increase) (Figure 2j, and 2k).

In all serial transplantations, we detected myeloid and lymphoid engraftment. Interestingly, at each serial transplantation, engraftment of myeloid progenitors CMP, GMP, and MEP was increased in mice serially transplanted with cells from 1° Ptn+/+ and Ptn−/− recipients (Figure S4b,h,k). This finding suggests a progressive dominance of myeloid and a loss of lymphoid engraftment (Figure S4f,i,l).
Thus, the loss of Ptn in the environment of 1° recipients in vivo results in a persisting improvement in HSC maintenance in serial transplantations associated with an increased ability to regenerate both Cd34- LSK and MMP compartments in lethally irradiated recipients.

**Loss of microenvironmental Ptn enhances stem cell repopulation ability in vitro.** Since a detailed study of the molecular mechanisms of the effects of the primary Ptn−/− recipient environment is not practical in the above in vivo experiments, we decided to return to our study of the co-cultures we described above (Figure 3a). A closer look at the two-week cultures revealed an increased amount of hematopoietic cells in co-cultures with PtnKD stroma cells compared to pLKO.1 (Figure 3b). Moreover, in the co-cultures on PtnKD stromal cells myeloid cells were increased as well as the population of MMP (Figure 3c, d).

To find out whether the co-cultures with PtnKD stromal cells cause a similar increase in HSC repopulation ability as found in the transplants of Ptn−/− 1° recipients (Figure 2), whole cultures were transplanted into lethally irradiated WT mice. Sixteen weeks after transplantation, only 6 out of 25 mice which received cells from pLKO.1 cultures were positive (estimated frequency 1 in 9100 [95% confidence interval: 1 in 4080-20300]). In contrast, 17/27 (estimated frequency 1 in 2200 [95% confidence interval: 1 in 1330-3620]) of mice transplanted with cells from co-cultures on PtnKD stromal cells were positive (Figure 3e). Taken the average of all transplanted animals, the earliest donor cell populations do not appear to be affected (Figure 3f), suggesting that the loss of stromal Ptn in co-cultures mainly affects the regenerative capacity of the more mature progenitor populations, as is also suggested by analysis of the cultures themselves, where an obvious increase in MMP compartment is noticeable.
(Figure 3d). In line with the transplantation experiments in primary Ptn
recipient mice, we detected an increase in myeloid and concomitant decrease in lymphoid engraftment in the transplanted mice (Figure 3g). In addition, HSC co-cultured on PtnKD stromal cells engrafted the bone marrow of 2° WT recipients at significantly higher levels as compared to HSC co-cultured on pLKO.1 stromal cells (Figure 3h). Also, in these 2° recipients, the population of donor-derived CD34− LSK as well as the CMP were more than twice as high as in mice which received cells from PtnKD co-cultures than in pLKO.1 mice (Figure 3j, Figure S5d). However, number of MMPs was not statistically different in mice which received cells from PtnKD co-cultures (Figure 3k).

These results indicate that the knockdown of Ptn expression in stromal cells enhances the maintenance of the number of stem cells in vitro. As such, the co-culture results imply that the increased HSC repopulating ability of cells transplanted into Ptn−/− mice (Figure 2) is due to the lack of Ptn in the stromal cell compartment.

**Enhanced stem cell repopulation ability is caused by a direct effect on donor LSK cells.** In the above experiments, the 1° Ptn−/− recipients present with a higher bone marrow reconstitution. Hence, it is possible that this increase may be carried-over to subsequent secondary 2° and further serial transplants and, thus, explaining the increased repopulating ability. In fact, when we calculate the carry-over of donor cells, the 3° and 4° recipients receive a significantly larger donor transplant from the 1° Ptn−/− recipients (Figure S6a), whereas the estimated number of donor marrow cells generated per Cd34− LSK cells (Figure S6b) remains similar. The reconstitution itself is therefore not a measure of stem cell quality per sé and changes in stem cell quality may be masked by changes in cell number.
To test whether stem cell quality is altered in the 1° recipients, we sorted donor LSK cells from 1° Ptn\(^{+/+}\) or Ptn\(^{-/-}\) recipients (Figure 4a) and transplanted these in equal numbers (1000 per animal) into 2° WT recipients. To include competitive ability in the study of stem cell quality, we performed this experiment in a 1:1 competitive manner (1x10\(^5\) Ly5.1 donor cells, and 1x10\(^5\) competitor cells). These experiments confirmed that LSK cells from 1° Ptn\(^{-/-}\) recipients show a tendency for higher engraftment, as well as increased myeloid and decreased lymphoid engraftment after 16 weeks in the 2° WT recipients (Figure 4c,d). Interestingly, we noted a significantly decreased LSK population in mice receiving LSK cells from 1° Ptn\(^{-/-}\) recipients. However, more mature progenitors, like the MMP and CLP were not different from those in 2° recipients transplanted with LSK cells from 1° WT recipients (Figure 4e). These results indicate that the Ptn\(^{-/-}\) environment directly modulates the repopulation behavior of HSC.

