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Functions of Pleiotrophin in the regulation of hematopoiesis

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Zusammenfassung

Hämatopoetische Stammzellen halten sich in einer spezialisierten Mikroumgebung auf, die essentiell für die strikte Regulation der Stammzellfunktionen ist. In dieser Arbeit wurde die Rolle des Zytokins Pleiotrophin (Ptn) bei der Regulation von Hämatopoese und Stammzellfunktionen untersucht. Nachdem die Expression von Ptn in den Stromazelllinien UG26-1B6 und EL08-1D2 herunterreguliert wurde konnte in Co-Kulturen mit Wildtypzellen ein Anstieg der Zahl koloniebildender Zellen beobachtet werden. Transplantationsexperimente zeigten eine erhöhte Anzahl von Stammzellen aus Co-Kulturen mit Ptn-defizienten Stromazellen. Dieser erhöhte Stammzellerhalt *in vitro* wurde mit Sekundärtransplantationen bestätigt. Mit Ptn-defizienten Mäusen wurde untersucht, ob der Verlust von Ptn ähnliche Effekte *in vivo* nach sich zieht. Die Hämatopoese von Ptn-defizienten Mäusen mit verschiedenen Stammhintergründen wurde in dieser Studie charakterisiert. Ptn-defiziente Mäuse mit einem 129-Hintergrund wiesen verschiedene Veränderungen in primitiven und reifen hämatopoetischen Zellpopulationen durch den Verlust von Ptn auf. Dies wurde von einer veränderten Proliferationsrate während Stress-induzierter Hämatopoese begleitet. In (129xB6)F2 genau wie in rederivierten 129-Ptn-defizienten Mäusen wurden dagegen keine deutlichen Veränderungen der Hämatopoese gefunden. In Repopulationsassays wurde gezeigt, dass der Verlust der Ptn-Expression keine intrinsischen Effekte auf die Stammzellfunktion hat. Dagegen zeigten Wildtyp-Stammzellen, die primäre $Ptn^{-/-}$ Mäuse repopuliert hatten, in seriellen Transplantationen eine erhöhte Repopulationskapazität. Dies deutet, ähnlich wie die Ergebnisse der Co-Kulturen, auf einen erhöhten Stammzellerhalt in einer Ptn-defizienten Mikroumgebung hin. In den seriellen Transplantationen wurden zudem in Mäusen, die Zellen aus einer Ptn-defizienten Umgebung erhalten hatten, eine erhöhte Anzahl von Cd34- LSK-Zellen sowie eine dominante Regeneration von myeloiden Zellen beobachtet. In einer Analyse der frühen Veränderungen von Genexpression und Proteinmengen in Co-Kulturen mit Stromazelllinien wurde entdeckt, dass Ccnd1, Cebpa sowie Pparg in LSK Zellen auf

Ptn-defizientem Stroma unabhängig von Ctnnb1 verändert reguliert waren. Zusätzlich zu den Funktionen von Pleiotrophin in der frühen Hämatopoese wurde gezeigt, dass Ptn die Apoptose von Granulozyten über einen bisher noch nicht bekannten Mechanismus beeinflusst. Die Ergebnisse dieser Studie zeigen, dass sekretierte Moleküle wichtige Faktoren für Stammzellerhalt und Gewebehomöostase darstellen.

Summary

Hematopoietic stem cells (HSC) reside in specialized microenvironments, which are crucial for the strict regulation of HSC function. In this study, the role of the cytokine Pleiotrophin (Ptn) in the regulation of hematopoiesis and HSC functions was investigated. In stable knockdowns of Pleiotrophin in the stromal cell lines UG26-1B6 and EL08-1D2, co-cultures with wild-type lineage-negative (lin-) cells revealed an increase of colony-forming cells. In transplantation experiments, an increased number of HSC from co-cultures on Ptn-knockdown stromal cells was detected. Secondary transplants confirmed the increased maintenance of stem cells *in vitro*. Ptn KO mice were investigated to find out whether the lack of Ptn *in vivo* would produce similar results. The hematopoiesis of Ptn KO mice on different backgrounds was characterized in the process of this project. Ptn KO mice on a 129 background showed several alterations in primitive and mature hematopoietic populations due to the loss of Ptn expression accompanied by alterations in proliferation in stress-induced hematopoiesis. However, no gross changes in hematopoiesis were found in (129xB6)F2 or rederived 129 Ptn KO mice compared to controls. It was shown in repopulation assays that the loss of Ptn expression has no intrinsic effects on HSC function. However, in line with the co-culture results, it was shown that wild-type HSC engrafted in primary $Ptn^{-/-}$ mice showed increased subsequent engraftment in serial transplantation experiments, thus pointing to an enhanced maintenance of HSCs in a Ptn-deficient microenvironment. Moreover, this was associated with increased numbers of Cd34- LSK cells and a dominant regeneration of myeloid cells. In a more detailed study of the early changes in gene expression and protein levels in stromal co-cultures, an early Ctnnb1-independent regulation of Ccnd1, Cebpa and Pparg in LSK cells cultured on Ptn knockdown stromal cells was found. In addition to the functions of Ptn in early hematopoiesis, it was shown that Ptn regulates granulocyte apoptosis through an as yet unidentified mechanism. The results of this project show that secreted factors are important in stem cell maintenance and tissue homeostasis.

1. Introduction

1.1. Hematopoietic stem cells and their regulation

1.1.1. The hematopoietic system

The hematopoietic system comprises many different cell types with varied functions. The immune defence of the mammalian organism consists of white blood cells or leukocytes, consisting of granulocytes, monocytes/macrophages and lymphocytes. Red blood cells, the erythrocytes, are responsible for transporting oxygen and carbon dioxide. Platelets are needed for blood coagulation after injuries. Even in a healthy organism, there is a constant need to replenish the number of these cells since they only have a limited lifespan. In case of an injury or exposure to toxic agents, the hematopoietic system needs to be able to produce an even higher amount of blood cells in a rather short time. A single subset of cells in the bone marrow is responsible for the generation of all blood cells: the hematopoietic stem cells (HSC). One single HSC is capable of repopulating the whole bone marrow compartment through a sequence of proliferation and differentiation steps. Another important characteristic of HSCs is their ability to self-renew, meaning that after division one or even both daughter cells remain in the multipotent HSC status instead of differentiating. This feature of HSCs prevents an exhaustion of these cells and assures the upholding of hematopoiesis over the lifetime of the organism (Akala and Clarke 2006).

1.1.2. The hematopoietic hierarchy

The adult hematopoietic hierarchy in the mouse has been well studied. The characterization of the constituent cells of the hematopoietic system has mainly been carried out by flow cytometry (Figure 1). Using this methodology, the hematopoietic hierarchy can be organized in distinct subpopulations of cells, based on the expression, or lack thereof, of surface antigens. At the top of this hematopoietic hierarchy stands the long-term hematopoietic stem cell (LT-HSC). These cells are able

to provide a long-lasting ability to restore hematopoiesis in a clonal manner and maintain hematopoiesis throughout a lifetime (Weissman *et al.* 2001). Beside lacking the expression of lineage markers (lin), LT-HSCs were shown to express Sca1 (Ly-6A/E) (Spangrude *et al.* 1988) as well as the tyrosine kinase receptor c-Kit (CD117) (Ikuta and Weissman 1992). Furthermore, LT-HSCs were recently found to express the surface markers CD150 (Slamf1) (Kiel *et al.* 2005b) and the EPC receptor (Balazs *et al.* 2006). But LT-HSC lack the expression of Cd34 (Osawa *et al.* 1996), Flk2 (CD135) (Christensen and Weissman 2001) and Cd48 (Slamf2) (Kiel *et al.* 2005b). In contrast to the generally quiescent LT-HSCs, activated HSCs are only able to reconstitute the hematopoietic system over a few weeks time (Weissman *et al.* 2001). These so-called short-term HSCs (ST-HSC) can be distinguished from the LT-HSC by the expression of Cd34 (Wilson *et al.* 2007). ST-HSCs further differentiate into multipotent progenitors; at this stage the separation of lymphoid and myeloid lineages is believed to take place (Lai and Kondo 2008).

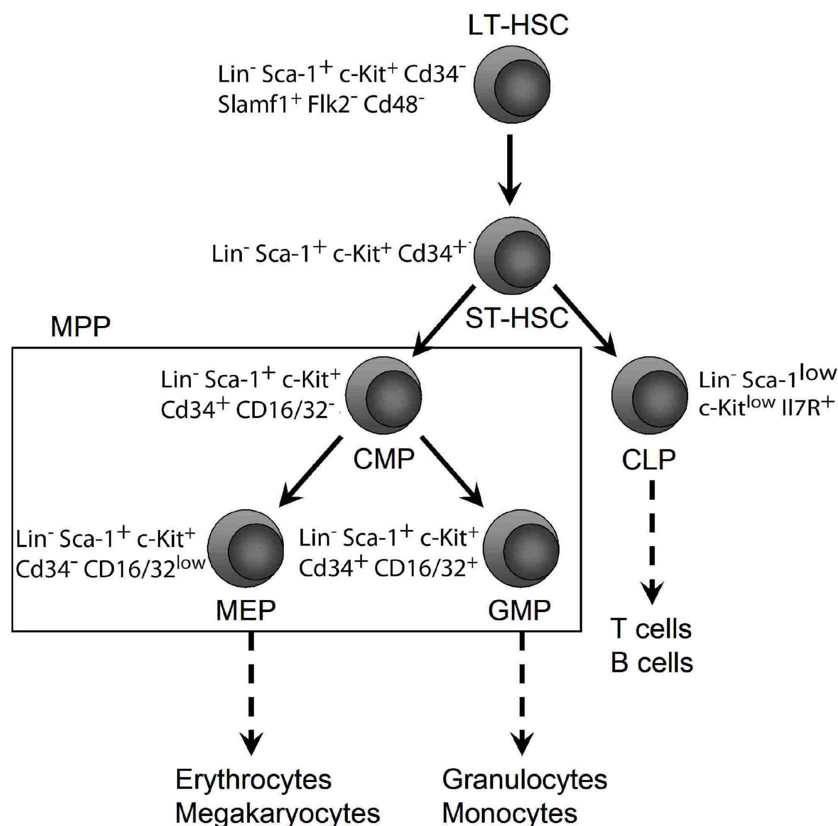


Figure 1. The hematopoietic hierarchy in the adult mouse. LT-HSC: long-term hematopoietic stem cell; ST-HSC: short-term hematopoietic stem cell; MPP: multipotent progenitor; CLP: common lymphoid progenitor; CMP: common myeloid progenitor; MEP: megakaryocyte-erythrocyte progenitor; GMP: granulocyte-monocyte progenitor

Recent findings, however, indicate that the HSC population itself might be heterogenous and can be subdivided into myeloid-biased and lymphoid-biased stem cells with different properties (Muller-Sieburg *et al.* 2002). Myeloid-biased stem cells, which preferentially differentiate to myeloid cells, have been reported to show improved engraftment and self-renewal capacities compared to lymphoid-biased stem cells (Dykstra *et al.* 2007). The separation of lymphoid and myeloid lineages might therefore take place even earlier than at the multipotent progenitor stage.

The multipotent progenitors split into common lymphoid and common myeloid progenitors (CLP and CMP, respectively). CLPs are characterized by the expression of interleukin 7 receptor (Il7R) and a low expression of both Sca-1 and c-Kit (Kondo *et al.* 1997). These cells then further differentiate into the lymphoid lineages, such as T and B lymphocytes and NK cells. CMPs lack the expression of Sca-1, but they are positive for the surface markers c-Kit and Cd34. In an additional differentiation step, they turn into granulocyte-monocyte progenitors (GMP) and megakaryocyte-erythroid progenitors (MEP). GMPs are defined by the expression of Cd34 and Fcγ Receptor (CD16/32), while MEPs show no expression of Cd34 and low expression of CD16/32 (Akashi *et al.* 2000). These progenitors then differentiate into mature myeloid cells cycling in the peripheral blood and populating the other hematopoietic organs like the spleen or the lymph nodes. It was long thought that the lymphoid and the myeloid lineages are strictly separated, but recent reports showed that under special conditions lymphoid progenitors are able to grow to cells belonging to the myeloid lineage, indicating that the separation of the lineages might not be as clear cut as thought before (Bell and Bhandoola 2008; Wada *et al.* 2008).

1.1.3. The hematopoietic stem cell niche

As it was proposed decades ago, HSCs do not reside in the bone marrow by themselves, but are associated with other cells regulating their behaviour (Schofield 1978). These cells together form a special microenvironment, referred to as the hematopoietic stem cell niche (Figure 2) (Fuchs *et al.* 2004; Morrison and Spradling 2008). The niche is essential for the regulation of self-renewal and differentiation of

HSCs. The tightness of this regulation is crucial for the maintenance of a functional blood cell system throughout the lifetime of an organism. Hence, there needs to be enough differentiation to maintain all the cells of the hematopoietic system, even after several insults. In addition, self-renewal is necessary to conserve the stem cell pool itself. Also, even the slightest dysregulation in the survival and/or proliferation of cells can lead to the production of such a huge amount of progeny that it could lead to leukemia. The cells of the niche achieve this surveillance of the HSCs by controlling the cell cycle as well as the adhesion of the HSC to its niche (Yin and Li 2006).

Which cells actually form the niche, however, is still a matter of debate. Two niches have been proposed: the endosteal niche, located in the trabecular bone (Nilsson *et al.* 2001), and the perivascular niche (Heissig *et al.* 2002), with the HSCs residing in close proximity to the sinusoidal endothelium (reviewed in: Kiel and Morrison 2008). HSCs (detected by SLAM markers) have been found in both places (Kiel *et al.* 2005b). Several studies suggest that osteoblasts are an important part of niches located at the endosteal bone surface (Taichman and Emerson 1994; Calvi *et al.* 2003; Zhang *et al.* 2003a). *In vivo* ablation of osteoblasts leads to hematopoietic failure (Visnjic *et al.* 2004). However, the same is also observed when endothelial cells are ablated (Yao *et al.* 2005). It has also been suggested that there might be a "maintenance niche" with resting HSCs which then move to the "expansion niche" in order to proliferate and differentiate (Lo Celso *et al.* 2009; Xie *et al.* 2009). However,

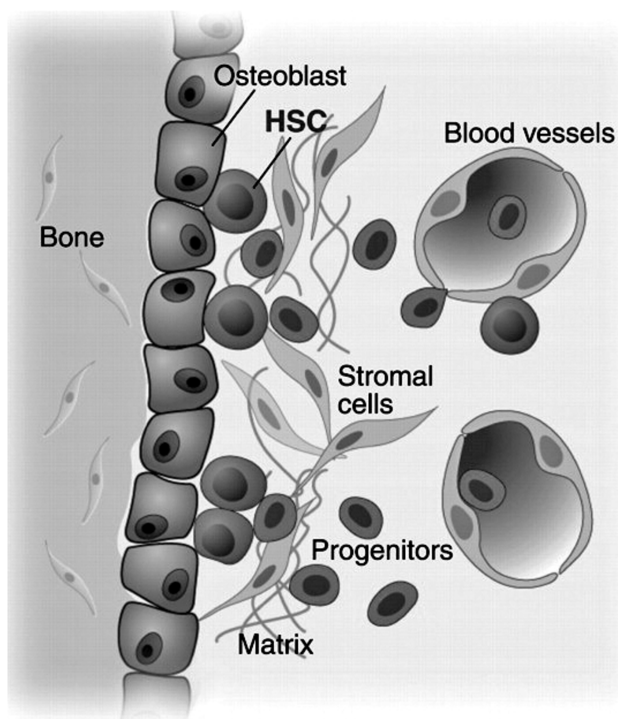


Figure 2. Schematic diagram of hematopoietic and niche cellular components in the bone marrow (Moore and Lemischka 2006).

it is not clear if there really exist two different niches or if there is only one niche which comprises both cell types (Garrett and Emerson 2009).

1.1.4. Cell cycle regulation of HSC

An important characteristic of an HSC is its quiescence. Most of the time, HSCs are in the G0 or G1 phase of the cell cycle, and proliferate only infrequently in order to restock the progenitor pool and to maintain the HSC population (Cheshier *et al.* 1999). Decreased proliferation would lead to a decline in HSCs and progenitors and subsequently in mature cell numbers. Enhanced proliferation, on the other hand, retrieves the danger of disturbing the tissue homeostasis by huge numbers of daughter cells as well as risking the accumulation of oncogenic events due to mistakes in DNA replication or mitosis (Martinez-Agosto *et al.* 2007). Moreover, HSCs would not be able to maintain permanent proliferation for the lifetime of an organism, as is seen in serial transplantations where HSCs are repeatedly forced to reconstitute the whole hematopoietic system. In the first, the primary transplantation, HSCs should be able to reconstitute all the blood cells. But, it has been noted that after several serial transplantations, wt HSCs become exhausted (Siminovitch *et al.* 1964; Bell and Van Zant 2004). Nevertheless, enhanced proliferation is needed in times of hematopoietic stress. Hence, HSCs must be able to respond to injuries or other disturbances of the hematopoietic system, but on the other hand need to return to G0/G1 as soon as homeostasis is reached (Wilson *et al.* 2008). The mechanisms of this return to G0/G1, however, are widely unknown at present.

Models of stem cell exhaustion

Experiences with different mouse inbred strains as well as different genetic models suggest that an enhanced proliferation of HSCs leads to their progressive loss of function (reviewed in: Orford and Scadden 2008; Wilson *et al.* 2009). Comparing different mouse inbred strains, a negative correlation was found between the proliferative rate of its HSCs and the longevity of the strain (de Haan *et al.* 1997).

As one of the first of different genetic models showing similar phenotypes, HSCs deficient for the cell-cycle regulator Cdkn1a (cyclin-dependent kinase inhibitor 1A, p21) were shown to act poorly in serial transplantation because of an accelerated exhaustion. It was found that, compared to wildtype controls, a greater percentage of HSCs had left the quiescent G0/G1 phase of the cell cycle (Cheng *et al.* 2000).

Another example are mice deficient for the zinc-finger repressor growth factor independent 1 (Gfi1). HSCs of these mice proliferated at a higher rate than wildtype controls and were able to reconstitute the whole bone marrow compartment. Nevertheless, in a challenge with wildtype HSCs, they failed to reconstitute the peripheral blood, showing severe loss of stem cell function. Cdkn1a expression in $Gfi^{-/-}$ HSCs was largely reduced, so it is not surprising that these mice develop similar phenotypes (Hock *et al.* 2004). A reduced expression of Cdkn1a was also found in Tcf3 (Transcription factor 3)-deficient mice. Consequently, quiescence of LSK cells was disturbed in these mice (Yang *et al.* 2008).

mTORC1 (mammalian target of rapamycin complex 1) is an important part of different signalling pathways. An increase in mTORC1 activity leads to enhanced HSC proliferation as noticed in various genetic models deficient in negative regulators of mTORC1 (Pten (phosphatase and tensin homolog) (Yilmaz *et al.* 2006; Zhang *et al.* 2006a), TSC1 (tuberous sclerosis 1) (Chen *et al.* 2008), PML (promyelocytic leukemia) (Ito *et al.* 2008), Fbw7 (F-box and WD-40 domain protein 7) (Thompson *et al.* 2008)). Like in the $Cdkn1a^{-/-}$ or $Gfi1^{-/-}$ mice, this increase in HSC proliferation leads to a progressive loss of HSC function (Gan and DePinho 2009).

HSCs with deficiencies for the transcription factor FoxO3a (forkhead box protein 3a) (Miyamoto *et al.* 2007), the developmental regulator Pbx1 (pre B-cell leukemia transcription factor 1) (Ficara *et al.* 2008) or the compound knockout of the 3 retinoblastoma family members Rb (retinoblastoma 1), p107 (Rbl1, retinoblastoma-like 1) and p130 (Rbl2, retinoblastoma-like 2) (Viatour *et al.* 2008) all show similar defects to the ones mentioned above: Proliferation of these distorted HSCs is enhanced, leading to a loss of HSC numbers and function over time.

There are, however, genetic models contradicting these results: in these mice, the enhanced proliferation of HSCs apparently does not consequently lead to their exhaustion.

Gene	KO/over-expr.	Cell-cycle entry	Stem cell function	Reference
Cdkn1a	KO	increased	reduced repopulation capacity in SRA	(Cheng *et al.* 2000)
Fbw7	Cond. KO	increased	reduced repopulation capacity in CRA	(Thompson *et al.* 2008)
FoxO1, 3 and 4	KO	increased	reduction in HSC function	(Tothova *et al.* 2007)
FoxO3a	KO	increased	reduced repopulation capacity in CRA	(Miyamoto *et al.* 2007)
Gfi1	KO	increased	reduced repopulation capacity in CRA	(Hock *et al.* 2004)
Lnk	KO	decreased	increased HSC numbers increased engraftment in CRAs	(Bersenev *et al.* 2008)
Elf4	KO	decreased	increased HSC number and function	(Lacorazza *et al.* 2006)
Pbx1	Cond. KO	increased	reduced repopulation capacity in SRA	(Ficara *et al.* 2008)
Pml	KO	increased	reduced repopulation capacity in CRA	(Ito *et al.* 2008)
Pten	KO	increased	short-term increase in immunophenotypic HSCs long-term loss of HSCs	(Zhang *et al.* 2006a)
Rb, p107 and p130	Cond. KO	increased	reduced repopulation capacity in RA	(Viatour *et al.* 2008)
SHIP	KO	increased	increased HSC numbers reduced repopulation capacity	(Desponts *et al.* 2006)
Stat5	Cond. KO	increased	depletion of HSC pool	(Wang *et al.* 2009)
Tal1	KO	increased	impaired potential in RAs	(Lacombe *et al.*)
Tsc1	Cond. KO	increased	reduced repopulation capacity in CRA and SRA	(Chen *et al.* 2008)
Txnip	KO	increased	HSC exhaustion	(Jeong *et al.* 2009)
c-Cbl	KO	increased	increased HSC numbers increased repopulation capacity in CRA	(Rathinam *et al.* 2008)
Cdkn2c	KO	increased	increased HSC number and function	(Yuan *et al.* 2004)
HoxB4	Over-expr.	increased	highly competitive in CRAs ex vivo expansion of HSCS	(Antonchuk *et al.* 2001)
Slug	KO	increased	increased repopulation potential in CRA and SRA	(Sun *et al.* 2010)

Table 1. Summary of genetic models with altered HSC cell cycle entry. CRA: competitive repopulation assay, HSC: hematopoietic stem cell, KO: knockout, over-expr.: over-expression, RA: repopulation assay, SRA: serial repopulation assay (modified from: Orford and Scadden 2008).

Models of stem cell increase

HSCs deficient for the cell cycle regulator Cdkn2c (cyclin-dependent kinase inhibitor 2C, p18Ink4c) were increased in numbers and proliferation rate. In competitive transplantation assays these HSCs reconstituted the hematopoietic system more efficiently than wildtype HSCs, even after tertiary transplantation (Yuan *et al.* 2004; Yu *et al.* 2006).

Similar effects were seen in HSCs with an enforced expression of the transcription factor Hoxb4 (homeobox B4) (Thorsteinsdottir *et al.* 1999; Antonchuk *et al.* 2001) or a loss of the E3 ubiquitin ligase c-Cbl (Casitas B-lineage lymphoma) (Rathinam *et al.* 2008). In both models, HSC proliferation was enhanced, leading to an advantage in competitive repopulation assays with wildtype HSCs.

Thus, an enhanced proliferation rate of HSCs mostly, but not always, leads to their exhaustion.

1.1.5. Regulation of HSC function by the microenvironment

For the regulation of HSCs through the microenvironment, it is important that the HSC is in close proximity to the cells of its niche. This contact is achieved by different pairs of adhesion molecules, including N-cadherin (Cdh2)/β-catenin (Ctnnb1), vascular cell adhesion molecule 1 (Vcam1)/integrin and osteopontin (Spp1, secreted phosphoprotein 1)/β_1 integrin (Itgb1) (Yin and Li 2006). The precise functions of these molecules has yet been clarified, but adhesion seems to be an important part of the regulation by the microenvironment, as an untimely release of HSCs or progenitors from the niche can lead to severe deregulations of the hematopoietic system as seen in mice with forced expression of c-Myc (myelocytomatosis oncogene) in HSCs. Overrepresentation of c-Myc in the stem cells represses the expression of adhesion molecules. This leads to the exit of the stem cells from the niche and subsequently to an enhanced differentiation of these cells at the cost of an reduced self-renewal, so that HSCs are progressively lost (Wilson *et al.* 2004). Another example are mice with a conditional knockout of Cdc42 (cell division cycle 42 homolog). The loss of Cdc42 caused an impaired adhesion of the HSCs to their niches, causing massive egress of

HSCs from the bone marrow and subsequently resulting in engraftment failure (Yang *et al.* 2007).

Several factors and pathways have been identified as being important in the niche-HSC interaction (reviewed in: Renstrom *et al.* 2009b). To begin with, osteoblasts and bone marrow stromal cells secrete a number of growth factors like G-CSF (Csf3, colony stimulating factor 3) (Taichman and Emerson 1994), GM-CSF (Csf2, colony stimulating factor 2) (Kittler *et al.* 1992), SCF (stem cell factor, Kit-Ligand), Sdf1 (stromal cell-derived factor 1α, Cxcl12), TGF-β (transforming growth factor β) (Yamazaki *et al.* 2009) or Il-6 (interleukine-6 (Taichman *et al.* 1997)). Several of these factors are required for motility, homing or docking of HSC to the niche. The interruption of the interaction of SCF with its receptor c-Kit, for example was shown to lead to hematopoietic failure, caused in an impaired HSC motility (Bernstein *et al.* 1991; Heissig *et al.* 2002). The interaction of Sdf1 with its receptor was found to be essential for maintaining HSC quiescence (Sugiyama *et al.* 2006; Nie *et al.* 2008). Likewise, Tie2 (Tek, endothelial-specific receptor tyrosine kinase) and its ligand Ang1 (Angpt1, angiopoetin1) are required for the maintenance of quiescence of HSC and for the adhesion of HSCs to their niches (Arai *et al.* 2004).

Another possible pathway for niche-HSC interaction is Notch signalling (for review: Weber and Calvi 2010). Notch signalling was shown to be active in the most primitive HSCs (Duncan *et al.* 2005) and it was established that Notch ligands Jagged1 and Delta-like1 are expressed in osteoblasts (Nobta *et al.* 2005). The interaction of Jagged1 (Jag1) and the receptor Notch1 (Notch homolog 1) was found to impair differentiation both *in vitro* and *in vivo* (Li *et al.* 1998; Stier *et al.* 2002). Furthermore, an enforced expression of Notch1 increased the self-renewal of LSK cells (Varnum-Finney *et al.* 2000). It was also reported that the inhibition of Notch signalling in HSCs led to the depletion of this population after transplantation (Duncan *et al.* 2005). On the contrary, conditional knockouts for Jagged1 or Notch1 were shown to leave steady-state hematopoiesis undisturbed (Mancini *et al.* 2005). Even the inhibition of all Notch receptors known in mammals (Notch 1-4) did not

impair the long-term repopulation capabilities of the HSCs (Maillard *et al.* 2008), leaving the role of Notch signalling uncertain so far.

Several genetic models suggest that Wnt (Wingless) signalling is important for the crosstalk of HSCs and their niches. Both the canonical as well as the non-canonical pathway seems to be involved in these processes. For example, Wnt3a, a ligand of the canonical pathway, was found to promote HSC self-renewal (Luis *et al.* 2009). This effect seems to be associated with the Notch pathway, since the inhibition of Notch signalling diminishes the enhanced self-renewal observed in the presence of Wnt3a (Duncan *et al.* 2005), pointing to a crosstalk between the two pathways. On the other hand, a promotion of HSC self-renewal was also detected by a ligand of the non-canonical pathway, Wnt5a (Murdoch *et al.* 2003; Nemeth *et al.* 2007). Ctnnb1 is one of the key targets of Wnt signalling. Enhanced Ctnnb1 levels in HSCs lead to increased HSC numbers, but also to a differentiation block (Kirstetter *et al.* 2006; Scheller *et al.* 2006). Nevertheless, the conditional knockout of Ctnnb1 leaves the HSC pool undisturbed (Cobas *et al.* 2004). Beside these intrinsic functions of Ctnnb1, its function in the extrinsic regulation of HSCs has been investigated. It was found that the enforced expression of Ctnnb1 in bone marrow cells leads to enhanced HSC maintenance and engraftment (Kim *et al.* 2009; Nemeth *et al.* 2009). It has also been shown that mice overexpressing the canonical inhibitor Dkk1 (dickkopf homolog 1) in stromal cells suffer from a progressive loss of HSCs (Fleming *et al.* 2008). Furthermore, during the course of this project, we have shown that Sfrp1 (secreted frizzled related protein 1) secreted by the microenvironment regulates maintenance of HSCs (Renstrom *et al.* 2009a).

Although some aspects of the regulation of HSC by the microenvironment have been revealed, many mechanisms still remain unclear. Therefore, it was so far not possible to expand HSCs in stroma cell free cultures. It was long though that stromal cells and HSCs in culture have to be in direct contact for maintaining the HSC function, but Robert Oostendorp established two stromal cell lines, EL08-1D2 and

UG26-1B6, which were able to maintain HSCs even without direct contact (Oostendorp *et al.* 2005). For investigating the soluble factors responsible for this HSC maintenance, RNA arrays were prepared comparing these two cell lines with a couple of stromal cell lines not capable of HSC maintenance without direct contact. One of the genes which were overrepresented in the two supportive cell lines was the cytokine Pleiotrophin (Oostendorp *et al.* 2005).

1.2. Pleiotrophin

Pleiotrophin (Ptn) is a small (18 kDa) heparin-binding cytokine, also called HB-GAM (heparin-binding growth-associated molecule), Osf-1 (Osteoblast stimulating factor 1), HARP (heparin-affin regulatory peptide) or HBNF (heparin-binding neurite-promoting factor). It was first purified in 1989 from neonatal rat brain as a neurite outgrowth promoting factor (Rauvala 1989) and in the same year independently discovered as a growth-promoting factor for fibroblasts, found in bovine uterus (Milner *et al.* 1989).

Ptn has been found in various mammals so far and has been shown to be highly conserved between the species, actually being one of the highest conserved cytokines known so far (reviewed in: Deuel *et al.* 2002). The human, mouse, rat, bovine and chicken proteins each consists of 136 amino acids plus an N-terminal signal sequence for secretion. The murine Ptn thereby differs from the human protein by only four amino acids.

Pleiotrophin is part of a small family with only one other member, Midkine (Mk) (Kovesdi *et al.* 1990). The amino acid sequence of Midkine is about 50% identical with Ptn, and both proteins have to some extent overlapping functions (reviewed in: Muramatsu 2002). Since screening of the human genome sequence has not led to the discovery of further family members, it is so far assumed that Ptn and Mk might be the only two members of this family (Deuel *et al.* 2002).

The structure of Ptn has been clarified, revealing that Ptn contains two beta-sheet domains connected by a flexible linker. These beta-sheet domains are homologous to the thrombospondin type I repeat (TSR), a domain often occurring in proteins that

take part in cell/extracellular matrix or cell/cell interactions. The N- and C-terminal regions of Pleiotrophin, being very rich in lysine residues, appear to form random coils (Kilpelainen *et al.* 2000). Ptn contains 10 cysteine residues, which are all forming disulfide bonds (Hulmes *et al.* 1993). The complexity of the formation of these disulfide bridges might be an explanation for the insufficient biological activity and improper folding often observed for Ptn synthesized in prokaryotic or insect cell expression systems (Deuel *et al.* 2002).

During development, Ptn mRNA is expressed in many neuroectodermal and mesodermal lineages, exhibiting a distinct pattern, specific for time point and cell-type. After birth, Ptn expression faded in most locations, leading to constitutive expression in the adult, restricted to specific celltypes such as bone progenitor cells or different cell types in the nervous system and the reproductive tract (Vanderwinden *et al.* 1992).

1.2.1. Functions of Pleiotrophin

As the name implies, Pleiotrophin has various functions, thereby performing distinct and often opposing tasks in dependence of the type of target cell. For example, it has been shown that Ptn is acting as a mitogen for fibroblasts (Milner *et al.* 1989; Fang *et al.* 1992), endothelial (Fang *et al.* 1992; Laaroubi *et al.* 1994; Papadimitriou *et al.* 2000) and epithelial cells (Fang *et al.* 1992; Delbe *et al.* 1995) as well as for carcinoma cells (see below), but it is inhibiting proliferation in neural stem cells (Hienola *et al.* 2004). It was also found to be a survival factor for human embryonic stem cells (Soh *et al.* 2007) and for premalignant SW13 and serum-starved NIH 3T3 cells (Bowden *et al.* 2002). On the other hand, after myocardial injury, Ptn enhances the apoptosis of cardiomyocytes at the injured site (Li *et al.* 2007).

Pleiotrophin is known to act as an angiogenic factor. After ischemic injury in rats, Ptn expression is strongly and selectively enhanced in endothelial cells of the newly forming blood vessels as well as in the surrounding macrophages and activated astrocytes (Yeh *et al.* 1998). It was also directly shown *in vivo* an *in vitro* that Pleiotrophin is able to initiate the formation of functioning blood vessels

(Papadimitriou *et al.* 2001; Christman *et al.* 2005). Nevertheless, it was recently shown that Ptn is able to inhibit VEGF-induced angiogenesis (Heroult *et al.* 2004).

Pleiotrophin can perform its task as an angiogenesis initiator in part by inducing migration of endothelial cells and early endothelial progenitor cells to the sites where new vessels should form (Souttou *et al.* 2001; Heiss *et al.* 2008). It also induces haptotactic migration in glioblastoma cells (Ulbricht *et al.* 2003).

Apart from ischemic injury, Pleiotrophin also takes part in other repair mechanisms, namely it was found to be involved in the repair mechanisms after peripheral nerve injury (Takeda *et al.* 1995; Blondet *et al.* 2005) as well as in liver regeneration after partial hepatectomy (Ochiai *et al.* 2004) and to be upregulated in the dermis after occurrence of an incisional wound in rat skin (Deuel *et al.* 2002). Pleiotrophin is also involved in fracture healing. It was shown that after tibia fracture, Ptn was highly expressed in osteoblasts, endothelial cells and fibroblasts in the newly formed bone (Petersen *et al.* 2004). In old Ptn overexpressing mice, however, fracture healing was impaired, attended by an delayed callus formation and remodelling (Li *et al.* 2005a).

Besides being involved in repair after nerve injury, Pleiotrophin also has other important roles in the nervous system. Long-term potentiation in the hippocampus is suppressed by Ptn, showing that the protein is involved in the regulation of synaptic plasticity (Lauri *et al.* 1998; Amet *et al.* 2001; Pavlov *et al.* 2002). In culture, Ptn triggers neurite outgrowth from various neuronal cell types including glial progenitor cells or embryonic neurons (Rauvala 1989; Yanagisawa *et al.* 2009).

Ptn is highly expressed by osteoblasts and this has given Ptn the alternative name of osteoblast-stimulating factor 1 (Tezuka *et al.* 1990; Petersen and Rafii 2001). It was shown that Ptn is able to promote adhesion, expansion, and differentiation of human osteoprogenitor cells (Yang *et al.* 2003). Ptn can also act as a chemotactic agent for osteoblastic cells (Li *et al.* 2005b). It is not clear yet, which Ptn signalling pathway is responsible for the migratory effect on osteoblasts, since both RPTP β/ζ as well as N-syndecan have been designated to be the receptor that mediates the effect of Ptn in

osteoblasts (Imai *et al.* 1998; Tare *et al.* 2002b; Schinke *et al.* 2008). Moreover, the addition of Ptn to cultures of human osteoblast-like osteosarcoma cell lines led to an enhanced attachment of these cells, indicating that Ptn might promote osteoblast attachment to the extracellular bone matrix (Gieffers *et al.* 1993).

Pleiotrophin has varying effects on bone formation which are dependent on its concentration and time of expression. This was shown in a study with mouse bone marrow cells (Tare *et al.* 2002a). Osteogenic differentiation of these cells was stimulated by low Ptn concentration, whereas higher concentrations showed no effect. The presence of Ptn together with rhBMP-2 (recombinant human bone morphogenetic protein 2) during osteoinduction in premyoblastic C2C12 cells inhibited the formation of osteocytes. When Ptn was added after osteoinduction had taken place, however, it stimulated the osteogenic differentiation. Bone formation in mice with enhanced expression of Ptn was also disordered. The mice showed an increased bone thickness in females (Imai *et al.* 1998; Hashimoto-Gotoh *et al.* 2004) and an higher calcium content compared to controls (Tare *et al.* 2002b). Ptn knockout mice were first reported having no bone phenotype at all, including normal numbers and functioning of osteoblasts, osteoclasts and osteocytes (Lehmann *et al.* 2004). More recently, however, it was found that Ptn knockout mice show osteopenia and a decrease in osteoblast activity (Imai *et al.* 2009).

Not much is known about the role of Pleiotrophin in hematopoiesis. It was found that Ptn acts as a proliferation factor for quiescent human peripheral blood mononuclear cells (Achour *et al.* 2001). Furthermore, it induces the expression of inflammatory cytokines including TNF-α, IL-1β, and IL-6 in these cells (Achour *et al.* 2008). The expression of Ptn in monocytes was shown to lead to a downregulation of monocytic cell markers and at the same time an upregulation of endothelial cell markers (Sharifi *et al.* 2006). Beside all that, Pleiotrophin was found to negatively regulate adipogenesis (Gu *et al.* 2007).

Pleiotrophin and cancer

Pleiotrophin is known as a proto-oncogene. Its forced expression is able to transform NIH 3T3 cells and leads to tumor formation in nude mice (Chauhan *et al.* 1993). However, the overexpression of Ptn is not sufficient for inducing breast cancer, but it promotes the progression of the cancer (Chang *et al.* 2007). Pleiotrophin was found to be overrepresented in various types of cancer, including melanomas (Wu *et al.* 2005), lung cancer (Jager *et al.* 1997), pancreatic cancer (Souttou *et al.* 1998; Weber *et al.* 2000), colon cancer (Souttou *et al.* 1998), testicular cancer (Aigner *et al.* 2003), prostate cancer (Vacherot *et al.* 1999), breast cancer (Fang *et al.* 1992) and multiple myeloma (Yeh *et al.* 2006). Moreover, its expression levels were shown to be correlated with disease status amongst others in multiple myeloma (Yeh *et al.* 2006), lung cancer (Jager *et al.* 2002) and pancreatic cancer (Klomp *et al.* 2002), presenting a possible marker for treatment success and prognosis. Ptn was also suggested as a possible target for treatment, as it was shown in melanoma (Czubayko *et al.* 1994; Grzelinski *et al.* 2005; Wu *et al.* 2005), breast cancer cell lines (Zhang *et al.* 1997; Duces *et al.* 2008), glioblastoma (Grzelinski *et al.* 2005) and multiple myeloma (Chen *et al.* 2007), that the expression of Ptn is rate-limiting for the malignant progression. As opposed to this, Ptn expression was found to be suppressed in NIH3T3 transformed by expression of various oncogenes like, for example, ras (Corbley 1997) as well as in colorectal cancer (Yamakawa *et al.* 1999).

An important role of Pleiotrophin in tumor progression is its ability to act as an angiogenesis factor (Souttou *et al.* 2001). In cancer, this angiogenic feature of Ptn leads to rapid tumor growth and a more aggressive tumor phenotype due to the formation of new blood vessels in the malignant tumor (Zhang *et al.* 2006b; Chang *et al.* 2007).

1.2.2. Signalling pathways of Pleiotrophin

Pleiotrophin was shown to bind different receptors and thus it takes part in various signalling pathways, raising thereby a possible explanation for the varying effects on its target cells.

On the surface of many cell types, Pleiotrophin binds the receptor protein tyrosine phosphatase (RPTP) β/ζ (reviewed in: Perez-Pinera *et al.* 2007a). It thereby binds with high affinity to the chondroitin sulphate portion of RPTP β/ζ and with low affinity to the protein portion (Maeda *et al.* 1996; Maeda *et al.* 1999). Notably, Ptn was the first natural ligand found to bind a receptor tyrosine phosphatase (Meng *et al.* 2000). Active receptor tyrosine phosphatases are important for obtaining steady levels of protein tyrosine phosphorylation of diverse substrates in the cell, which are constantly phosphorylated by different tyrosine kinases. The binding of Pleiotrophin to its receptor triggers the dimerization of RPTP β/ζ, leaving the active sites of the dimerized RPTP β/ζ no longer accessible for its substrates (Majeti *et al.* 1998; Fukada *et al.* 2006). The inactivation of RPTP β/ζ therefore leads to an ascending phosphorylation status of its various substrates. The first substrate found to be higher phosphorylated by this mechanism was Ctnnb1 (Meng *et al.* 2000). In fetal lung cells, the increased phosphorylation of Ctnnb1 due to Ptn signalling was shown to lead to its translocation to the nucleus; there, Ctnnb1 activated the expression of Dlk1 (Delta-like 1 homolog), a member of the Notch signalling pathway (Weng *et al.* 2009). Among the other substrates of RPTP β/ζ discovered so far are β-adducin (Add2) (Pariser *et al.* 2005b; Pariser *et al.* 2005c), Fyn (Fyn proto-oncogene) (Pariser *et al.* 2005a), p190RhoGAP (Tamura *et al.* 2006), Git1 (G protein-coupled receptor kinase-interactor 1) (Kawachi *et al.* 2001), Magi1 (membrane associated guanylate kinase, WW and PDZ domain containing 1) (Fukada *et al.* 2006) and ALK (anaplastic lymphoma kinase) (Perez-Pinera *et al.* 2007b). If Pleiotrophin, however takes part in signalling through ALK by the indirect action through RPTP β/ζ (Mathivet *et al.* 2007; Perez-Pinera *et al.* 2007b), or by direct contact with the ALK receptor (Stoica *et al.* 2001; Bowden *et al.* 2002; Yanagisawa *et al.* 2009), is still a matter of debate.