**Loss of Ptn alters cell division behaviour of LSK cells.** The above results demonstrate that Ptn-dependent effects on HSC maintenance could be caused by an altered regulation of the cell cycle. To find out whether cell division recruitment of WT LSK cells was affected by loss of Ptn in stromal cells, we studied cell division behavior of CFSE-labeled WT LSK cells (Figure 5a). These experiments demonstrated that cells from 3-day co-cultures on primary Ptn\(^{-/-}\) stroma lost their LSK phenotype more quickly than those cultured on WT stroma. (Figure 5b, f), which was associated with a smaller proportion of cells remaining undivided (Figure 5c, d). The LSK cells cultured on Ptn\(^{-/-}\) stroma did not only lose Sca-1 (Ly6a) expression more quickly, they also generated a higher proportion of cells expressing lineage markers (Figure 5e, f). These results show that LSK cells proliferate more quickly on Ptn\(^{-/-}\)
stromal cells, with increased expression of differentiation markers within the first 3 days of co-culture.

**Loss of Ptn in the stromal microenvironment modulates gene expression in hematopoietic progenitors.** Since cell division behavior of LSK cells on Ptn-deficient stromal cells was already affected in short-term cultures, we investigated the earliest gene expression changes in 3-day co-cultures and sorted LSK, CMP and GMP to study mRNA expression.

Real time PCR analysis of these cells showed an unaltered level of Ctnnb1 mRNA expression (Figure 4a) as well as Cdkn1 and Cdkn2 family members and other molecules known to be involved in hematopoietic regulation (Figure S7). Co-culture on Ptn\textsuperscript{KD} stromal cells caused a suppression of the transcription factor Pparg in all populations analysed (Figure 6a). Interestingly, Cyclin D1 (Ccnd1) and C/EBP\textalpha (Cebpa) were both higher expressed in LSK cells recovered from co-cultures on Ptn\textsuperscript{KD} stromal cells. But, in more mature populations (GMP), expression of both Ccnd1 and Cebpa decreased to significantly lower expression levels relative to co-cultures on pLKO. stromal cells (Figure S7). Similarly, although Hes1 and Dlk1 expression levels were unaltered in LSK cells recovered from co-cultures, their expression decreased in GMP recovered from co-cultures on Ptn\textsuperscript{KD} stromal cells (Figure S7a).

We also studied some selected genes expressed in cells recovered from co-cultures with primary Ptn\textsuperscript{−/−} stromal cells. We confirmed that Ctnnb1 expression remained unaltered, whereas we found a decrease in Pparg expression and both Ccnd1 and Cebpa were increased (Figure 6), confirming the regulation of these genes in both Ptn\textsuperscript{KD} and Ptn\textsuperscript{−/−} stromal cell co-cultures.
Loss of Ptn may cause a possible “priming” effect of the Ptn−/− environment (Figure S2). We therefore also looked at gene expression of selected genes in LSK cells recovered from steady-state WT and Ptn−/− mice. Interestingly, despite the lack of detectable changes in long-term function of Ptn−/− cells, we found clear modulation of Cebpa and Pparg in primary LSK cells from Ptn−/− mice. Most surprisingly, we observed a decrease of Ccnd1 in Ptn−/− LSK cells (Figure 6a), suggesting that Ccnd1 is differentially regulated during steady state hematopoiesis in vivo and the triggering of hematopoietic regeneration in vitro.