Another receptor of Pleiotrophin is N-syndecan (Syndecan-3), which seems to be an important receptor of Ptn in neuronal cells (Raulo *et al.* 1994). The binding of Ptn to the heparin sulphate side chains of N-syndecan was shown to be essential for the

induction of neurite outgrowth (Kinnunen *et al.* 1996), and deficiency in N-syndecan impairs neural migration in the brain (Hienola *et al.* 2006)

It was further reported that Pleiotrophin is able to bind to nucleolin on the cell surface. This binding leads to the translocation of Ptn to the nucleus (Take *et al.* 1994; Said *et al.* 2005).

1.2.3. Pleiotrophin and stem cells

So far, the effects of Pleiotrophin on stem cell function have barely been investigated. There are two fundamentally different classes of stem cells, the continuously proliferating (like embryonic stem cells) and the adult quiescent stem cells (neural stem cells, hematopoietic stem cell etc.). It was found that adding Ptn to feeder-free cultures of human embryonic stem cells (hESC) led to an enhanced retrieval of undifferentiated cells. The knockdown of the receptor RPTP β/ζ did not alter the proliferation of the hESCs but led to an increased apoptosis rate. It was therefore proposed that Ptn maintains hESCs mainly by protecting the undifferentiated cells from apoptosis (Soh *et al.* 2007).

For another study, transgenic mice were generated which expressed the residues 1-40 of Ptn. It had been shown before, that this mutant Ptn dimerizes with wt Ptn and thereby inhibits its functions (Zhang *et al.* 1997). In germ cells of male mice, the expression of this dominant-negative Ptn mutant led to a highly enhanced rate of programmed cell death at all stages of development (Zhang *et al.* 1999).

The effects of Pleiotrophin on neural stem cells have also been investigated. It was found that Ptn KO mice exhibit an increased neuronal density in the cerebral cortex. This was caused by enhanced stem cell proliferation in the embryo. There was no change in apoptosis rates detectable, neither in the embryo or post-natal. It was further found that adding Ptn to neurospheres led to the differentiation of the neural stem cells (Hienola *et al.* 2004; Jung *et al.* 2004).

1.3. Aim of this thesis

Pleiotrophin was found to be higher expressed by stromal cell lines, which are able to support HSC maintenance without direct contact with the HSCs in comparison to non-supporting cell lines (see 1.1.5). Moreover, Ptn is highly expressed in osteoblastic cells (Tezuka *et al.* 1990; Petersen and Rafii 2001), which are believed to form an important part of the HSC niche (Taichman and Emerson 1994; Calvi *et al.* 2003; Zhang *et al.* 2003a). In addition to that, Ptn has already been shown to take part in proliferation and differentiation mechanisms in neural stem cells. Nevertheless, the involvement of Pleiotrophin in the regulation of HSC function has never been investigated.

The aim of this thesis was therefore to analyse the effects of the loss of Pleiotrophin *in vivo* and *in vitro* in order to define the role of Ptn in maintenance of HSC function and hematopoiesis. Furthermore, we explored which molecular signalling pathways might be affected by Ptn.

2. Materials and methods

2.1. Materials

2.1.1. Mice

129 Ptn KO mice	129S6x129Ola HB-GAM$^{-/-}$ (Amet *et al.* 2001)
(129xB6)F2 Ptn KO mice	(129PtnKO x C57Bl/6.J)F2
rederived 129 Ptn KO mice	(129PtnKO x 129S2)F2
129S2	
C57BL/6.Pep3b (Cd45.1)	
C57BL/6.J (B6, B6.Cd45.2)	

2.1.2. Cell lines

UG26-1B6	stromal cell line derived from murine embryonic urogenital ridge
EL08-1D2	stromal cell line derived from murine embryonic liver
AM30-3F4	stromal cell line derived from murine embryonic AGM region
EL28-1B3	stromal cell line derived from murine embryonic liver
UG15-1B7	stromal cell line derived from murine embryonic urogenital ridge
◎NX (Phoenix) Eco	293T cell line with vectors for retroviral packaging and envelope protein infectious for murine cells

2.1.3. Antibodies

Surface staining for Flowcytometry

Antigen	Clone	Colour	Volume/1·10^6 cells	Company
CD3ε	145-2C11	biotinylated	0.5 µl	eBioscience
Cd4	GK1.5	PE, PE-Cy5	0.5 µl	eBioscience
Cd8a	53-6.7	PE, PE-Cy5	0.5 µl	eBioscience
Cd11b	M1/70	PE-Cy5	0.5 µl	eBioscience
CD16/32	93	PE, PE-Cy5.5 eFluor405	0.3 µl (PE, PC5.5) 0.6 µl (eFluor405)	eBioscience

Antigen	Clone	Colour	Volume/1·10^6 cells	Company
Cd34	RAM34	FITC, AlexaFluor647	0.5 µl (FITC) 1 µl (AlexaFluor647)	eBioscience
Cd43	S7	FITC	0.5 µl	BD Biosciences
Cd45.1	A20	PE, APC-eFluor780	0.8 µl (APC-eFluor780) 0.5 µl	eBioscience
Cd45.2	104	FITC	0.5 µl	eBioscience
CD45R (B220)	RA3-6B2	PE-Cy5, eFluor405	0.5 µl	eBioscience
Cd48	HM48-1	biotinylated	0.5 µl	eBioscience
CD71	R17217	biotinylated	0.5 µl	eBioscience
CD117 (c-Kit)	2B8	PE, APC, APC-eFluor780	0.7 µl (PE, APC) 1 µl (APC-eFluor780)	eBioscience
CD127 (IL7Rα)	A7R34	APC	0.7 µl	eBioscience
CD135	A2F10	PE-Cy5	1 µl	eBioscience
CD150	TC15- 12F12.2	PE	1 µl	BioLegend
CD161c (NK1.1)	PK136	FITC	0.7 µl	eBioscience
Gr-1	RB6-8C5	PE eFluor405 biotinylated	0.3 µl 0.5 µl (eFluor405)	eBioscience
IgM	DS-1	PE	0.5 µl	BD Biosciences
lineage (CD3ε, B220, Ter119, Gr-1, Cd11b)		biotinylated	1 µl	eBioscience
Sca-1	D7	PE-Cy5 PE-Cy7	0.4 µl	eBioscience
Ter119	TER-119	FITC PE	0.5 µl	BD Biosciences

Other primary antibodies

Antigen	Antibody	Application	Species/ Conjugation	Dilution	Company
Ccnd1	92G2	Flow cytometry	rabbit	1:100	Cell Signaling
Ctnnb1	L54E2	Single cell staining	AlexaFluor488	1:50	Cell Signaling
Cepba	2295	Single cell staining	rabbit	1:50	Cell signaling
Ptn	ab14025	Western blotting	rabbit	1:500	Abcam
Ctnnb1	6B3	Western blotting	rabbit	1:1000	Cell Signaling
β-actin	AC-74	Western blotting	mouse	1:1000	Sigma-Aldrich

Secondary antibodies

Flow cytometry (0.5 µl/1·10^6cells):

Streptavidin, AlexaFluor488 conjugated (S11223, Invitrogen)

Streptavidin, AlexaFluor610 conjugated (S32359, Invitrogen)

Streptavidin, PE-Cy5.5 conjugated (SA1018, Invitrogen)

Streptavidin, Pacific Blue conjugated (S11222, Invitrogen)

Streptavidin, APC conjugated (S868, Invitrogen)

Western blotting (dilution 1:10000):

Anti-rabbit IgG, HRP conjugated (31460, Pierce)

Anti-mouse IgG, HRP conjugated (31430, Pierce)

Single cell staining/intracellular flow cytometry (dilution 1:200):

Anti-rabbit IgG, AlexaFluor488 conjugated (4412, Cell Signaling)

2.1.4. Primer

Genotyping

Gene name		Sequence	Reference
Ptn	forward	GGAGAATGGCAGTGGAGTGT	
	reverse	TTCCAGTTGCAAGGGATCTT	
Neomycin	forward	GATCGGCCATTGAACAAGAT	
cassette	reverse	ATACTTTCTCGGCAGGAGCA	

Real time PCR

Gene name		Sequence	Reference
Adam17	forward	GTACGTCGATGCAGAGCAAA	
	reverse	AAACCAGAACAGACCCAACG	
C1galt1	forward	AAGGCCAAACATGTCAAAGC	
	reverse	TCTTCGCCCAAAGTAAATGG	
Cdkn1a	forward	TTCCGCACAGGAGCAAAGT	(Passegue *et al.* 2005)
	reverse	CGGCGCAACTGCTCACT	
Cdkn1b	forward	GGCCCGGTCAATCATGAA	(Passegue *et al.* 2005)
	reverse	TTGCGCTGACTCGCTTCTTC	
Cdkn1c	forward	CAGCGGACGATGGAAGAACT	(Passegue *et al.* 2005)
	reverse	CTCCGGTTCCTGCTACATGAA	
Cdkn2a	forward	CCCAACGCCCCGAACT	(Passegue *et al.* 2005)
	reverse	GTGAACGTTGCCCATCATCA	
Cdkn2b	forward	TCAGAGACCAGGCTGTAGCAATC	(Passegue *et al.* 2005)
	reverse	CCCCGGTCTGTGGCAGAA	
Cdkn2c	forward	AACCATCCCAGTCCTTCTGTCA	(Passegue *et al.* 2005)
	reverse	CCCCTTTCCTTTGCTCCTAATC	
Cdkn2d	forward	CGGTATCCACTATGCTTCTGGAA	(Passegue *et al.* 2005)
	reverse	CCGCTGCGCCACTCAA	
Cebpa	forward	GAACAGCAACGAGTACCGGGTA	(Iwasaki *et al.* 2005)
	reverse	CCCATGGCCTTGACCAAGGAG	
Cfl2	forward	TCTCAGCCACAGTTCCTTCA	
	reverse	TCCGGCTAATAGCAGAGAGC	
Cpm	forward	CGACTCCTACCTCACGAAGC	
	reverse	TGGCTTGGCATGAATTTGTA	
Ctnnb1	forward	ACCTTTCAGATGCAGCGACT	
	reverse	TGGCACACCATCATCTTGTT	
Ctsk	forward	CCAGTGGGAGCTATGGAAGA	
	reverse	AAGTGGTTCATGGCCAGTTC	

CyclinD1	forward	TGTTACTTGTAGCGGCCTGTTG	(Passegue *et al.* 2005)
	reverse	CCGGAGACTCAGAGCAAATCC	
CyclinD2	forward	CACGACTTCATTGAGCACATCCT	(Passegue *et al.* 2005)
	reverse	GCGGATCAGGGACAGCTTCT	
CyclinE1	forward	GCAGCGAGCAGGAGACAGA	(Passegue *et al.* 2005)
	reverse	GCTGCTTCCACACCACTGTCTT	
Dlk1	forward	CTGGAGAAAGGCCAGTACGA	
	reverse	AGGGGTACAGCTGTTGGTTG	
Eif2s3y	forward	CGTCAGGATCTTGCCACTTT	
	reverse	ATCGGACAGTGTGAACACCA	
Elavl2	forward	CTTATACGTCAGCGGGCTTC	
	reverse	CCTCAATCCGCTTGTCAAAT	
Gata1	forward	GGCCCCTTGTGAGGCCAGAGAG	(Iwasaki *et al.* 2005)
	reverse	CGCTCCAGCCAGATTCGACCC	
Gata2	forward	ACACACCACCCGATACCCACCTAT	(Iwasaki *et al.* 2005)
	reverse	CCATGGCAGTCACCATGCT	
Geminin	forward	ACGCTGAAGATGATCCAGCCTTCT	
	reverse	TAGCTGGTCATCCCAAAGCTTCCT	
Gorasp2	forward	CACTGGGTTCCCTGTACCAC	
	reverse	GATGCGACTCACAGAGACCA	
Gpx7	forward	ACTTCAAGGCGGTCAACATC	
	reverse	GGGAAGGCAAGCACATTAAA	
Hes1	forward	AAACCAAAGACGGCCTCTGA	
	reverse	GGAATGCCGGGAGCTATCTT	
Hivep3	forward	CTCAGACACACGCTCCAAAA	
	reverse	AAGCTGCCGTAGTCAAAGGA	
Hoxb13	forward	TCTTGCCGAGTATCCAGGAG	
	reverse	ACTGGCCATAGGCTGGTATG	
Mki67	forward	CATCCATCAGCCGGAGTCA	(Passegue *et al.* 2005)
	reverse	TGTTTCGCAACTTTCGTTTGTG	
Nme3	forward	GCCGGACATTGGCTATATGA	
	reverse	GACCGACTAGGTTGGGTTGA	

Olfml3	forward	ACCGAACAGTGGTGGATAGC	
	reverse	CGTTCTCTCTGGGACATGGT	
Pparg	forward	TTCGGAATCAGCTCTGTGGA	
	reverse	TGGAGCAGAAATGCTGGAGA	
Pten	forward	ACACCGCCAAATTTAACTGC	
	reverse	TACACCAGTCCGTCCCTTTC	
Ptn	forward	TGTCGTCCCAGCAATATCAG	
	reverse	ACTCCACTGCCATTCTCCAC	
Runx1	forward	TACCTGGGATCCATCACCTC	
	reverse	AGGCGCCGTAGTATAGATGG	
Serpina3g	forward	ACATTGATGGTGCTGGTGAA	
	reverse	AGTGCAGGACAGCTCCTCAT	
Sfpi1	forward	CAGAGCTCAGATGAGGAGGA	
	reverse	CTTGTGCTTGGACGAGAACT	
Slc29a1	forward	AACTCTCCACCCACCAACAG	
	reverse	TTCCACCTCAGCAGTCACAG	
Snx6	forward	TAGAAGCTCGGGTGTCTGCT	
	reverse	TGTCCAGTGCTTTATTGGCA	
Trp53	forward	AAGATCCGCGGGCGTAA	(Passegue *et al.* 2005)
	reverse	CATCCTTTAACTCTAAGGCCTCATTC	
Ythdf1	forward	TTGGTCCAGCCACAGTATCA	
	reverse	CAGGACAGGGTGGGATTCTA	

All primers were obtained from Sigma-Aldrich.

2.2. Methods

In vivo methods

2.2.1. Genotyping

Ptn knockout (KO), heterozygous (HE) and wildtype (wt) mice were distinguished from one another by means of the polymerase chain reaction (PCR).

Either small pieces of the ear (from ear clipping) or the tail were used as samples. The tissue samples were incubated over night in lysis buffer (100 mM Tris/HCl (Carl Roth), pH 8.3, 500 mM KCl (Sigma-Aldrich), 0.1 mg/ml gelatine (Carl Roth), 1% NP40 (Carl Roth), 1% Tween 20 (Carl Roth), with freshly added 200 µg/ml Proteinase K (Invitrogen)) at 50°C with constant shaking in order to decompose the tissue. The next day, the Proteinase K was inactivated by the incubation of the samples at 95°C for 5 min. After that, the samples were centrifuged at 15 000 g for 2 min and the supernatant was used for a PCR analysis.

For the PCR, the peqGOLD PCR Master Mix Y (Peqlab) was used:

10 µl peqGOLD PCR Master Mix Y

1 µl Primer Ptn genom (forward and reverse, 10 µM each)

1 µl Primer neo genom (forward and reverse, 10 µM each)

1 µl DMSO (Riedel-de Haën)

0.1 µl template

6.9 µl H_2O

20 µl

The samples were run in a PTC 200 thermal cycler (BioRad). Program:

95°C 5 min

95°C 45 s

56°C 45 s

72°C 1 min 40 cycles

72°C 4 min

5 µl of loading buffer (30% glycerol (Sigma-Aldrich), with Orange G (Sigma-Aldrich) added, till the colour turned a rich orange) were added to the samples and they were loaded on a 1% agarose gel made with NaB buffer (0,01 M di-sodium tetraborate (Sigma-Aldrich)) and ethidium bromide (0.5 µg/ml, Carl Roth). The gel was run with 300 V and analysed with a BioRad Gel-Doc XR Imaging System.

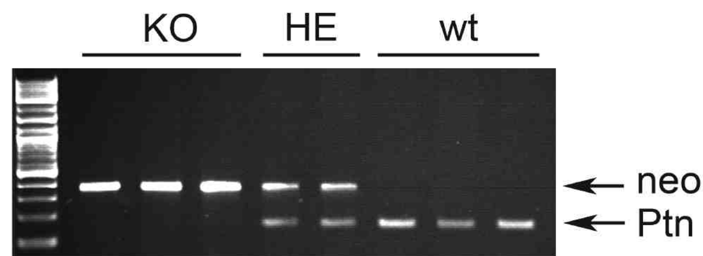


Figure 3. Genotyping of Ptn KO mice.

2.2.2. Preparation of murine tissues

In this project, the organs to be analysed were bone marrow, spleen, thymus and blood. For the bone marrow analysis, the 4 long bones (femurs and tibia) of both hind legs were extracted and flushed with 3 ml of HF2 buffer each (HBSS (Invitrogen), 10 mM HEPES (Invitrogen), 2 % FCS (PAA), 100 U Penicillin, 100 µg streptomycin (Invitrogen)). The cell suspension was filtered through a 30 µm filcon (BD Biosciences) and used for analysis.

For an analysis of spleen or thymus, the organs were squished and subsequently filtered through 30 µm filcons, too.

Blood was obtained from mice by punctuating the facial vein and collected in EDTA-coated vials. After sacrificing the mice, blood was taken directly from the heart. Blood samples were mixed with 10 x volume of ACK lysing buffer (Invitrogen) and incubated on ice for 15 min in order to lyse the erythrocytes. Samples were then centrifuged at 300 g for 5 min and resuspended in HF2 buffer. For analysis of the blood serum, blood was collected into Eppendorf tubes and incubated on ice for 30 min. After that, the samples were centrifuged at 15 000 g and 4°C for 10 min and the supernatant was transferred to fresh tubes.

2.2.3. Transplantation assays

Transplantation assays are important for studying HSC function *in vivo* because the donor HSCs have to restore the whole hematopoietic system and deficiencies in

HSC function can therefore be detected. For all transplantation assays the recipient mice were lethally irradiated with 9 Gy (KD2 Mevatron, Siemens).

For the transplantation of bone marrow cells, $2 \cdot 10^5$ donor bone marrow cells were injected in the tail vein of irradiated recipient mice. One week prior to irradiation and in the first 5 weeks after transplantation, recipients received 1 mg/ml neomycin sulphate (Sigma-Aldrich) and 500 units/ml Polymyxin B (Sigma-Aldrich) with the drinking water. The mice were bled after 5 and 10 weeks and sacrificed 16 weeks after the transplantation. For the secondary transplantation, bone marrow cells of the different mice in one group were pooled and $1 \cdot 10^6$ cells were injected into irradiated recipient mice. Like in the primary transplantation, the mice were bled after 5 and 10 weeks and sacrificed 16 weeks after the injection. The tertiary and quaternary transplantations were performed in a similar manner, except for the number of cells injected. For the tertiary transplantation $2 \cdot 10^6$ bone marrow cells were used and for the quaternary transplantation $5 \cdot 10^6$ cells.

For the transplantation of co-cultures of stromal cells and hematopoietic cells (see below under 2.2.7), half of a 3 cm dish, representing the input equivalent of 2500 lineage-depleted cells (lin^-) cells, were transplanted into congenic wild-type recipients. For secondary transplants $5 \cdot 10^6$ bone marrow cells were injected.

Bone marrow, spleen and blood of the recipient mice were analysed by flow cytometry. To distinguish donor and recipient cells, throughout the transplantation experiments mice with congenic expression of different Cd45.1 (Ly5.1) or Cd45.2 (Ly5.2) isotypes were used. After transplantations of cocultures mice were counted positive with at least 1 % donor cells in peripheral blood, and at least 1 % myeloid and lymphoid cells, respectively, in the donor cell population.

2.2.4. 5-FU and BrdU injections

Mice were injected intraperitoneally (i.p.) with 150 mg/kg 5-FU (Sigma-Aldrich). For regeneration kinetics, mice were sacrificed 4, 8, 12 or 16 days after injection, respectively. For proliferation analysis 50 mg/kg BrdU (BD Biosciences) was injected i.p. alone or 5 days after the injection of 5-FU and the mice were sacrificed 16 h later.

To assess the incorporation of BrdU in the DNA during the S-phase progression in different cell populations, bone marrow cells were first stained for surface markers and afterwards the protocol for the BrdU staining was performed following the manufacturer's instructions (FITC BrdU Flow Kit, BD Biosciences). In brief, the cells were fixed and permeabilized due to paraformaldehyde (fixation) and saponin (permeabilization) in the used Cytofix/Cytoperm buffer and Cytoperm plus buffer. After that, the cells were treated with DNAse for 1 h at 37°C in order to expose the epitopes of the BrdU molecules which had been integrated into the DNA during replication. Afterwards, the antibody against BrdU was added to the cells and incubated for 30 min at room temperature. The cells were then washed and analyzed on a Coulter EPICS XL (Beckman Coulter) or CyAn ADP Lx P8 (Coulter-Cytomation).

***In vitro* methods**

2.2.5. Cell culture

Stromal cell lines were cultured on 0.1 % gelatine-coated dishes in stroma medium (AlphaMEM (Invitrogen), 15 % FCS (PAA), 5 % HS (BioWhittaker), 100 U Penicillin, 100 μg streptomycin (Invitrogen), 8 μM folic acid (Sigma-Aldrich), 80 μM inositol (Sigma-Aldrich), 10 μM β-mercaptoethanol (Invitrogen)) with 20 % conditioned medium from the previous passage.

Phoenix Eco cells were cultured in DMEM (Invitrogen) with 10 % FCS (PAA).

2.2.6. Stable Knockdown of Ptn

Lentiviruses were used for the stable knockdown of Ptn in the stromal cell lines UG26-1B6 and EL08-1D2,. The vectors containing the shRNA sequence were packaged into lentiviral envelopes in a packaging cell line, namely ◎NX (Phoenix) Eco, with the help of two supplementary vectors. These lentiviruses subsequently infected the stromal cells and due to the lentiviral components, the shRNA sequences were stably integrated into the genome. Since the two vectors which were additionally transfected into the packaging cell lines were not present in the target cells, these cells were not able to produce virus particles themselves.

The evening before the transfection, 2·10^6 Phoenix Eco cells were seeded on 6 cm dishes. For the transfection 4 µg of the pLKO.1-shPtn (RMM3981-97060061, Openbiosystems) or the pLKO.1 vector (Openbiosystems) and 4 µg each of the supplementary vectors pMD2.G and psPax2 were transfected into the Phoenix Eco cells with the help of Lipofectamine 2000 (Invitrogen, protocol following the manufacturer's instructions). In brief, DNA and Lipofectamine were separately diluted with OptiMEM (Invitrogen) and subsequently mixed. The mixture was incubated for 30 min to allow complexes to form. These complexes are added to the Phoenix Eco cells. The positive charges on the surface of the liposomes then allow the fusion of the liposome/nucleic acid complex with the cell membrane, in this way the nucleic acid can enter the target cell. 6 h after the transfection the medium was changed to stroma medium. After that virus supernatant was harvested three times, with an interval of about 12 h between the harvesting steps. For harvesting the supernatant of the cells was removed and stored at 4°C, and fresh stroma medium was added to the Phoenix Eco cells. After the last harvesting step the supernatant was filtered (0.45 µm) and polybrene (Sigma-Aldrich) was added to a final concentration of 8 µg/ml. The stromal cells were thawed the day before infection and seeded in 6 cm dishes with 1·10^5 cells per dish. For infection, the supernatant of the stromal cells was removed and one third of the virus containing supernatant was added to the cells. The infection was repeated 12 and 24 h after the first infection. 12 h after the last infection the virus supernatant was replaced by fresh stroma medium. 24 h later 5 µg/ml puromycin was added to the culture for selection of infected cells. After 3 days of selection, the selective medium was replaced by fresh stroma medium.

2.2.7. Co-cultures of lin$^-$ with stromal cell lines

Lineage-depleted bone marrow cells (lin-) were co-cultured with 30 Gy irradiated confluent stromal cells. The lineage negative fraction (lin$^-$) was negatively selected from flushed bone marrow (Lineage depletion kit, Miltenyi Biotec) according to the manufacturer's instructions. In brief, bone marrow cells were first incubated with a

mixture of primary biotinylated antibodies (Cd5, B220, Cd11b, Gr-1 and Ter119) and subsequently with Streptavidin-coupled magnetic MicroBeads. The labelled cells were run through columns in a magnetic field. Cells coated with MicroBeads were restrained in the column, the lineage negative, unlabelled cells run through the column. 5000 lin^- cells were plated on the confluent and irradiated stromal cells in a 3 cm dish. The cells were cultured in long-term culture medium (M5300 (StemCell Technologies) with 200 µM Glutamax (Invitrogen) and 100 U Penicillin, 100 µg streptomycin (Invitrogen)). Each week half of the supernatant was replaced with fresh medium. For cell cycle analysis after 3 days, wild-type lin^- were grown in medium which was previously conditioned for one day on confluent irradiated stromal cells. For cell division tracking, the lin^- cells were stained with Carboxyfluorescein succinimidyl ester (CFSE) prior to culture. For that, the cells were resuspended in PBS ($1 \cdot 10^7$ cells/ml) and 5 µM CFSE (Invitrogen) was added. The cells were incubated for 10 min at 37°C and then washed twice with HF2 buffer.

2.2.8. RNA isolation

For the confirmation of array results or analysis of knockdown efficiency, about $5 \cdot 10^5$ stromal cells were used. RNA isolation was performed using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. In brief, pelleted cells were resuspended in RLT buffer containing guanidine-thiocyanate, the cells are thereby lysed and RNases are inactivated. Subsequently, the samples are homogenized by running through QIAshredder spin columns. The homogenized samples are mixed with ethanol to optimize binding conditions and loaded on RNeasy Mini spin columns where the RNA binds to the silica-based membrane. Contaminants were washed away by the use of the wash buffers RW1 and RPE and finally, the total RNA was eluted under low-salt conditions.

For gene expression analysis in progenitor cells, RNA was isolated with the Dynabeads mRNA DIRECT Micro Kit (Invitrogen), following the protocol given in the kit. In brief, pelleted cells were resuspended for cell lysis in Lysis/Binding buffer and Dynabeads Oligo $(dT)_{25}$ were added to the samples. The thymidine residues

connected with the Dynabeads binds to the poly-adenine tails of the mRNAs. The Dynabeads are magnetic, therefore the samples were placed in a magnetic field and the Dynabeads with the bound mRNA congregated at one side of the tube, which allowed the removal of the supernatant with its contaminants. After two washing steps, the Dynabeads with the bound mRNAs were resuspended in 10 mM Tris/HCl pH 7.5 and directly used for reverse transcription.

2.2.9. Reverse transcription

For array confirmation or analysis of the knockdown of Ptn in stromal cell lines, RNA was transcribed to cDNA by the use of the Omniscript RT Kit (Qiagen):

1 µg	RNA in 13 µl H_2O
2 µl	Nucleotide Mix (contained in the kit)
2 µl	anchored dT (Sigma-Aldrich)
2 µl	10 x RT buffer (contained in the kit)
0.5 µl	Omniscript (contained in the kit)
0.5 µl	RNaseOut (Invitrogen)
20 µl	

The mixture was incubated for 1 h at 37°C.

For gene expression analysis of cultured progenitor cells, RNA was transcribed with the QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's instructions.

2.2.10. Real time PCR

Real time PCR can be used to determine the expression levels of various genes. It measures how many cycles are needed before the amount of amplified DNA reaches a certain value. Under perfect conditions, the DNA strands with the "right" sequence (consistent with the primer sequences) should double in one cycle because all the sequences bind primers and get amplified. Samples with a two-fold difference in the concentration of the wanted sequence should therefore reach the threshold with a difference of one cycle. Since amplification efficiency depends on the primer

sequences chosen, both amplification efficiency and melting curves of the PCR amplicon should be determined prior to use. The melting curve analysis will also give an idea of whether primer pairs will amplify so-called primer pairs.

Confirmation of array results and knockdown analysis

Real time PCR analysis was done using the Power Sybr Green PCR Master Mix from Applied Biosystems. The samples were diluted 1:4 with H_2O after the reverse transcription. For examining the conditions, a mixture of the different stromal cell lines was used as a standard. The standards were serially diluted twofold and used for every primer to determine the amplification rate per cycle. From the previously published microarray results, several housekeeping genes were tested. At the end, Ythdf1 was chosen for use as housekeeping gene for normalization.

10 µl	Power Sybr Green PCR Master Mix
1 µl	Primer (forward and reverse, each 10 µM)
1 µl	template
8 µl	H_2O
20 µl	

The assays were done with an Applied Biosystems 7900HT.

Program:

50°C	2 min	
95°C	5 min	
95°C	15 s	
58°C	20 s	
72°C	30 s	40 cycles

Gene expression analysis of cultured progenitors

The same mixture was used for the gene expression analysis of LSKs, CMPs and GMPs after 3 days of culturing. Samples from LSKs and CMPs were diluted 1:6 with water after reverse transcription, samples from GMPs 1:8. For standards a mixture of reverse transcribed universal RNA (Stratagene) and bone marrow of 129S2 mice

were used. From published gene expression analyses of HSC and MPP (Kiel *et al.* 2005a), Gorasp2 and Nme3 were chosen for use as housekeeping genes.

Program:

50°C 2 min

95°C 10 min

95°C 15 s

60°C 1 min 40 cycles

2.2.11. Western blotting

For determining protein levels in supernatant or cell lysates SDS polyacrylamide gel electrophoresis (SDS-PAGE) was used, meaning that all the proteins were denaturated and surrounded with negatively charged SDS before loaded on the polyacrylamide gel. This leads to a separation according to the size of the proteins if potential is applied to the gel. The proteins can then be transferred to polyvinylidene difluoride (PVDF) membranes and detected with specific antibodies.

Preparation of samples

For determining the amount of Pleiotrophin in the supernatant of stromal cell lines, the stromal cells were grown to confluence. Fresh stroma medium was added to the cells and removed 24 h later. A small amount of this supernatant was mixed with 5x loading buffer (250 mM Tris/HCl (Carl Roth), pH 6.8, 50 % Glycerol (Sigma-Aldrich), 10 % (w/v) SDS (Sigma-Aldrich), 0.5 M DTT (Sigma-Aldrich), 0,125 % Bromophenolblue (Sigma-Aldrich)), heated 5 min at 95°C for denaturation and used for Western blotting.

For the analysis of protein levels in stromal cell line lysates, the cells were frozen as pellets without supernatant. The frozen pellets were then resuspended in lysis buffer with added protein and phosphatase inhibitors (50 mM HEPES (Invitrogen), 150 mM NaCl (Carl Roth), 1 mM EDTA pH 7.5 (Carl Roth), 2.5 mM EGTA pH 7.5 (Carl Roth), 0.1 % Tween 20 (Sigma-Aldrich), 1 mM PMSF (Sigma-Aldrich), 10 mM β-Glycerophosphate (Sigma-Aldrich), 5 mM NaF (Sigma-Aldrich), 0.1 mM Na_3VO_4

(Sigma-Aldrich)) and incubated on ice for 15 min. Afterwards the samples were sonificated (SONOPLUS HD 2070 (Bandelin), 10 pulses) and centrifuged at 15 000 g for 2 min. The supernatant was transferred into a fresh tube and protein concentrations were measured with the help of the Protein assay Kit from BioRad, according to the manufacturer's instructions. 30 µg of protein were mixed with loading buffer and used for Western blotting.

Western blotting

For polyacrylamide gels the Mini protean system from BioRad was used. The Precision Plus Protein Dual Color Standard (BioRad) was used as a molecular weight marker. The samples were loaded onto the gel and the gel was run with 120 V. Afterwards, the proteins were blotted on PVDF membranes (0.45 µm, Millipore) with 100 mA for 30-60 min (30 min for Ptn, 60 min for larger proteins). For detection of Pleiotrophin, the membrane was incubated with 2 % horse serum (BioWhittaker) and 0.1 % Tween 20 in PBS (Invitrogen), for other proteins, the blocking was done with 5 % milk powder (Carl Roth) and 0.1 % Tween 20 in PBS. The membranes were blocked for 1 h and then transferred into 50 ml falcons containing the primary antibody diluted in blocking buffer and incubated over night. The next day, the membranes were washed twice with PBS with 0.1 % Tween 20 and then secondary antibody was added diluted in blocking buffer. The membranes were incubated for 1 h at room temperature and then washed twice with PBS with 0.1 % Tween 20 and finally twice with PBS. Detection was done with SuperSignal West Dura or Femto solutions from Pierce.

2.2.12. Colony forming assay

Colony forming assays can be used to determine the number of progenitors in cell suspensions or cultures. M3434 from StemCell Technologies was used for the colony forming assays in this project. This methylcellulose contains several cytokines which promote the growth of myeloid progenitors to colonies.

For a colony forming assay with fresh bone marrow cells, 25 000 cells in 250 µl long-term culture medium were added to 2.25 ml methylcellulose. 1 ml at a time of this mixture was plated on a 3 cm dish and cultured at 37°C for 10 days. For colony forming assays after cultures half of the cells contained in one well of a 6 well plate were added to the methylcellulose. Colonies were counted according to size and lineage as: burst-forming units erythroid (BFU-E), granulocytic/monocytic colony-forming cells (GM-CFC), and granulocytic/erythroid/megakaryocytic/monocytic colony-forming cells (GEMM-CFC).

2.2.13. ELISA

An ELISA was used for the analysis of the concentration of immunoglobulins in the serum of mice. To do so, a 96 well plate was coated with 10 µg/ml anti-mouse Ig capture antibody (Southern Biotech) diluted in PBS over night at 4°C. The next day, the antibody was removed and 1 % BSA (Sigma-Aldrich) in PBS was added for 1 h at room temperature for blocking unspecific binding sites. After washing the samples and, as a standard, known concentrations of the wanted immunoglobulin (Southern Biotech) were added to the plate and incubated for 1 h at room temperature. After the incubation the wells were washed and the antibody specific for the wanted immunoglobulin (AP-labelled goat-anti-mouse Ig screening antibody, AP-labelled goat-anti-mouse IgM antibody or AP-labelled goat-anti-mouse IgG2a antibody (all Southern Biotech) was added (diluted 1:250 in blocking buffer). After 1h of incubation the plate was washed again several times with PBS and then the substrate (1 mg/ml p-nitrophenyl phosphate in 250 µM $MgCl_2$, 9.6 % (v/v) diethanolamine, pH 9.8) was added which led to a change of colour. After 10-25 min (depending on the depth of the colour in the plate) the extinction at 405 nm was measured.

2.2.14. Flowcytometry staining

For the staining of surface markers the cells were resuspended in 100 µl staining buffer (0.5 % BSA (Sigma-Aldrich) in PBS (Invitrogen)) with primary antibodies. For the staining of mature populations, $1 \cdot 10^6$ cells were used, for progenitor and HSC

stains $5 \cdot 10^6$ cells were stained. In most of the cases 0.5 µl antibody was used per $1 \cdot 10^6$ cells, exceptions are noted in 2.1.3. The cells were incubated with the antibodies for 15 min at 4°C and after that washed with staining buffer. If necessary, a secondary antibody was added (also diluted in blocking buffer, 0.5 µl antibody per $1 \cdot 10^6$ cells). The cells were incubated again for 15 min at 4°C and then washed with staining buffer. Finally, the stained cells were resuspended in staining buffer with 1 µg/ml propidium iodide (PI, Invitrogen) and analysed on a Coulter EPICS XL (Beckman Coulter) or CyAn ADP Lx P8 (Coulter-Cytomation). Sorting of cell populations was performed on a BD FACSAria IIu (Becton Dickinson).

For the staining of apoptotic cells, the cells were stained as above. At the last washing step, however, cells were resuspended in 500 µl annexin buffer (10 mM HEPES pH 7.4 (Invitrogen), 140 mM NaCl (Carl Roth), 2.5 mM $CaCl_2$ (Sigma-Aldrich)) with added annexin-V-FITC (1:200, Invitrogen) and PI. The cells were incubated at 4°C for 15 min before measurement.

Intracellular staining

For intracellular staining the cells were paraformaldehyde-fixed and permeabilized using a saponin-containing buffer after surface staining by resuspension in 100 µl Cytofix/Cytoperm (BD Biosciences). After 30 min incubation at 4°C the cells were washed with 1 x Perm/Wash buffer (BD Biosciences) and resuspended in staining buffer with primary antibody added. The samples were incubated at room temperature for 1 h and then again washed with Perm/Wash buffer. A secondary antibody was diluted in staining buffer as well and added to the cells for 30 min. Subsequently, the cells were washed again, resuspended in staining buffer and analysed on a CyAn ADP Lx P8 (Coulter-Cytomation).

2.2.15. Single cell staining assays

For single cell staining assays, 500 cells were directly sorted or spotted onto poly-L-lysine coated slides and incubated for 30 min on ice. Subsequently, the spotted cells were fixed with 4 % paraformaldehyde (PFA, Sigma-Aldrich) for 15 min. The fixed

cells were then blocked with 10 % FCS (PAA) and 0,1 % Triton-X (Sigma-Aldrich) in PBS (Invitrogen) for 1 h at room temperature. Cells were stained overnight at 4°C with the primary antibody diluted in blocking buffer. The next day, the cells were washed, and if necessary, secondary antibody was added, diluted in blocking buffer for 1 h at room temperature. The cells were washed again and counterstained with DAPI (4,6-diamino-2-phenylindole dihydrochloride) (Invitrogen). A Leica DM RBE fluorescent microscope (Leica) was used for detection. Fluorescence intensities of stained cells were quantified using ImageJ (NIH). Nuclear and cytoplasmic regions were differentiated using the DAPI staining.

2.2.16. Apoptosis assay

For apoptosis assays supernatant of shPtn and control UG26-1B6 was used. The supernatant was collected from cultures which had grown to confluency. For each condition and timepoint $1 \cdot 10^6$ freshly isolated wt bone marrow cells were resuspended in 2 ml of supernatant with added 50 µg/ml cycloheximide (Sigma-Aldrich) and, if applicable, other additives. The samples were incubated at 37°C for 4, 8, 12 and 24 h, respectively. Then the cells were stained for surface markers and the apoptosis marker annexin-V.

Concentrations of additives:

4 µM MG-132 (Calbiochem)

50 µM 5-FU (Sigma-Aldrich)

40 µM Gossypol (Sigma-Aldrich)

1 µM Nutlin-3 (Calbiochem)

100 nM Wortmannin (Calbiochem)

50 µM LY294002 (Calbiochem)

50 µM Caspase3-Inhibitor II (Calbiochem)

2.2.17. Statistics

For statistical analysis unpaired and paired Student's t-tests were used where appropriate. L-Calc software (StemCell Technologies) was used for calculating the HSC frequency after coculture.

3. Results

It has long been known that several stromal cell lines are able to maintain HSCs in *in vitro* co-cultures, in which stromal cells and HSC are in direct contact to each other (Wineman *et al.* 1996; Moore *et al.* 1997). Before the start of this project, Robert Oostendorp generated two stromal cell lines – UG26-1B6 and EL08-1D2 – which were able to maintain HSCs even without direct contact (Oostendorp *et al.* 2005). It thus became clear that secreted factors may play important roles in the regulation of HSC functions. To identify these factors, macro arrays were performed comparing the two supporting cell lines with several other stromal cell lines that fail to maintain HSCs in non-contact cultures, namely AM30-3F4, EL28-1B3 and UG15-1B7 (Oostendorp *et al.* 2005). One of the factors that was overexpressed in both supporting cell lines was Pleiotrophin, whose effects on HSC maintenance and hematopoiesis were therefore analyzed during this project.

3.1. Confirmation of array results

At the beginning of the project it was important to first confirm the results of the array analysis. For that reason, real time PCR was used to compare the expression levels of different secreted factors in UG26-1B6, EL08-1D2 and a mixture of the non-supporting cell lines AM30-3F4, EL28-1B3 and UG15-1B7. Amongst the expression levels to be confirmed were mRNA overexpressed in both UG26-1B6 and EL08-1D2 compared to a mixture of non-supporting cell lines according to the array analysis. But there were also genes overexpressed in only one of the supporting cell lines and also several mRNAs which were underrepresented in both UG26-1B6 and EL08-1D2. It was found that the real time PCR results almost completely confirmed the results of the arrays (Figure 4). The real time PCR also validated the overexpression of Ptn by both UG26-1B6 and EL08-1D2, which had also been shown in the array analysis. According to real time PCR results, Pleiotrophin was more than 4000 fold overexpressed in UG26-1B6 and even more than 8000 fold overexpressed in EL08-1D2 compared to a mixture of AM30-3F4, EL28-1B3 and UG15-1B7 (Figure 5).

expression relative to mixture of non-supporting stromal cell lines

Serpina3g Ctsk Slc29a1 Olfml3

C1galt1 Gpx7 Hivep3 Isl1

Cpm Eif2s3y

Elavl2 Dlk1 Hoxb13

Cfl2 Adam17 Snx6

□ EL08 Array
■ EL08 real time PCR
■ UG26 Array
■ UG26 real time PCR

Figure 4. Confirmation of array results. Comparison of array results and real time PCR results for various genes. Shown is the expression of the gene in EL08-1D2 and UG26-1B6 as obtained from array or real time PCR analysis in comparison to the expression in a mixture of the non-supporting stromal cell lines AM30-3F4, EL28-1B3, UG15-1B7.