Since mRNA levels do not always correlate to protein levels, we also studied the expression of selected proteins in sorted LSK cells from steady-state Ptn−/− animals and cells co-cultured on PtnKD or Ptn−/− stromal cells. In flow cytometry experiments, we found that Ccnd1 high-expressing cells showed a similar increase as compared in the real time PCR analysis (Figure 6a,b). In single-cell immunofluorescence staining of sorted LSK and GMP from co-culture experiments, we found that, in line with the unchanged transcriptional regulation of Ctnnb1, the total protein level of Ctnnb1 was not affected in either LSK or GMP, nor was its intracellular localization (Figure S8). In addition, these sorting experiments demonstrated that the transcriptional increase in Cebpa was mirrored by an increase at the protein level in LSK cells from primary Ptn−/− mice (Figure 6d,e), as well as LSK cells co-cultured on PtnKD UG26-1B6 or on primary Ptn−/− stroma (Figure 6e, Figure S9). Downregulation of Ccnd1 protein in LSK cells from primary Ptn−/− mice (Figure 6f, g), and upregulation in co-cultures on Ptn−/− stromal cells were also confirmed. Thus, the gene expression changes we found at the transcriptional level were also represented at the protein level in sorted LSK cells from primary mice, or stromal co-cultures.
Discussion

The secreted 17 kDa cytokine Ptn is known to be involved in diverse cellular functions in various cell types. However, the role of Ptn in niche-associated regulation of hematopoiesis has, so far, not been explored. We show that Ptn is not required for maintenance of the HSC pool in steady-state hematopoiesis. In contrast, the knockdown of Ptn in stromal cells increases the production of hematopoietic progenitors and, more importantly, the number of HSC maintained in co-cultures. This functional observation is associated with an upregulation of Ccnd1 and Cebpa in LSK cells. In the HSC-downstream CMP and GMP, we find further deregulation of a number of cell cycle-related genes, but survival-associated genes (Trp53, Pten) were not altered in any of the cell populations sorted from the co-cultures. Interestingly, our experiments suggest a long-lasting enhancement of donor stem cell regeneration and self-renewal in primary Ptn-deficient recipients and on Ptn KD stromal cells. In addition, we show that the lack of environmental Ptn increases the number of donor cells generated per stem cell, as well as promoting myeloid engraftment.

Pleiotrophin is known to bind and inhibit the receptor tyrosine phosphatase RPTP β/ζ (Ptprz1). Thus, Ptn increases the phosphorylation status of various substrates of Ptprz1, including Ctnnb1. We and others have previously shown that Ctnnb1 is an important regulator of HSC self-renewal and differentiation. However, co-culture with Ptn-knockdown stromal cells did not affect Ctnnb1 expression, neither at the mRNA, nor at the protein level, nor in the distribution between cytoplasm and nucleus in sorted LSK, CMP or GMP from stromal co-cultures. Hence, in line with another study of the in vitro effects of Ptn on HSC cells, the effects of Ptn...
knockdown in stromal cells on HSC does not involve regulation of Ctnnb1 levels in HSC.

Nevertheless, Ccnd1, an important regulator of G1/S cell cycle transition, is upregulated in cultured LSK cells, suggesting that in the absence of stromal Ptn Ccnd1 is upregulated independent of Ctnnb1 regulation. This finding may be related to our observation that Pparg expression is strongly downregulated not only in progenitors, but also in LSK cells. Since HSC stimulation through the non-canonical Wnt signalling (which represses Pparg transcription), enhances self-renewal,26,27 our findings support the idea that the absence of Ptn may promote non-canonical Wnt signalling pathways normally associated with enhanced self-renewal. Further support for this notion is the reported observation that Pparg induces cell cycle withdrawal.28 Thus, the suppression of Pparg expression contributes to enhanced cycling through upregulation of Ccnd1 expression. Since Pparg regulates Ccnd1 through Creb rather than Ctnnb1,29 canonical Wnt signalling may not be required for the regulation of Ccnd1.

Another pathway which is potentially affected by Ptn is Notch signalling.14,30 Deregulation of Notch signalling might be involved in our observations, since gene expression of Hes1 and Dlk1, which are both known as targets of Notch signalling,31,32 is reduced in GMPs (and for Hes1, also in CMPs) cultured with Ptn knockdown stroma cells. The effects of this transcriptional regulation may be related to the recent observation that Hes1 confers self-renewal activity on CMP and GMP carrying the Bcr-Abl oncogene.33 Since we observe that Hes1 and Dlk1 expression are unchanged in LSK cells, the downregulation of these molecules would be consistent with the idea that Ptn acts, instead, more on Notch signals in the
differentiated cells, like CMP and GMP, and prevents self-renewal of these progenitors.