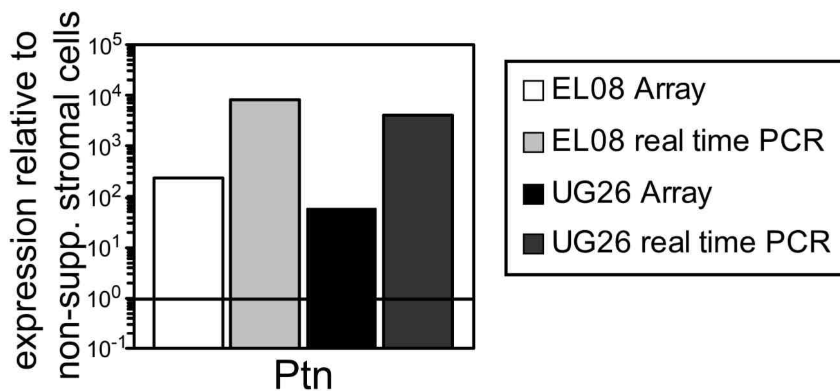


Figure 5. Confirmation of Pleiotrophin array results. Shown is the expression of Pleiotrophin in EL08-1D2 and UG26-1B6 as obtained from array and real time PCR analysis relative to the expression level of Ptn in a mixture of non-supporting stromal cell lines AM30-3F4, EL28-1B3, UG15-1B7.

3.2. Knockdown of Pleiotrophin in supporting stromal cell lines

In order to investigate if the Pleiotrophin secreted by the supporting stromal cell lines plays an important role in co-cultures with HSCs and progenitor cells, Ptn expression was downregulated in UG26-1B6 and EL08-1D2, using a shRNA-generating vector (pLKO.1) packaged in lentiviruses. With the help of the lentiviral components, the vector was integrated into the genome. There, its transcription led to constant generation of shRNAs and therefore a stable downregulation of Ptn. The efficiency of the knockdown was assessed by real time PCR, revealing a strong reduction of Pleiotrophin in both UG26-1B6 (96.8 % reduction) as well as in EL08-1D2 (96.0 % reduction). The transfection of the empty control vector did not alter the expression of Ptn (Figure 6a).

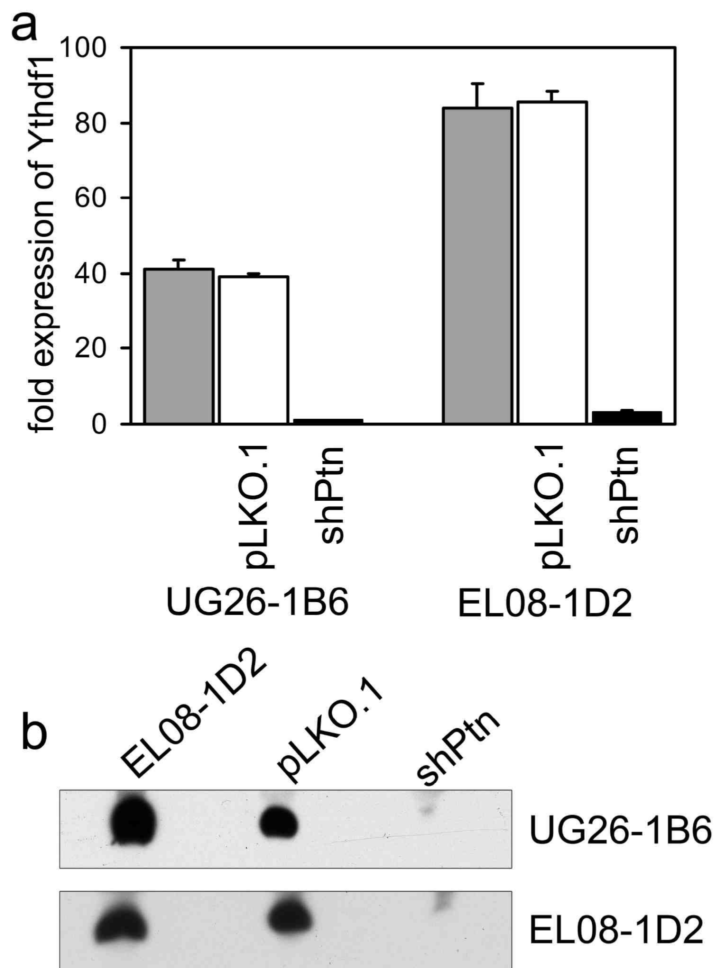


Figure 6. Knockdown of Pleiotrophin in UG26-1B6 and EL08-1D2. a) Knockdown of Ptn in supportive stromal cell lines as measured by real time PCR. Shown is expression relative to Ythdf1. b) Western blot of Ptn expression, using supernatant of confluent monolayer of stromal cell lines. Mean ± SEM, n=4.

This result was additionally verified on the protein level: Supernatant of confluent cultures of UG26-1B6 and EL08-1D2 with and without knockdown was used for Western blotting. Similarly to the mRNA levels, the introduction of shPtn led to a strong reduction of the Ptn protein concentration in the supernatant of UG26-1B6 and EL08-1D2 cultures (Figure 6b).

UG26-1B6 cells, which were used for most experiments, were checked for gross changes due to the strong decrease in Ptn expression. There were no morphological changes observed after the Ptn knockdown (Figure 7).

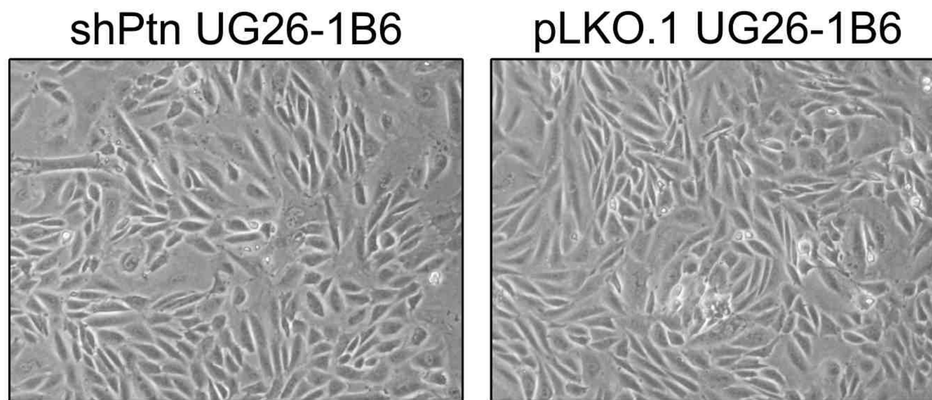


Figure 7. Morphology of shPtn and pLKO.1 UG26-1B6.

3.3. Progenitor frequency in co-cultures lacking Ptn

The UG26-1B6 and EL08-1D2 cells with shPtn and pLKO.1, respectively, were used for co-cultures with wildtype ($Ptn^{+/+}$) lineage negative (lin^-) bone marrow cells, which present a population including HSCs and progenitor cells, but are depleted of cells belonging to the myeloid, lymphoid or erythroid lineages. These cells were at first co-cultured in contact with the irradiated stromal cell lines. After two weeks of co-culture the cells were harvested and seeded in cytokine-containing methylcellulose, in which hematopoietic progenitor cells form colonies. An analysis after ten days revealed that the lin^- cells cultured on shPtn stromal cells formed considerably more colonies than the lin^- cultured on control (pLKO.1) stromal cells. The number of colonies was 3.2 fold higher after culture on shPtn UG26-1B6 compared to the control culture; after culture on shPtn

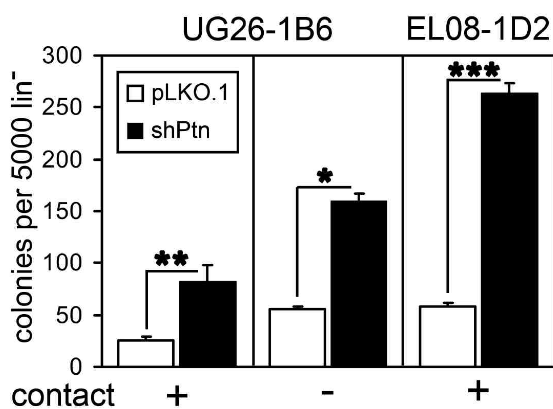


Figure 8. Progenitor frequency in co-cultures of wt lin^- cells with shPtn and pLKO.1 UG26-1B6 and EL08-1D2. Colony number of wt lin^- after co-culture of 2 weeks on shPtn and control stromal cell lines, n=10 (UG26-1B6 contact), n=4 (UG26-1B6 non-contact, EL08-1D2). Mean ± SEM, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

EL08-1D2 the number was even 4.6 fold higher than in the control (Figure 8). This shows that the absence of secreted Pleiotrophin in co-cultures of stromal cells with lin$^-$ cells increases the number of hematopoietic progenitor cells in the culture. This might for example be the case because of an enhanced proliferation of HSCs and progenitor cells. Alternatively, though, it might be that the stromal cells are not able to maintain the HSC function anymore and that the HSCs are thus starting to differentiate, thereby producing more progenitors. This question will be addressed later.

Non-contact co-cultures

First, there is another question to be answered: Does Ptn determine the condition that direct contact is not required for HSCs or progenitors in their communication with stromal cells? In other words, does the observed effect in Figure 8 really dependent on a secreted factor? To answer this question, non-contact co-cultures of shPtn and pLKO.1 UG26-1B6 with wildtype lin$^-$ were done using transwells to separate stromal and lin$^-$ cells from each other. The non-contact co-cultures showed the same results as the ones carried out in contact: lin$^-$ cultured on shPtn UG26-1B6 formed significantly more colonies than lin$^-$ cultured on control stromal cells (Figure 8). This shows that this alteration is independent from contact of the lin$^-$ cells with the stromal cells and that therefore a secreted factor (like Pleiotrophin) must be responsible for the effect.

Co-cultures with Ptn$^{-/-}$ lin$^-$

The results so far suggest that Ptn regulates progenitor cell production in an extrinsic manner. However, the possibility that Pleiotrophin also has intrinsic effects, meaning that the lin$^-$ cells themselves are no longer able to express Ptn and their functions are therefore changed, remains. This question was addressed by using Pleiotrophin knockout (KO) lin$^-$ cells for co-cultures. Wildtype and Ptn KO lin$^-$ cells were cultured on UG26-1B6 shPtn and pLKO.1, respectively. Ptn KO lin$^-$ cells showed the same effect after co-culture as wildtype lin$^-$, forming 3.1 fold more

colonies after being cultured on shPtn than on pLKO.1 UG26-1B6. There was no significant difference, however, between cultures with wildtype or Ptn KO lin$^-$ (Figure 9). This shows that, at least *in vitro*, the loss of Ptn in the hematopoietic cells themselves has no effect on progenitor production.

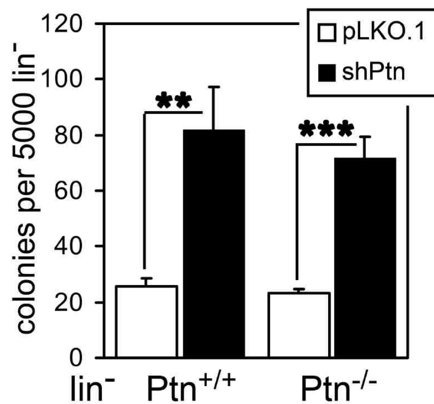


Figure 9. Progenitor frequency in co-cultures of wt and Ptn$^{-/-}$ lin$^-$ cells with shPtn and pLKO.1 UG26-1B6. Colony number of wt and Ptn$^{-/-}$ lin$^-$ after co-culture of 2 weeks on shPtn and control UG26-1B6, n=4. Mean ± SEM, **p<0.01, ***p<0.001.

3.4. Characterization of 129 Ptn KO mice

The results of the experiments with the loss of secreted Ptn in stromal cells strongly suggest that Ptn regulates early haematopoiesis. Hence, the question is whether hematopoiesis is also altered in mice deficient for Ptn. Such mice were previously generated from E14 ES cells (129/OlaHsd strain (=129P2)) (Amet *et al.* 2001). Therefore, hematopoiesis in 129 Ptn KO mice, and age- and sex-matched controls was compared using flowcytometry, as well as colony forming assays and ELISA.

At first, the cell numbers of the hematopoietic organs were assessed. It was found that bone marrow and spleen cell numbers were reduced in Ptn KO mice, while the cell numbers of thymus and blood were unaltered (Figure 10), suggesting that in the absence of Ptn, alterations in blood cell production occur.

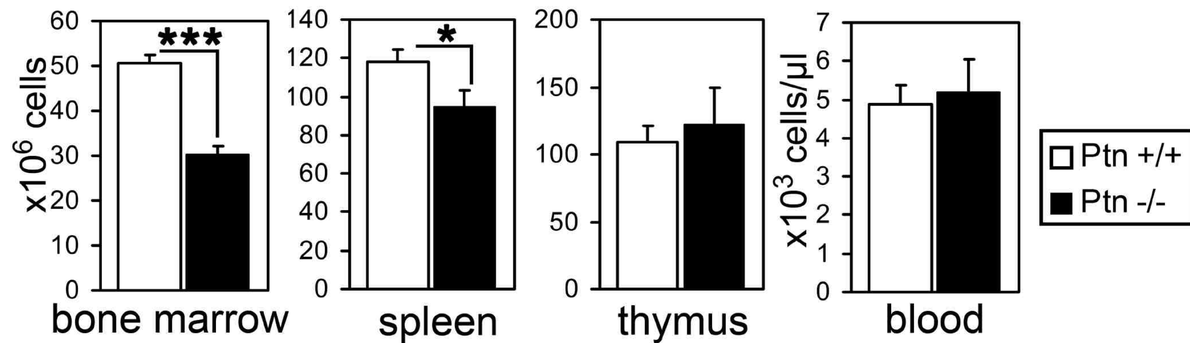


Figure 10. Organ cell numbers of 129 Ptn KO mice and controls. Cell counts from bone marrow (BM, four long bones: two femurs and two tibia per mouse), spleen, thymus, and peripheral blood, bone marrow: n=16 (+/+), n=19 (-/-), spleen, thymus, blood: n=9 (+/+), n=11 (-/-). Mean ± SEM, *p<0.05, ***p<0.001.

HSCs and early progenitors

To investigate whether these alterations originate in early or late hematopoiesis, the number of cells belonging to the early stages of the hematopoietic hierarchy was determined by flow cytometry. The gating strategy used here can be seen in Figure 11.

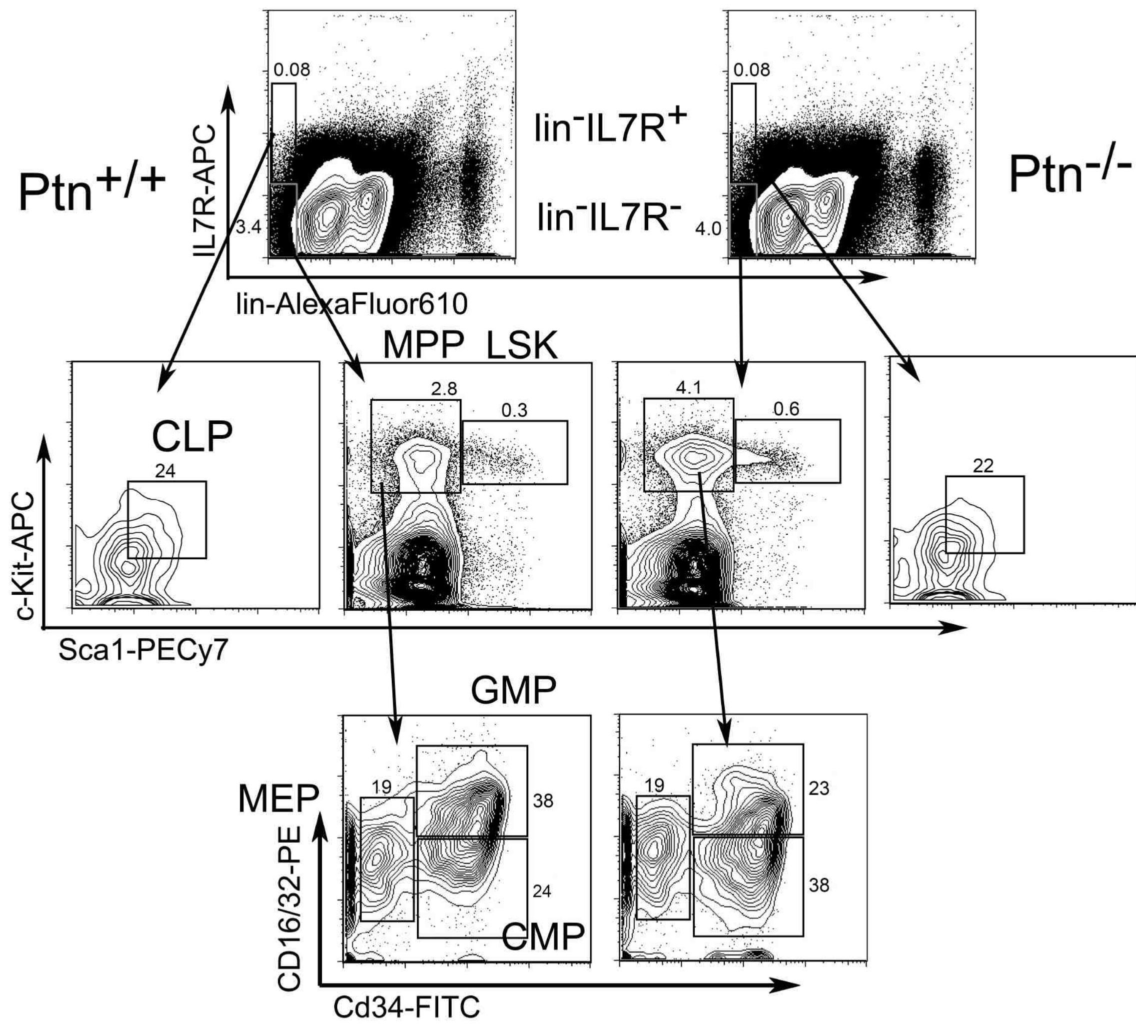


Figure 11. gating strategy for flow cytometry staining of progenitor populations.

The analysis of bone marrow of Ptn KO mice showed a reduction in the absolute numbers of lin^{-} cells, of CLPs and MPPs. LSK numbers, however, were not changed (Figure 12).

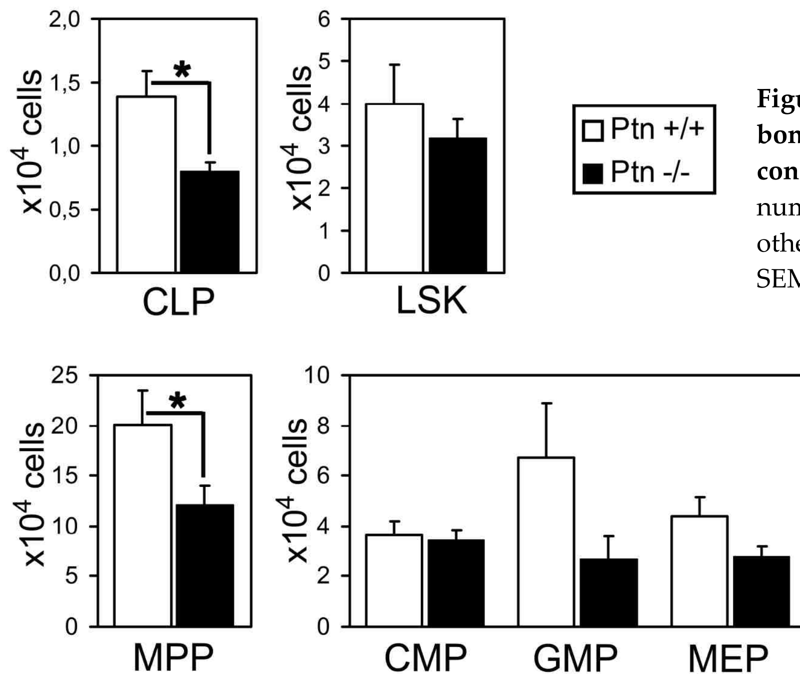


Figure 12. Progenitor populations in bone marrow of 129 Ptn KO and control mice. Shown are absolute numbers per 4 long bones, CLP: n=4, other: n=10 (+/+), n=12 (-/-). Mean ± SEM, *p<0.05.

A histogram showing the Cd34 expression of Ptn KO and wt LSK cells revealed a notably higher expression of this marker in the Ptn KO LSK cells (Figure 13). Since Cd34 expression is reduced or absent of true long-term repopulating HSC (Osawa *et al.* 1996), this result suggests a relative expansion of short-term repopulating LSK cells in the absence of Ptn.

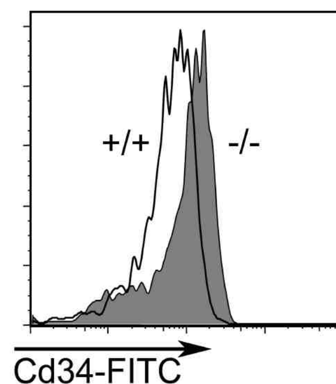


Figure 13. Expression of Cd34 in the LSK population of 129 Ptn KO and control mice.

It was then studied whether the changes in phenotype were mirrored by corresponding alterations in functional capacity of hematopoietic progenitors. Colony forming assays (CFA) were used for determining the number of progenitors in a functional analysis. The number of colony forming cells per 4 long bones was reduced in Ptn KO mice compared to wt mice, like the number of granulocyte-monocyte colony forming cells. The number of cells forming erythrocyte colonies and

the number of cells able to form mixed colonies, meaning colonies containing cells of both the erythroid and the myeloid lineages, however, were increased in the bone marrow of Ptn KO mice (Figure 14).

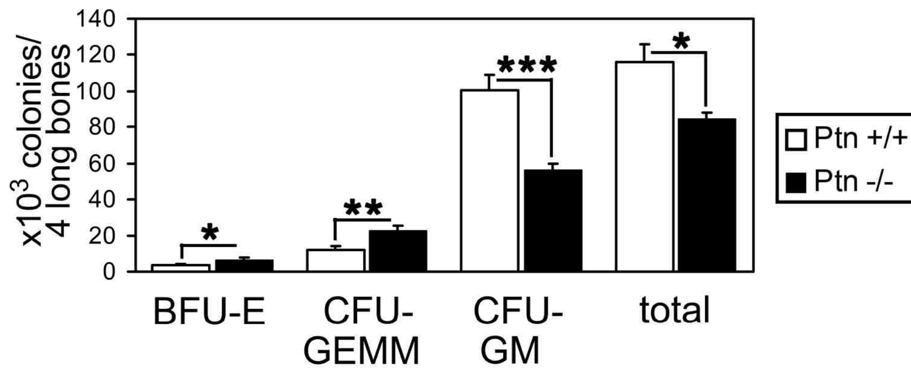


Figure 14. Colony forming assay of 129 Ptn KO and control mice. Shown are the total number of colonies per 4 long bones, n= 11 (+/+), n=10 (-/-). Mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.

B cells

The impact of the loss of Pleiotrophin was also assessed in more mature hematopoietic populations by means of flow cytometry. B cell numbers were determined with the surface markers B220, IgM and Cd43 (Figure 15).

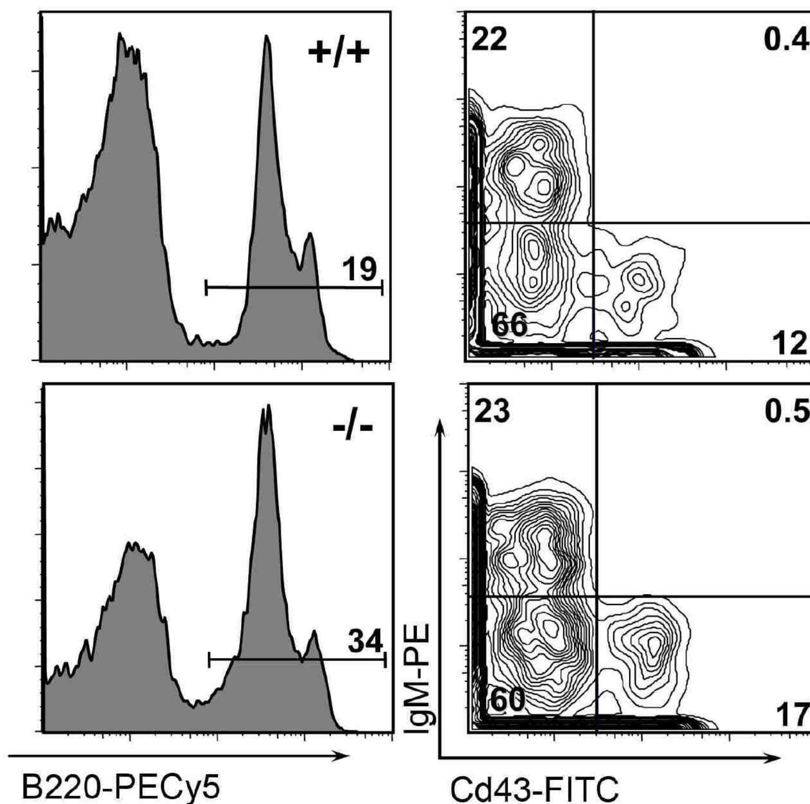


Figure 15. Gating strategy for B cell populations.

While B220 (CD45RA) is expressed in all B-lineage cells, Cd43 is only expressed in pre- and pro-B cells. IgM, on the other hand is mainly expressed in

mature B cells. B cell lymphopoiesis in the Ptn KO mice was undisturbed, there was no change in any population evaluated (Figure 16).

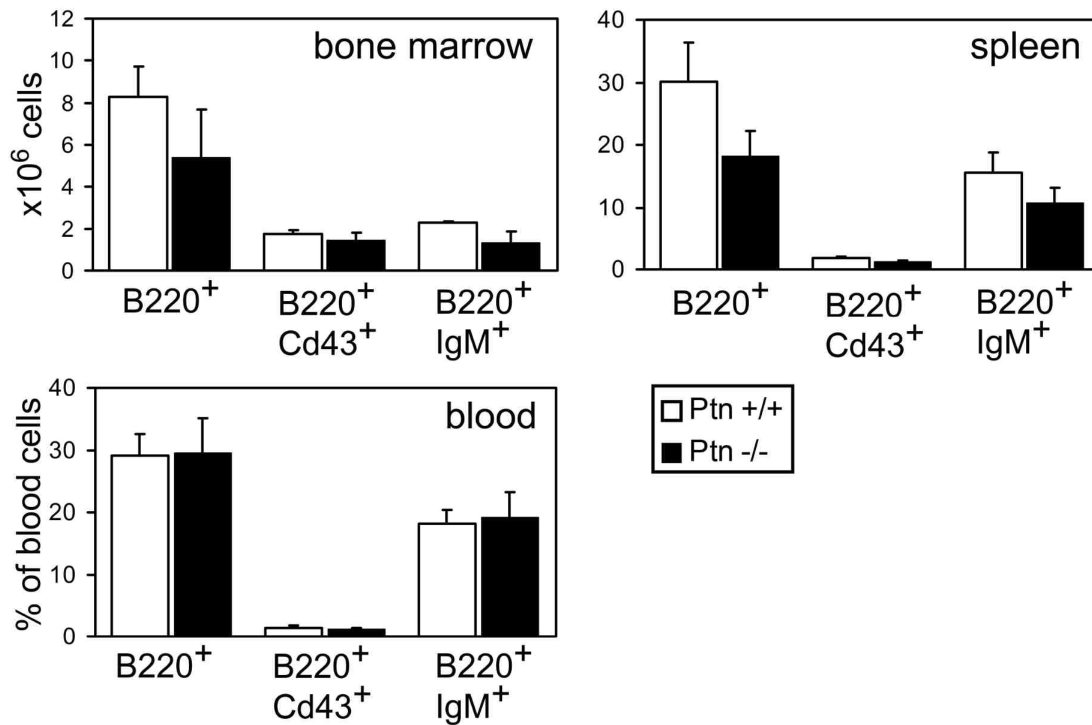


Figure 16. B cell populations in 129 Ptn KO and control mice. Absolute numbers of B cell populations are shown for bone marrow and spleen (bone marrow: number of cells per 4 long bones, spleen: total number of cells). For the peripheral blood, frequencies are shown, n=6 (+/+), n=7 (-/-). Mean ± SEM.

This was further confirmed by the ELISA analysis of soluble immunoglobulins in the peripheral blood. Neither in the concentration of total immunoglobulins nor in IgM- nor IgG2a-concentration in the blood serum was any difference detected between control and $Ptn^{-/-}$ mice (Figure 17).

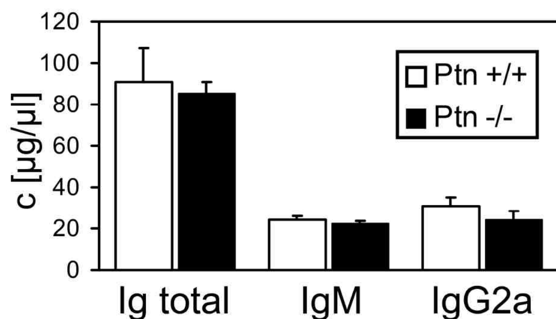


Figure 17. Immunoglobulins in the serum of 129 Ptn KO and control mice. Shown are the concentrations of immunoglobulins in the serum of the mice as determined by ELISA, n=5. Mean ± SEM.

T cells

T cells were detected using antibodies for Cd4 and Cd8a. Cd4 is a marker for helper T cells, while Cd8a is expressed on cytotoxic T cells. During their development in the thymus, T cells pass through a Cd4⁺ Cd8a⁺ stage which was also detected by flow cytometry (Figure 18). There were no significant differences found in the number of T cells in bone marrow or peripheral blood. Likewise, all T cell subsets in the thymus of Ptn KO mice were unchanged. In the spleen, however, the numbers of Cd8a⁺ were reduced, while the helper T cells were unchanged (Figure 19).

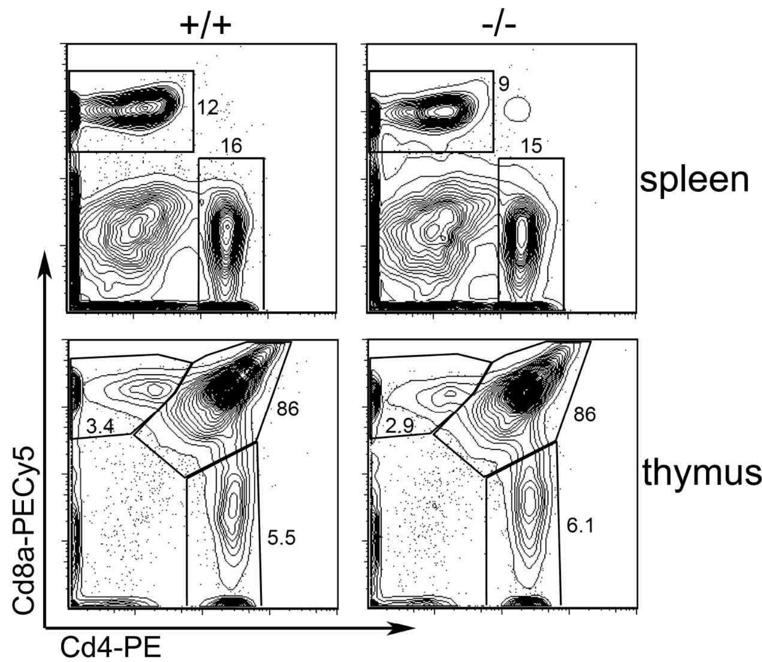


Figure 18. Gating strategy for T lymphocytes.

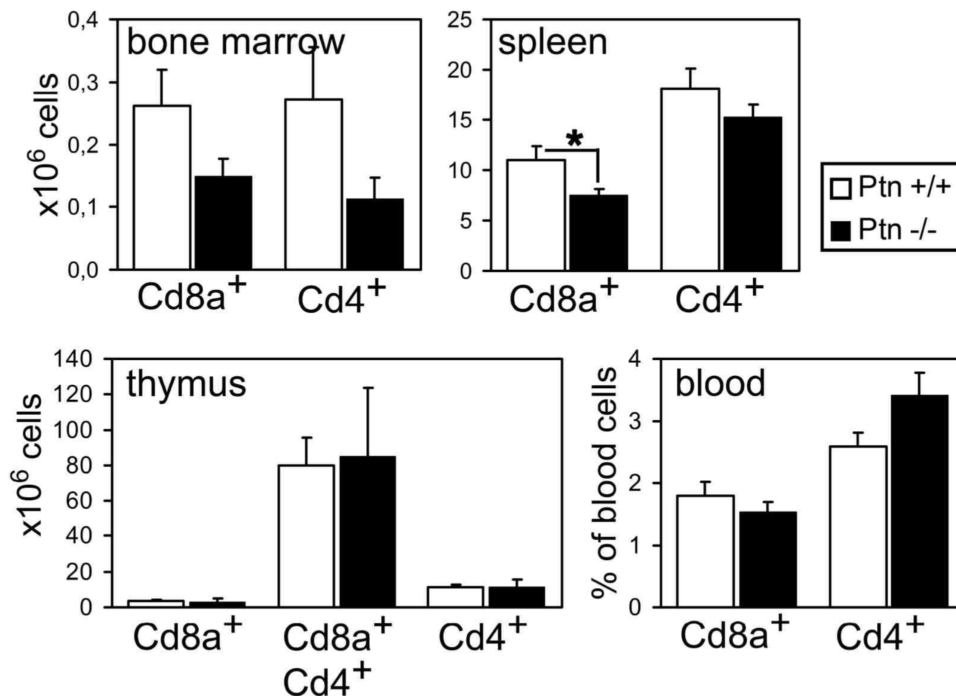


Figure 19. T cell populations in 129 Ptn KO and control mice. Absolute numbers of T cell populations are shown for bone marrow, spleen and thymus (bone marrow: number of cells per 4 long bones, spleen, thymus: total number of cells). For the peripheral blood, frequencies are shown, n=6 (+/+), n=7 (-/-). Mean ± SEM, *p<0.05.

NK cells

NK cells, on the other hand, detected by the absence of the T cell marker CD3 and the expression of NK1.1 marker, were notably reduced in bone marrow and spleen of Ptn KO mice. In the blood, by contrast, the levels of $CD3^{-}NK1.1^{+}$ cells were normal (Figure 20).

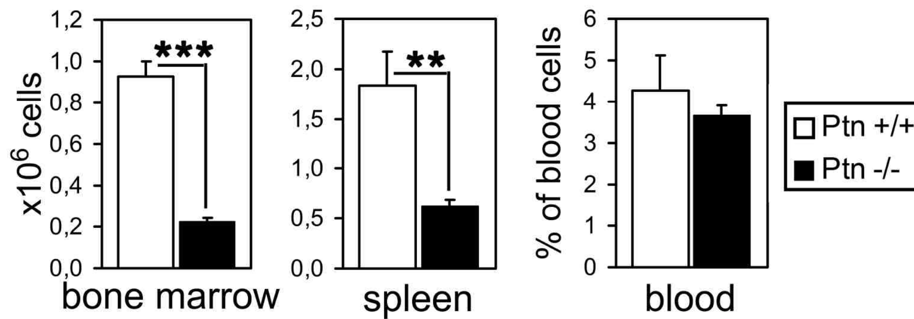


Figure 20. NK cells in 129 Ptn KO and control mice. Absolute numbers of NK cells are shown for bone marrow and spleen (bone marrow: number of cells per 4 long bones, spleen: total number of cells). For the peripheral blood, frequencies are shown, n=6 (+/+), n=7 (-/-). Mean ± SEM, $^{**}p<0.01$, $^{***}p<0.001$.

Erythroid cells

The development of the erythroid cells takes place in the bone marrow as well as in the spleen. During their development cells, committed to the erythroid lineage first start to express the marker Cd71, followed by the expression of Ter119. In the later development, the Cd71 expression then fades again (Zhang *et al.* 2003b) (Figure 21).

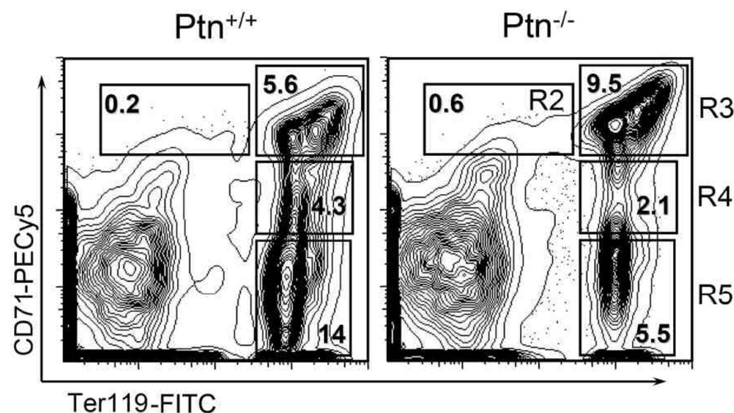


Figure 21. Gating strategy for erythroid cells.

Cells in the latest stage of this progression, the reticulocytes (R5), were reduced in the bone marrow of Ptn KO mice. In spleen however, none of the populations was significantly changed (Figure 22).

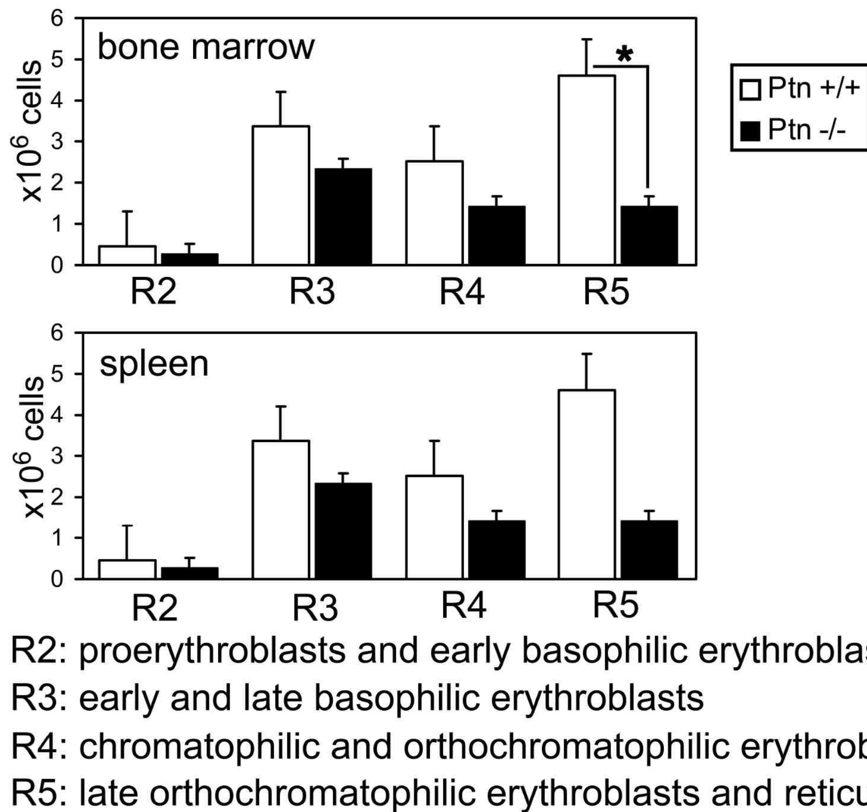


Figure 22. Erythroid cell populations in 129 Ptn KO and control mice. Absolute numbers of erythroid cell populations are shown for bone marrow and spleen (bone marrow: number of cells per 4 long bones, spleen: total number of cells), n=6 (+/+), n=7 (-/-). Mean ± SEM, *p<0.05.

Myeloid cells

The cells of the myeloid lineage, granulocytes and monocytes, are defined by the expression of the surface markers Gr-1 and Cd11b (Mac-1). Granulocytes highly express both markers, while monocytes are also strongly expressing Cd11b, but they show only a low expression of Gr-1 (Figure 23). The myeloid

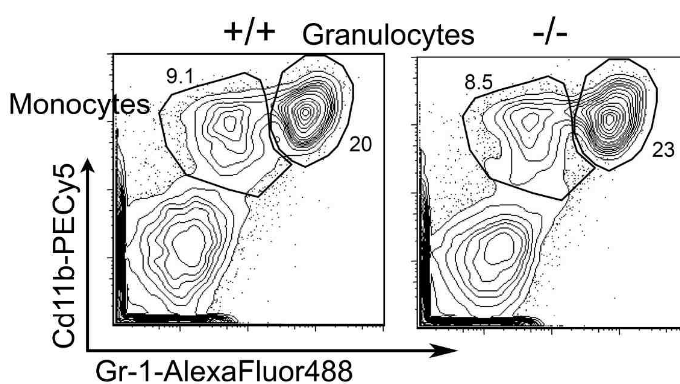


Figure 23. Gating strategy for myeloid cells.

populations were significantly changed in the Ptn KO mice. While monocytes are reduced in the bone marrow, both populations, monocytes and granulocytes were notably enlarged in the peripheral blood (Figure 24).

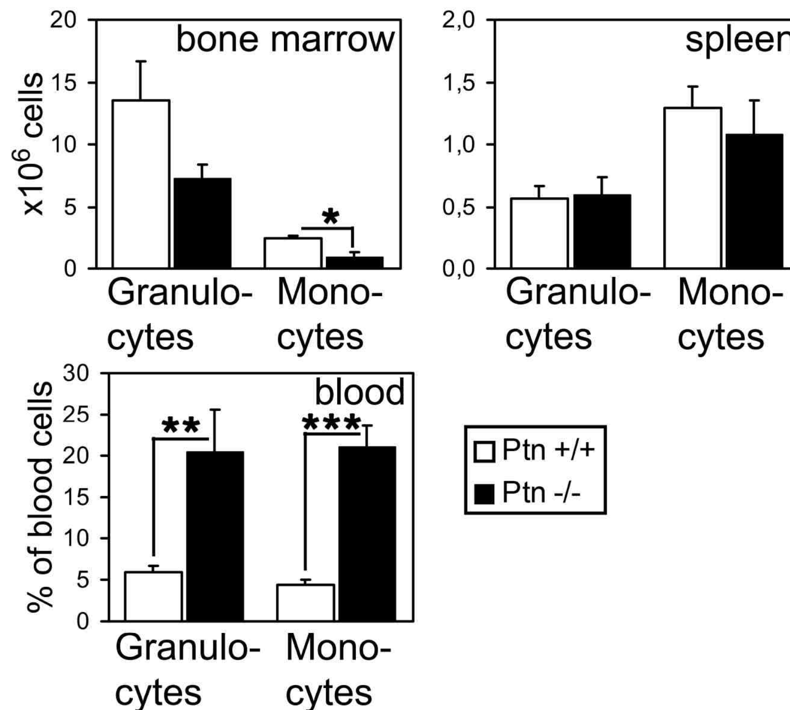


Figure 24. Myeloid cell populations in 129 Ptn KO and control mice. Absolute numbers of Granulocytes and Monocytes are shown for bone marrow and spleen (bone marrow: number of cells per 4 long bones, spleen: total number of cells). For the peripheral blood, frequencies are shown, n=6 (+/+), n=7 (-/-). Mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.