Since Ptn was overexpressed in cell lines supporting HSC in long-term cultures, and addition of Ptn to in vitro cultures increases HSC cell numbers, we anticipated that the loss of stromal Ptn would have a negative effect on HSC behavior. However, our observations do not support such a view, since HSC co-cultured on Ptn\textsuperscript{KD} stromal cell or transplanted into Ptn\textsuperscript{-/-} deficient recipients, show increased donor cell repopulation which is progressively skewed towards the myeloid lineage and is consistently associated with an increase donor-derived MMP and other myeloid progenitors. At the same time, 16 weeks after transplantation, the relative number of Cd34\textsuperscript{-} LSK cells in mice is increased. These in vivo data suggest that the hematopoietic hierarchy generated from HSC previously primed by a Ptn\textsuperscript{-/-} environment, may be more shallow. Direct comparisons with current reported data from others, with regard to myeloid skewing or HSC expansion, is complicated by the use of different experimental systems. Since in the present study, all experiments were performed in the presence of stroma, it is highly likely, that Ptn may exert the effects we have observed in part through regulation of other microenvironmental cells.

We have found that the loss of Ptn under steady-state conditions or after triggering a repopulation response lead to different outcomes. It is known, that under steady-state conditions, HSC are present mainly in a quiescent or dormant state, and that upon total body irradiation, chemostatic treatment or transplantation, HSC are rapidly recruited into cell cycle. We found that under steady-state conditions, \textit{Ccnd1} was expressed at a lower level in Ptn\textsuperscript{-/-} LSK cells, whereas \textit{Ccnd1} was upregulated under conditions triggering HSC into cycle. Our findings that the absence of Ptn in stromal
cells increases the amount of cell divisions in short term cultures, may well be related to the upregulation of \( Ccnd1 \). Although entry into cell cycle is obviously required for self-renewal to occur, cell cycle kinetics are, by themselves, not reliable predictors of self-renewal. In most published knockout models which affect HSC cell cycle kinetics, entry into cell cycle leads to HSC exhaustion.\(^{34} \) However, in a few models, HSC engraftment and enhanced self-renewal has also been demonstrated, for example in HSC deficient in \( Cdkn2c \).\(^ {35,36} \) However, our results cannot be explained by a secondary downregulation of \( Cdkn2c \), since none of the \( Cdkn1 \) and \( Cdkn2 \) family members we examined showed alterations in expression when cultured on Ptn-knockdown cells. Hence, our results suggest that Ptn differentially regulates \( Ccnd1 \) under steady-state conditions and HSC activation.

The cause of the myeloid skewing is not clear from our observations and several hypotheses can be formulated which would explain our findings. Firstly, as described above, they could be the result of the observed regulation of \( Ccnd1 \) and \( Cebpa \) which are both involved in regulation of HSC self renewal.\(^ {41,42} \) Also, \( Cebpa \) is known as a master regulator of myeloid differentiation\(^ {43} \). This does, however, not explain why myeloid engraftment is still favored in quaternary recipients, nor why we observed a significant peak of this behavior in 3° recipients. Secondly, our observations of myeloid skewed engraftment and increase in marrow \( Cd34^-\) LSK are consistent with those reported for ageing HSC,\(^ {44-47} \) a phenomenon which may depend on soluble factors emanating from niche cells. However, our observations are not entirely consistent with these data because aged HSC show a functional defect in engraftment which is not detectable in our experiments. A third explanation could also be that stromal \( Ptn \)-deficiency differentially affects HSC subsets. Myeloid-skewed engraftment is also observed in studies with the myeloid-biased \( \alpha \) subset of
HSC, which show robust engraftment and self-renewal capacity compared to lymphoid-biased (γ- and δ) HSC\textsuperscript{48,49}. Thus, like recently shown for Tgfb1,\textsuperscript{50} the absence of Ptn in the microenvironment, may favor maintenance of α-type, myeloid/biased HSC. In this case, the lack of Ptn could be interpreted as an imbalance in the regulation of myeloid- and lymphoid-biased HSC.