In summary, it can be stated that the early hematopoiesis was altered in 129 Ptn KO mice, the loss of Ptn led to an enhanced Cd34 expression by the LSK cells and a reduction of MPPs. Since Cd34 is known to be upregulated in LT-HSCs when they are activated into active proliferating ST-HSCs (Wilson *et al.* 2007), the enhanced Cd34 expression might indicate an increased activation of HSCs in Ptn KO mice. In the mature populations, the most strongly affected lineages by the loss of Ptn are the NK cells and the myeloid cells, where NK cells are reduced in bone marrow and spleen and myeloid cells increased in the circulation. The lymphoid B and T cells and the erythroid lineage seem to be mostly undisturbed.

3.5. Characterization of old 129 Ptn KO mice

If the enhanced Cd34 expression in the LSKs of the Ptn KO mice really points to an enhanced HSC activation, this most probably leads to an exhaustion of HSCs over time (see 1.1.4). Accordingly, it should be expected that changes observed in young mice will be amplified when the mice grow older. To find out whether this is indeed the case in $Ptn^{-/-}$ mice, old 129 Ptn KO mice (1 year old) and age- and sex-matched controls were used for a flow cytometry analysis of HSCs and early progenitors. Like in the young mice, the old Ptn KO mice showed a decreased bone marrow cell number, but an unchanged white blood cell count in the peripheral blood (Figure 25). None of the observed HSC and progenitor populations were significantly changed in the Ptn KO mice in comparison with controls (also due to the small number of old mice available). It is known that the Cd34- fraction of LSK increases with age. All in all, however, the expression of Cd34 in the LSK cells was increased, similar to the young 129 Ptn KO mice (Figure 26). It appears, therefore, that the HSCs in Ptn KO mice are not more activated than in the control animals. Another explanation might be that the loss of Ptn, with the corresponding enhanced proliferation of HSCs does not lead to an exhaustion of HSCs. This question can be addressed by means of serial transplantations. In case of normal HSC proliferation and function there would be no difference between Ptn KO mice and controls, but if HSC proliferation is increased without exhaustion, the engraftment in Ptn KO mice would be consistently higher in these mice than in control mice.

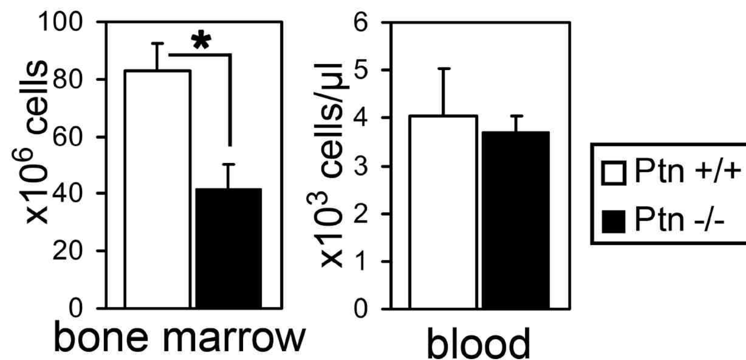


Figure 25. Organ cell numbers of old 129 Ptn KO mice and controls. Cell counts from bone marrow (BM, four long bones: two femurs and two tibia per mouse) and peripheral blood, n=4. Mean ± SEM, *p<0.05.

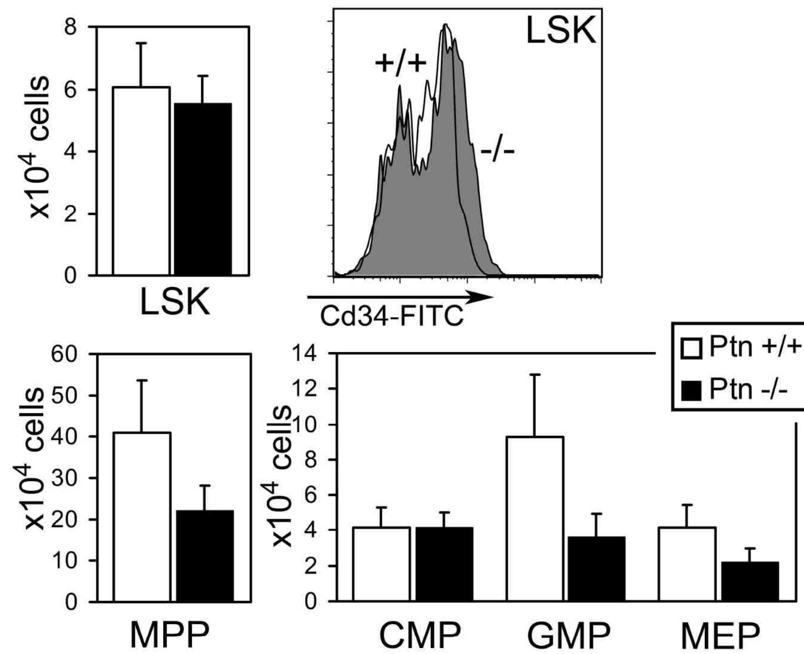


Figure 26. Progenitor populations and expression of Cd34 in the LSK population in the bone marrow of old 129 Ptn KO and control mice. Shown are absolute numbers per 4 long bones, n=4. Mean ± SEM.

3.6. Characterization of (129xB6)F2 mice

The easiest way to follow donor cells in a recipient mouse after transplantation is to use mice differing in the expression of Cd45.1 and Cd45.2. The donor cells can then be retrieved by flow cytometry. Most mouse strains express Cd45.2, as do the Ptn KO mice. Since mice which express Cd45.1 were only available on a C57Bl/6J (B6) background and not on a 129 background, the Ptn KO mice were crossed with B6 to generate Ptn KO mice on a mixed 129/B6 background which could be transplanted with B6 cells (Cd45.2) as well as with congenic B6.SJL-Ptprc^{a} Pep3^{b}/BoyJ (Cd45.1) without rejection.

In order to check their phenotype, the (129xB6)F2 littermates were analysed. Very surprisingly, it was found that the organ cell numbers, especially the bone marrow cell numbers, of the Ptn KO mice did not differ from the control mice anymore (Figure 27), suggesting that crosses into the B6 background had diminished the phenotype we had observed above in Ptn KO mice on a 129 background.

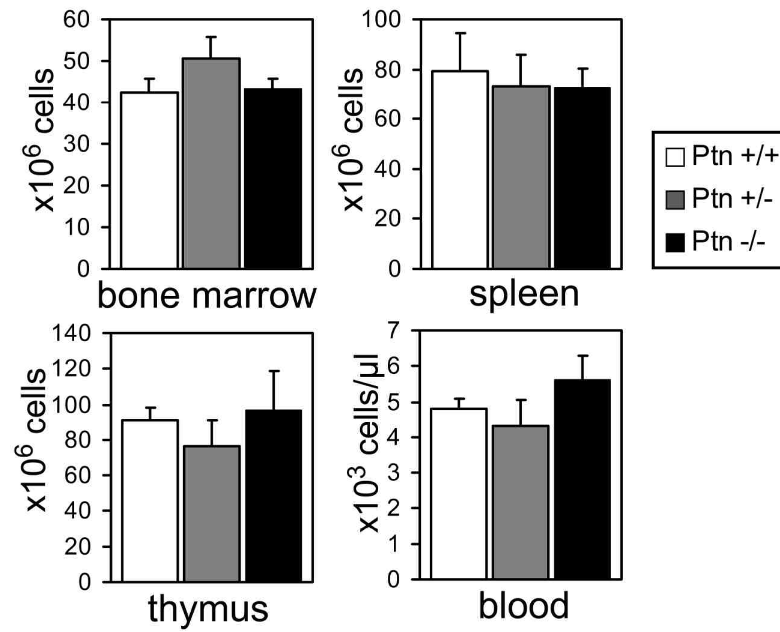


Figure 27. Organ cell numbers of (129xB6)F2 Ptn KO mice and controls. Cell counts from bone marrow (BM, four long bones: two femurs and two tibia per mouse), spleen, thymus, and peripheral blood, bone marrow, blood: n=6 (+/+), n=8 (+/-, -/-), spleen, thymus: n=4 (+/+), n=5 (+/-, -/-). Mean ± SEM.

In addition to that, no changes were found in the HSC and progenitor populations of the (129xB6)F2 $Ptn^{-/-}$ mice compared to wt mice (Figure 28).

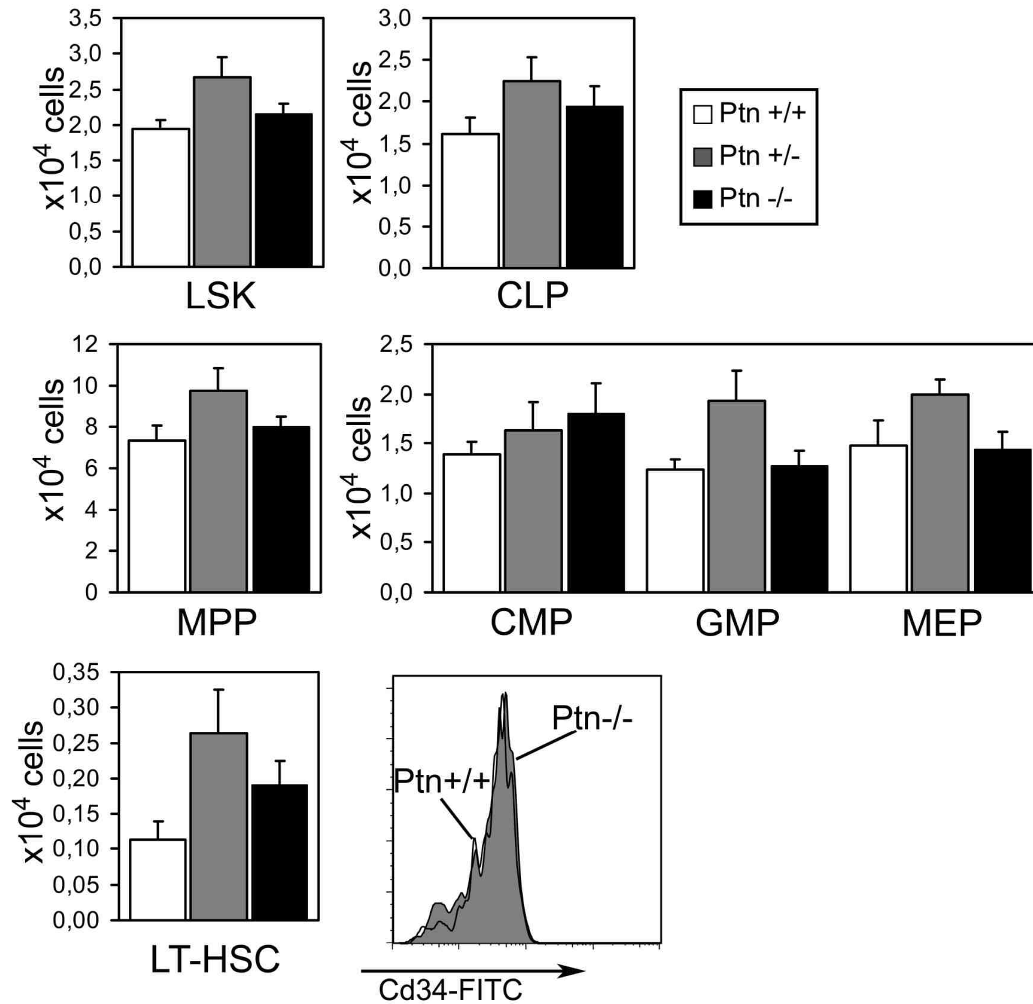


Figure 28. Progenitor populations in bone marrow of (129xB6)F2 Ptn KO and control mice. Shown are absolute numbers per 4 long bones, n=6 (+/+), n=8 (+/-, -/-) Mean ± SEM.

This was confirmed by colony forming assays, which showed no difference between $Ptn^{-/-}$ and $Ptn^{+/+, +/-}$ (Figure 29).

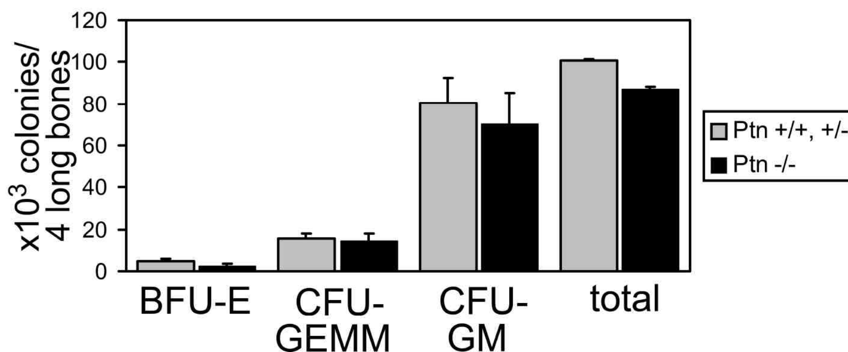


Figure 29. Colony forming assays of (129xB6)F2 Ptn KO and control mice. Shown are the total number of colonies per 4 long bones, n=3. Mean ± SEM.

The only alterations found in the hematopoietic populations of the (129xB6)F2 Ptn KO mice compared to $Ptn^{+/+}$ were a reduction of $CD3^+$ cells (T cells) in the bone

marrow and, which was similar to the 129 Ptn KO mice, enlarged myeloid populations in the peripheral blood (Figure 30).

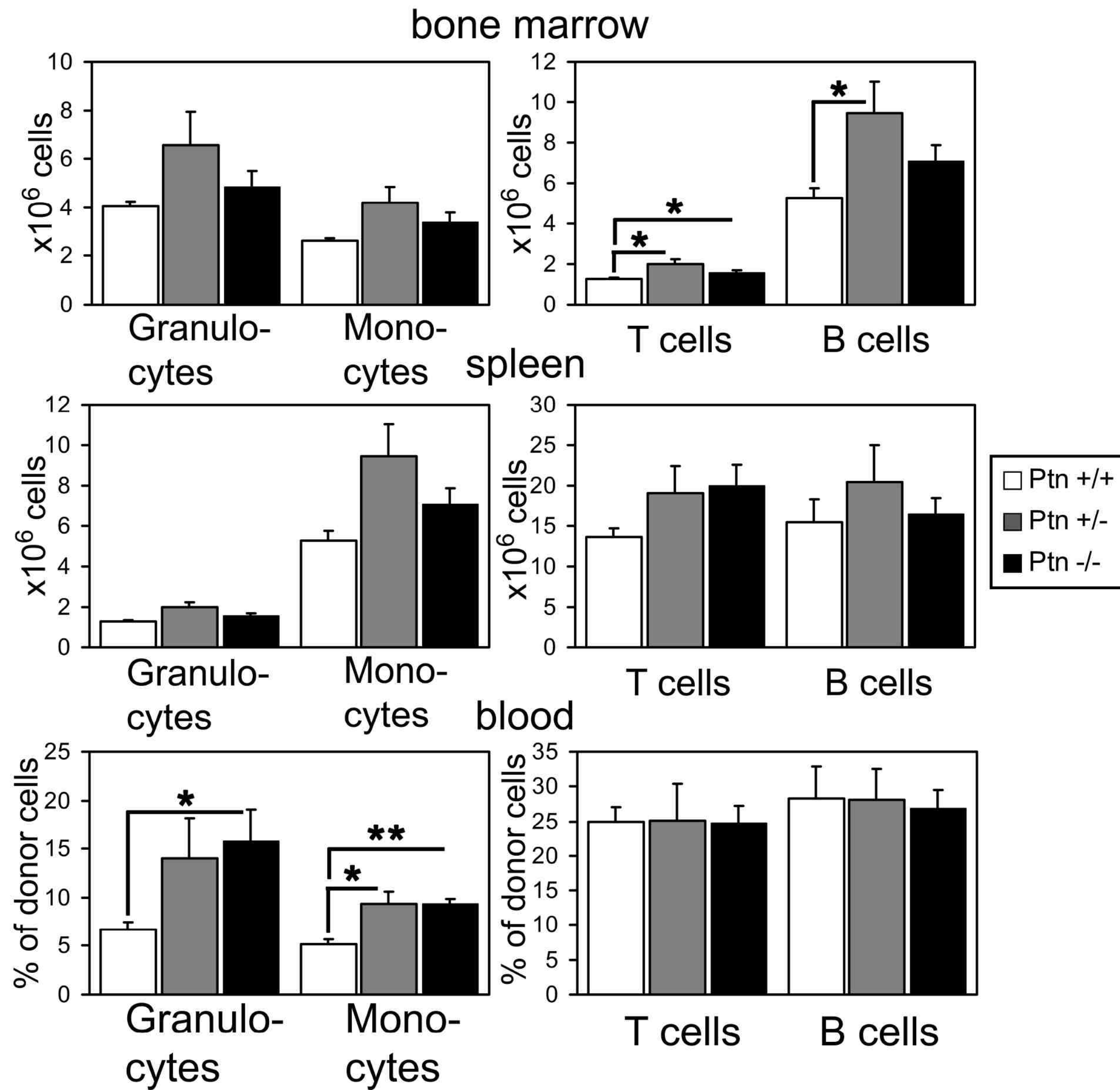


Figure 30. Mature cell populations in (129xB6)F2 Ptn KO and control mice. Absolute numbers of B cell, T cell and myeloid populations are shown for bone marrow and spleen (bone marrow: number of cells per 4 long bones, spleen: total number of cells). For the peripheral blood, frequencies are shown, bone marrow, blood: n=6 (+/+), n=8 (+/-, -/-), spleen: n=4 (+/+), n=5 (+/-, -/-). Mean ± SEM, *$p<0.05$, **$p<0.01$.

The surprising difference in phenotype between Ptn KO mice on a 129 and a (129xB6)F2 background gives the impression that the phenotype of the Ptn KO mice might depend on the mouse strain. This phenomenon is rare, but similar phenomena have already been observed in other genetic models, like, for example, in Cdkn1a

(p21[WAF1]) KO mice, which have already been mentioned above (see 1.1.4) (van Os *et al.* 2007). In an attempt to find out whether the different phenotypes in 129 and (129xB6)F2 were related to changes as those reported for the mice deficient for the cell cycle repressorCdkn1a KO, in the next step the proliferation of the hematopoietic cells in the two different mouse strains was examined.

3.7. Proliferation in 129 and (129xB6)F2 Ptn KO mice

In order to find an explanation for the strain-dependent phenotype of the Ptn KO mice, the rates of cell proliferation were checked in these mice in steady-state hematopoiesis as well as under stress.

In order to compare cell proliferation in the bone marrow of Ptn KO and control mice, BrdU analysis was used. BrdU, which resembles thymine, is integrated in the DNA during replication and can afterwards be detected by intracellular staining. Those cells, which have gone through the S phase in a certain timeframe (the time in which BrdU was available), can therefore be observed as BrdU positive cells.

Steady-state hematopoiesis

BrdU was injected into 129 Ptn KO mice and controls and the mice were sacrificed 2 h later. Although only a very small number of mice were used for this experiment, the results showed that there was no indication for an enhanced proliferation of bone marrow cells (Figure 31). Almost the same experiment was done with (129xB6)F2 Ptn$^{-/-}$ mice and their Ptn$^{+/-}$ littermates – the only difference being that the mice were only sacrificed 16 h after BrdU injection. Nevertheless, the result was the same: There was no indication of an altered proliferation of bone marrow cells under steady-state conditions (Figure 32).

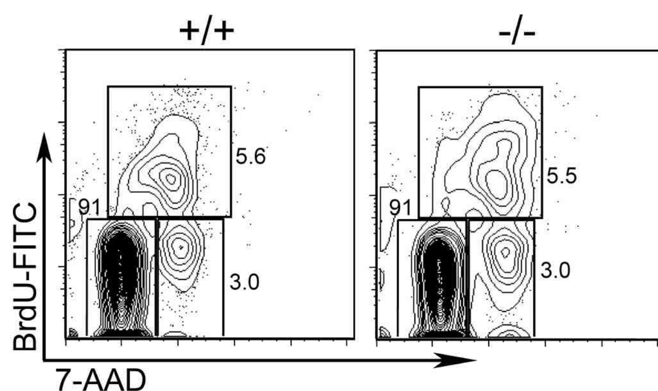


Figure 31. BrdU analysis of 129 Ptn KO mice and wt control. The mice were sacrificed 2h after BrdU injection, n=2 per genotype.