In conclusion, the present study shows that steady-state hemostasis, maintenance, of HSC in stromal co-cultures, and hematopoietic engraftment of myeloablated recipients are subject to different regulatory mechanisms. The loss of Ptn in the steady-state environment does not affect the ability of HSC to uphold normal hemostasis throughout the lifetime of a mouse. In contrast, activation of a regenerative hematopoietic response in co-culture, or after transplantation into myeloablated recipients, modulates HSC repopulation behavior towards a more proliferative, myeloid-favoring behavior, which results in an enhanced engraftment of donor HSC initially exposed to a Ptn-deficient environment. We show that loss of stromal Ptn leads to subtle changes in gene expression which have profound and late-acting effects on HSC repopulation behavior, detectable even after quaternary transplantation. The dominance of myeloid engraftment and the accumulation of Cd34\textsuperscript{-} LSK detectable in serial transplants indicates, that the secreted factor Ptn is required in an environmental context to maintain the balance of myeloid and lymphoid potential of regenerating HSC.
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Conflict of Interest Disclosures

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Figure legends

Figure 1. Loss of Ptn affects hematopoiesis in vitro. a) Expression of Ptn in UG26-1B6 and EL08-1D2 in comparison with a mixture of non-supportive stromal cell lines (UG15-1B7, EL28-1B3, AM30-3F4) as detected by real time PCR. Shown is expression relative to housekeeping gene Ythdf1. b) Knockdown of Ptn in supportive stromal cell lines as measured by real time PCR. Shown is expression relative to Ythdf1. Grey bars represent the unmanipulated parental stromal cells, white bars represent the stromal cells transduced with empty pLKO.1 vector (pLKO.1) and shPtn-transduced cells (PtnKD) are shown in black bars. c) Western blot of Ptn expression, using supernatant of confluent monolayer of stromal cell lines. d) Colony number of WT Lin- after 2 week co-culture on PtnKD and pLKO.1 stromal cell lines, n=10 (UG26-1B6 contact), n=4 (UG26-1B6 non-contact), n=3 (EL08-1D2, contact). e) The colony number of Ptn+/+ and Ptn-/- Lin- after 2 week co-culture on PtnKD and pLKO.1 UG26-1B6 (n=4). All values mean ± SEM, *p<0.05.

Figure 2. Loss of microenvironmental Ptn leads to enhanced stem cell maintenance in vivo. a) Experimental design of serial transplantation experiments. 2x10^5 WT Ly5.1 bone marrow cells were transplanted into 9 Gy-irradiated Ptn-/- and Ptn+/+ littermate (Ly-5.2+) recipients. Sixteen weeks after transplantation, recipients were sacrificed and 1x10^6 bone marrow cells serially transplanted into WT secondary recipients. Again, mice were sacrificed 16 weeks after transplantation and 2x10^6 BM cells were serially transplanted into tertiary irradiated WT mice. Finally, 5x10^6 BM cells of these tertiary recipients were again transplanted into irradiated quaternary WT recipients. b) Engraftment levels as percentage of Ly-5.1+ donor cells in bone marrow of 1° recipients, n=5 (+/+), n=10 (-/-) c) Primary transplantation, engraftment
levels in peripheral blood 16 weeks after transplantation. d) Secondary transplantation, engraftment levels in bone marrow 16 weeks after transplantation, percentage of donor cells in total cell count of 4 long bones, n=10 (Ptn⁺/⁺), n=8 (Ptn⁻/⁻). 
e) Secondary transplantation, engraftment levels in peripheral blood 16 weeks after transplantation. f) Tertiary transplantation, engraftment levels in bone marrow 16 weeks after transplantation as percentage of donor cells, n=7 (Ptn⁺/+), n=5 (Ptn⁻/-). g) Tertiary transplantation, engraftment levels in peripheral blood 16 weeks after transplantation. h) Quaternary transplantation, engraftment levels in bone marrow 16 weeks after transplantation, as percentage of donor cells, n=5 (Ptn⁺/+), n=6 (Ptn⁻/-). i) Quaternary transplantation, engraftment levels in peripheral blood 16 weeks after transplantation. j) Calculated absolute numbers of Cd34-LSK and MMP cells per 4 long bones in the serial transplantations, based on the number of transplanted bone marrow cells. k) Flow cytometry analysis of bone marrow of quaternary transplants. LSK and MMP are gated in lineage negative cells, Cd34-LSK were gated in the LSK cells. All values mean ± SEM. *p<0.05.

Figure 3. Loss of microenvironmental Ptn leads to enhanced stem cell maintenance in vitro. a) Scheme of co-culture experiment. In brief, Lin⁻ from Ly-5.2⁺ WT bone marrow, were co-cultured with PtnKD and pLKO.1 stroma in contact. After three weeks, cultures were transplanted into irradiated WT mice. Recipients were sacrificed 16 weeks after injection and 5x10⁶ bone marrow cells were again transplanted into irradiated WT recipients. b) Total number of hematopoietic cells as percentage of the total cell number (hematopoietic + stromal cells) recovered from the culture. Statistics were performed using a paired Student’s t-test. c) Example of the flow-cytometric analyses after co-culture. d) Hematopoietic subpopulations in
stromal co-cultures. Myeloid cells were gated as $\text{Gr1}^{\text{low-high}}$, CD11b+. LSK and MMP were gated as Lin$^{-}$ Ly6a$^{+}$ Kit$^{+}$ cells and Lin- Ly6a- Kit+ cells, respectively. Statistics were performed with a paired Student’s t-test. e) Engraftment in peripheral blood 16 weeks after primary transplantation. Animals were counted positive with at least 1% engraftment in blood, and donor cells containing at least 1% myeloid and lymphoid cells, respectively. f) Enumeration of Cd34- LSK and MMP populations in bone marrow 16 weeks after primary transplantation of co-cultured cells. Shown are the results of mice with at least 1% engraftment in bone marrow, n=4 (white bars, pLKO.1 stroma in co-cultures), n=8 (black bars, Ptn$^{\text{KD}}$ stroma in co-cultures). g) Frequency of lymphoid and myeloid cells within the donor cell population in peripheral blood of transplanted mice, 5, 10, and 16 weeks after transplantation (pLKO.1=6; Ptn$^{\text{KD}}$=8). h) Secondary transplantation, donor engraftment levels in bone marrow (n=9), and i) in peripheral blood. j) Secondary transplantation, total number of donor-derived Cd34- LSK in bone marrow, 16 weeks after the start of secondary transplantation, as well as k) donor-derived MMP in bone marrow. All values mean ± SEM, *p<0.05.

**Figure 4. Lack of Ptn modulates repopulating behavior of HSC.** Changes in self-renewal and stem cell quality can be masked by alterations in proliferation. (a) Experimental design: to address this issue, we here sorted Lin$^{-}$ Kit$^{+}$ donor (Ly-5.1) cells from primary 129S2B6 (Ly-5.2) Ptn$^{+/+}$ or Ptn$^{-/-}$ recipients (see Figure 2a) and transplanted equal numbers of LSK cells into 2° WT recipients in a competitive manner (that is: together with 2x10$^5$ recipient-type bone marrow cells). (b) FACS plots from the pool of bone marrow from Ptn$^{+/+}$ and Ptn$^{-/-}$ primary recipients. (c) Level of engraftment, 16 weeks after transplantation of 1000 LSK cells from 1° recipients. (d) Relative contribution of donor myeloid cells (non-T, non-B, Gr1+) and lymphoid
cells (T plus B) to the total population in the 2° recipient mice. (d) Relative numbers of LSK, MMP, and IL-7R⁺ CLP within the donor Lin- population of 2° recipients, 16 weeks after transplantation.

**Figure 5. Lack of Ptn promotes cell division recruitment.** In the experiments presented here, LSK cells were first sorted from WT donors and then labeled with CFSE. Cells were cultured overnight in long-term culture medium. The next day, a tight peak of CFSE⁺ cells was sorted to facilitate detection of single divisions⁵¹ co-cultured in the presence of primary stromal cells (a). After three days of co-culture, cells were analyzed for the presence of Lin⁻, Kit⁺ and Ly6a⁺ cells (b). Figure (c) show a representative histogram of the cell division kinetics of Kit⁺ Cells. The fraction of undivided and divided cells was then determined in co-cultures on either Ptn⁺/+ or Ptn⁻/- stromal cells, and compared using a paired student’s t-test. The ratio of these fractions (fraction of division n in Ptn⁻/-) / (fraction of division n in Ptn⁺/+)) was then calculated. These fractions are shown per division in (d). (e) shows the acquisition of lineage markers of the LSK cells per division and (f) the loss of Ly6a in those divisions in LSK cells co-cultured with Ptn⁺/+ and Ptn⁻/- stromal cells. A total of 6 independent repeats of this experiment were performed (d) of which representative plots and histograms are shown. The experiment in figures (e) and (f) was performed independently three times, a representative example is shown.

**Figure 6. Altered gene expression and protein levels in stem cells and progenitors lacking microenvironmental Ptn.** (a) Gene expression analysis in sorted LSK :from Ptn⁻/- bone marrow (white bars); from co-cultures with Ptn⁺⁺⁻⁻ (n=5)
(grey bars), or from co-cultures on primary Ptn^{+/−} stromal cells (n=3, black bars). Gene expression was normalized relative to the housekeeping gene Gorasp2. Shown is the ratio of the normalized gene expression to Ptn^{+/+}, pLKO.1 and primary Ptn^{+/+} stroma.

(b) Ccnd1 protein levels in 3-day co-cultures on Ptn^{KD} or pLKO.1 UG26-1B6 obtained by intracellular flow cytometry. Shown is a representative example. (c) Percentages of Ccnd1^{high} cells in LSK cells recovered from 3-day co-cultures on pLKO.1- or Ptn^{KD} UG26-1B6 cells. (d) Cebpa protein levels in LSK cells sorted from the BM of Ptn^{+/+} and Ptn^{−/−} mice. (e) Pixel quantitation of stained LSK cells from Ptn^{−/−} mice (white bars, n=3), co-cultures on Ptn^{KD} (grey bars, n=6) or primary Ptn^{−/−} stromal cells (black bars, n=3). Shown is the ratio of pixels to Ptn^{+/+}, pLKO or WT stroma, respectively. (f) and (g) similar measurements of Ccnd1 protein levels in LSK cells from the BM of Ptn^{−/−} mice, co-cultures on primary Ptn^{−/−} stromal cells. Co-cultures on Ptn^{KD} stroma could not be analyzed. All values mean ± SEM, statistics were performed using the paired student’s t-test: *p<0.05.
Figure 2

**Panel a**: Schematic diagram showing the experimental setup for serial transplantation of bone marrow cells into recipients of different genotypes.

1. **1° in Ptn^+/+**: 2x10^5 BM cells are transplanted into Ptn^+/+ recipients.
2. **1° in Ptn^+-**: 1x10^6 BM cells are transplanted into Ptn^+- recipients.
3. **2°**: 2x10^6 BM cells are transplanted into wt recipients.
4. **3°**: 5x10^6 BM cells are transplanted into wt recipients.
5. **4°**: 4x10^6 BM cells are transplanted into wt recipients.

**Panels b-i**: Bar graphs showing the percentage of donor cells in bone marrow and blood from different genotypes at 1°, 2°, 3°, and 4°.

**Panel j**: Graph showing the absolute number of cells per four long bones per million transplanted BM cells over serial transplantation generations.

**Panel k**: Flow cytometry analysis showing the expression of Kit and Ly6a in bone marrow samples.
Figure 5

(a) Sorted CFSE\(^+\) LSK

\[
\begin{align*}
\text{3 days} & \quad \text{cultured on primary stroma} \\
\downarrow & \\
\text{FACS}
\end{align*}
\]

(b) cultured on Ptn\(^{+/+}\)  cultured on Ptn\(^{-/-}\)

(c) Lin\(^-\)Kit\(^+\)

\[
\begin{array}{c}
\text{CFSE} \\
\end{array}
\]

(d) Ratio co-culture on Ptn\(^{+/+}\)/Ptn\(^{-/-}\)

\[
\begin{align*}
p = 0.02 \\
0.1 & \quad 1 & \quad 3 & \quad 4 \quad > 4
\end{align*}
\]

(e) cultured on Ptn\(^{+/+}\)  cultured on Ptn\(^{-/-}\)

(f) Lin\(^-\)Lineage  Ly6a

\[
\begin{array}{c}
4.0 & \quad 1.8 & \quad 17 & \quad 2.1 \\
31 & \quad 63 & \quad 40 & \quad 41 \\
6.3 & \quad 66 & \quad 22 & \quad 47 \\
21 & \quad 11 & \quad 28 & \quad 4.2
\end{array}
\]
Stromal pleiotrophin regulates repopulation behavior of hematopoietic stem cells

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