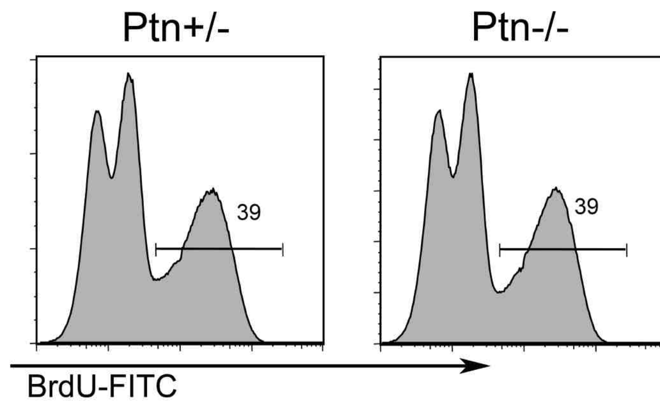


Figure 32. BrdU analysis of (129xB6)F2 Ptn$^{-/-}$ and Ptn$^{+/-}$ mice. The mice were sacrificed 16 h after BrdU injection, n=2 (+/-), n=3 (-/-).

Stress-dependent hematopoiesis

For the investigation of stress-dependent hematopoiesis, mice were injected with 5-Fluorouracil (5-FU). 5-FU can be integrated into DNA and RNA during synthesis because of its similarity with thymine and uracil, resulting in non-functional nucleic acids. Therefore, the injection of 5-FU leads to the cell death of dividing cells such as hematopoietic progenitors and mature cells. Since most of the HSCs are quiescent, mice do not die due to an injection of 5-FU. The eradication of the main part of the hematopoietic system, however, leads to the activation of the HSCs which have to regenerate and compensate for the loss of cells. Firstly, 5-FU was injected into 129 Ptn KO and control mice and the mice were sacrificed at day 4, 8, 12 or 16 after the injection. It can be noted that after 4 days, the bone marrow cell numbers were reduced to about 10%, but the numbers already recovered at day 8 and had almost come back to normal after 16 days (Figure 33). The Ptn KO mice had similar bone marrow cell numbers at day 4 in comparison with the control mice, but at day 8 after injection, the bone marrow cell numbers of the Ptn KO mice were 2.2 fold higher than the bone marrow cell numbers of the controls. This effect might be a result of a higher

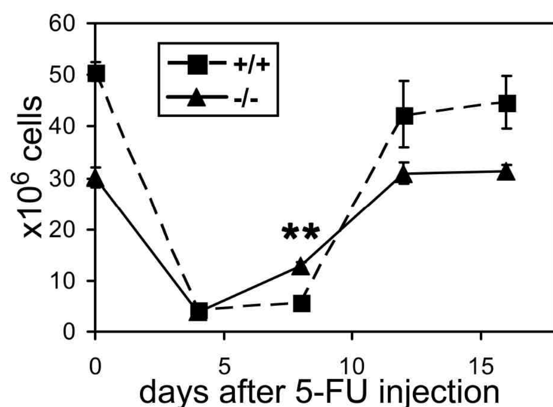


Figure 33. Recovery of 129 Ptn KO and control mice after injection of 5-FU. Shown are bone marrow cell numbers per 4 long bones, n=3 per timepoint. Mean ± SEM, **p<0.01.

recovery capability due to an increased cell cycling activity. Another explanation might be a reduced sensitivity of Ptn KO cells to 5-FU, maybe due to a reduced proliferative rate.

To further investigate this question, 129 Ptn KO mice were injected with 5-FU and subsequently, 5 days later, they were injected with BrdU. The mice were finally sacrificed 16 h after the injection of BrdU. Comparing the frequency of BrdU positive cells of the Ptn KO mice with the controls reveals that the fraction of dividing cells is notably higher in the Ptn KO mice (Figure 34).

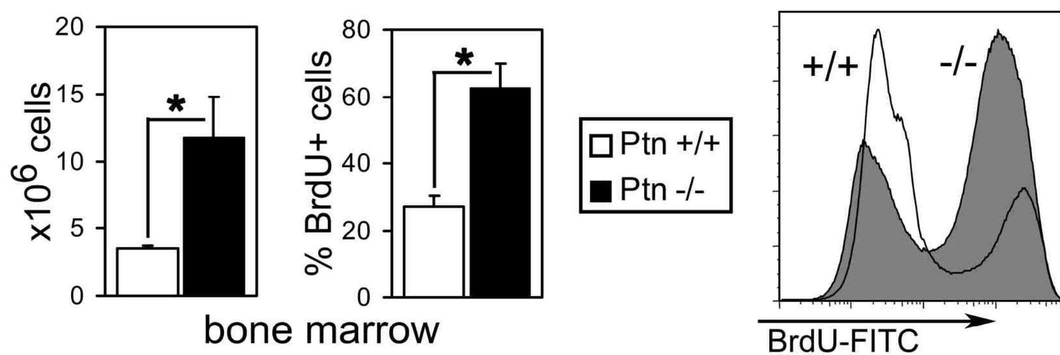


Figure 34. Proliferation after 5-FU injection in 129 Ptn KO mice and controls. Analysis of bone marrow cell numbers (per 4 long bones) and the percentage of BrdU-positive cells 6 days after 5-FU injection and 16h after injection of BrdU. n=3. Mean ± SEM, *p<0.05.

The same experiment (BrdU injection five days after 5-FU treatment) was performed with (129xB6)F2 $Ptn^{-/-}$, $Ptn^{+/-}$ and $Ptn^{+/+}$ mice. In contrast to the results in the 129 Ptn KO mice, there was no difference found in the frequency of dividing cells between the different groups (Figure 35), though the levels of BrdU+ cells in both 129 and (129xB6)F2 controls were very similar (compare open bars in Figures 33 and 34).

Figure 35. Proliferation after 5-FU injection in (129xB6)F2 Ptn KO mice and controls. Analysis of bone marrow cell numbers (per 4 long bones) and the percentage of BrdU-positive cells 6 days after 5-FU injection and 16h after injection of BrdU. n=3 (+/+), n=5 (+/-, -/-), Mean ± SEM.

This indicates that the divergent effects observed by the loss of Ptn in the two different mouse strains might indeed be caused by the unequal proliferation rates of the two strains. In addition to this, this experiment indicates that in the 129 Ptn KO mice, an enhanced proliferation leads to the accelerated recovery of the bone marrow cell numbers after 5-FU injection.

3.8. Characterization of rederived 129 Ptn KO mice.

Because of too little space and several occurring infections in the animal facility in which the 129 Ptn KO mice were kept, these mice were rederived and afterwards kept in individually ventilated cages. During the rederivation, embryos of the mouse line in question are transferred to pseudopregnant females, known to be free of pathogens. Since the embryos are not infected by most of the pathogens this leads to the acquisition of animals with the same health status as the females they were transferred into.

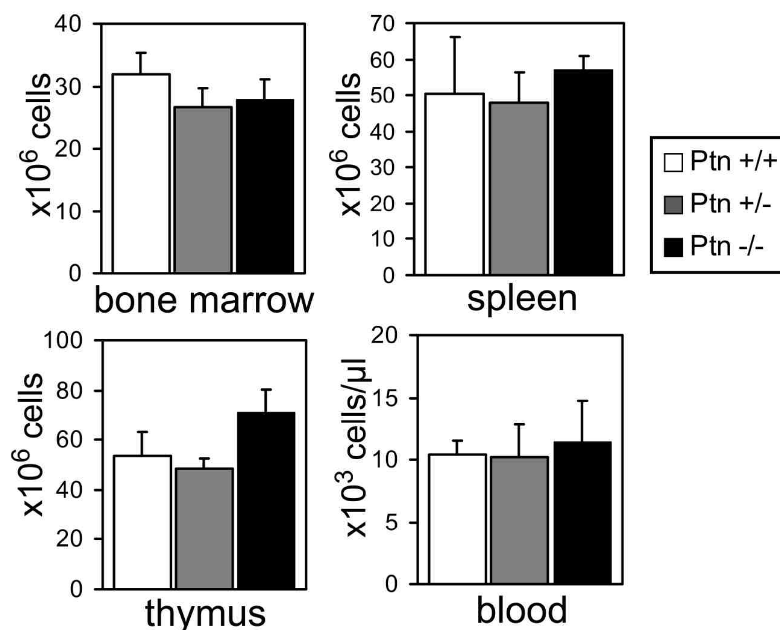


Figure 36. Organ cell numbers of rederived 129 Ptn KO mice and controls. Cell counts from bone marrow (BM, four long bones: two femurs and two tibia per mouse), spleen, thymus, and peripheral blood, n=3 (+/+, +/-), n=4 (-/-). Mean ± SEM.

At this point, the question of which strain would produce which phenotype became slightly more complex. There are 15 different 129 sub strains (Simpson *et al.* 1997). The Ptn KO mice were originally established in E14 ES cells (129P2 strain) and crossed with 129S6 mice (Amet *et al.* 2001). However, neither 129P2 nor 129S6 strains were available to us for backcrossing purposes after rederivation. Only the 129S2 strain was available from European commercial providers of experimental animals (Charles River). Hence, the original 129P2x129S6 mixed background Ptn KO mice

were backcrossed to the 129S2 strain ((129P2x129S6)F?x129S2)Fx. For the experiments described underneath, F2 mice were used.

After the rederivation, the mice were analysed in order to check if they still show the same phenotype as before. Similar to the (129xB6)F2, the rederived 129 Ptn KO mice showed no differences in organ cell numbers (Figure 36). There were also no alterations detected in the progenitor populations (Figure 37).

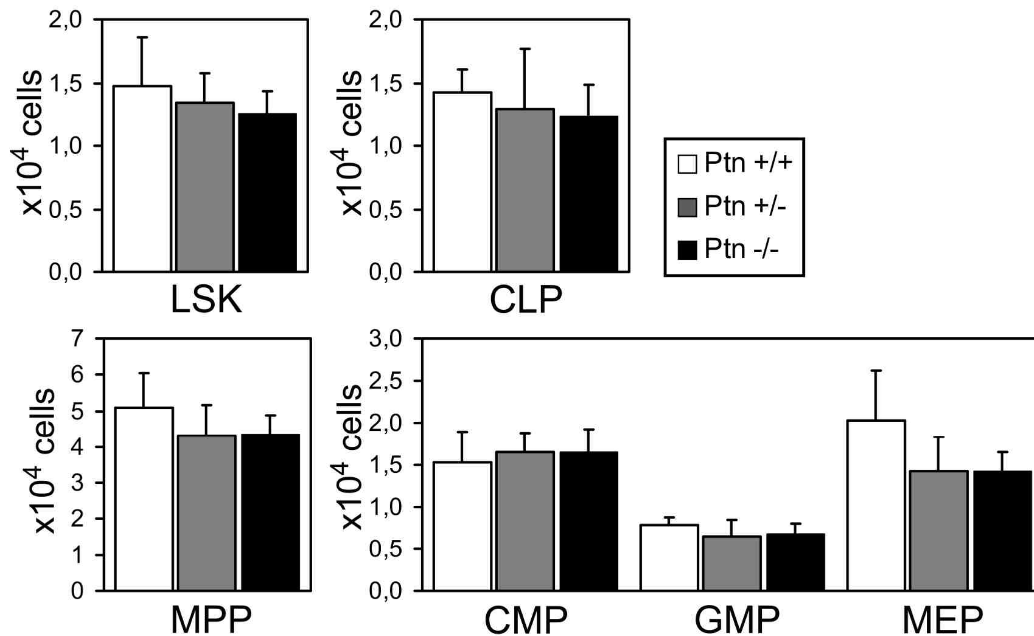


Figure 37. Progenitor populations in bone marrow of rederived 129 Ptn KO and control mice. Shown are absolute numbers per 4 long bones, n=3 (+/+, +/-), n=4 (-/-) Mean ± SEM.

For the HSCs, an extended stain was used, including staining of the markers CD150, Flk2 and Cd48 (see 1.1.2, Figure 38).

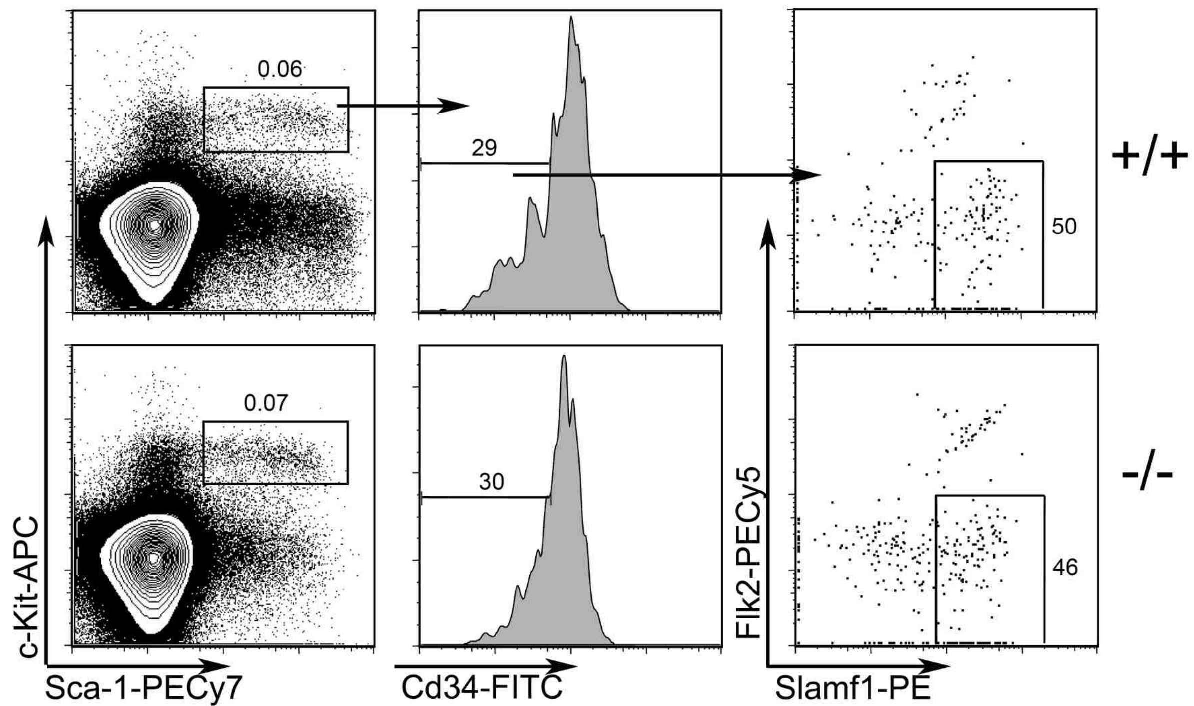


Figure 38. Gating strategy for LT-HSCs

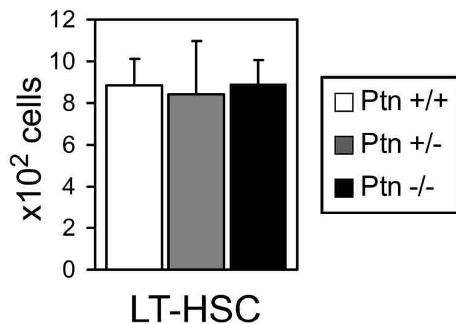


Figure 39. LT-HSC numbers in bone marrow of rederived 129 Ptn KO mice and controls. Cells per 4 long bones, n=4 (+/+, +/-), n=6 (-/-), Mean ± SEM.

Nevertheless, there was no difference found between the $Ptn^{-/-}$ mice and their $Ptn^{+/-}$ and $Ptn^{+/+}$ littermates (Figure 39). Likewise, the mature populations in the rederived 129 Ptn KO mice were unchanged (Figure 40). (Only the most important lineages were shown but the same populations were checked as during the first characterization of the 129 Ptn KO mice, but there was no difference anyway. Also, data from spleen and thymus is not shown here, but was obtained and also showed no differences.)

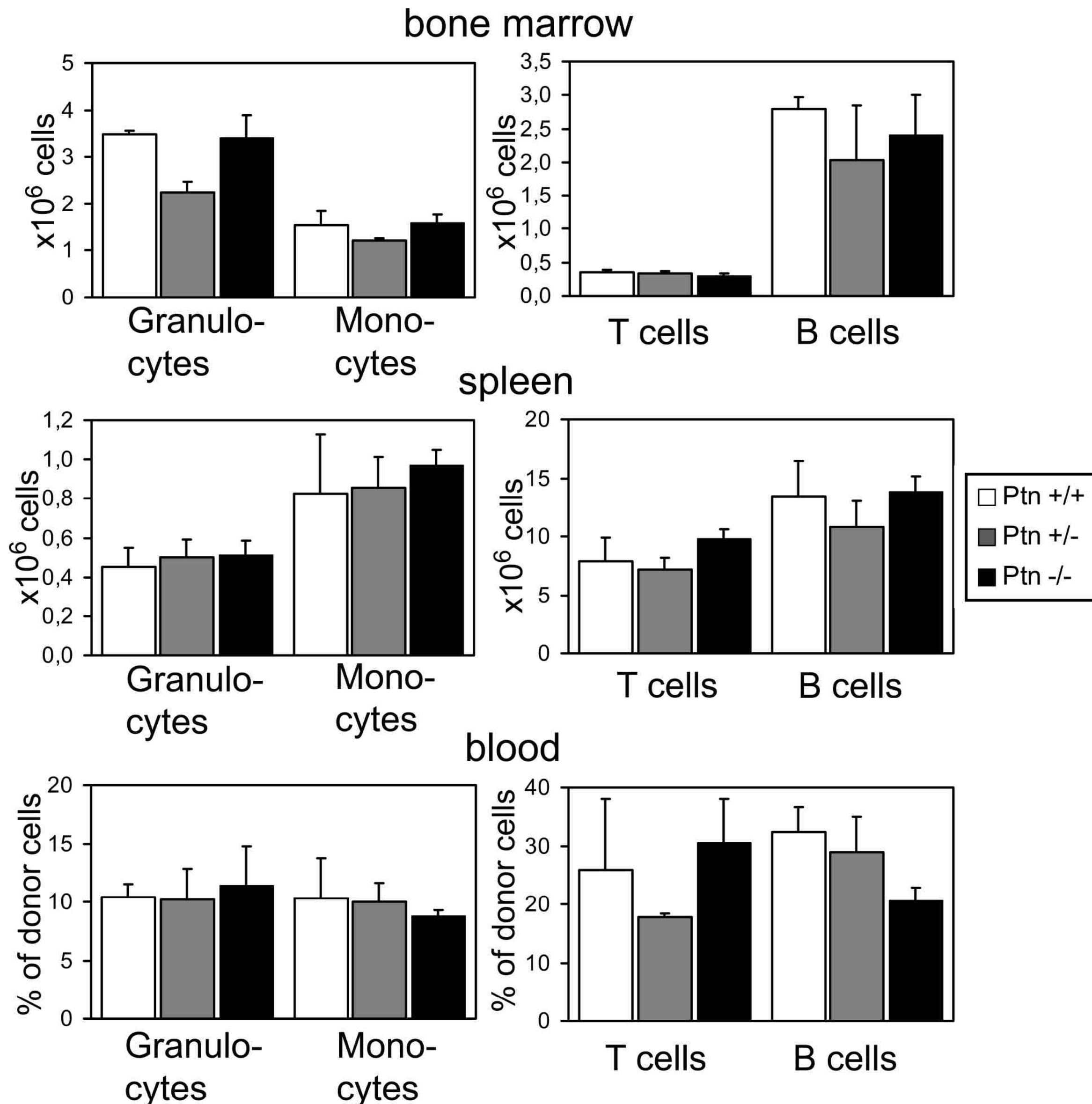


Figure 40. Mature cell populations in rederived 129 Ptn KO and control mice. Absolute numbers of B cell, T cell and myeloid populations are shown for bone marrow and spleen (bone marrow: number of cells per 4 long bones, spleen: total number of cells). For the peripheral blood, frequencies are shown, n=3 (+/+, +/-), n=4 (-/-). Mean ± SEM.

In conclusion, the rederived mice crossed onto the 129S2 background phenotypically resembled the (129xB6)F2 mice that had been used prior to the rederivation.

For further experiments in this thesis, the rederived 129S2 Ptn KO mice (F2 generation) and (129S2xB6)F2 Ptn KO mice were used as indicated.

3.9. Transplantation of Ptn deficient HSCs

The results with co-cultures of stromal cells and lin$^-$ bone marrow cells indicated that the loss of Ptn in stromal cells increases the production of colony-forming progenitors. Since these experiments were performed with cells derived from 129S2 as well as from B6 mice (Figure 8), and the results of these experiments were the same as those performed with (129P2x129S6) Ptn KO cells (Figure 9), these results do not show the same dependency on mouse strain as the steady state and 5-FU experiments described above. Thus, it remains of interest to investigate the role of Ptn in progenitor production, and reconstitution/engraftment of myeloablated recipients.

In the *in vitro* co-cultures of shPtn and pLKO.1 UG26-1B6 with wt or Ptn$^{-/-}$ lin$^-$ cells, there was apparently no difference between the usages of wt or Ptn$^{-/-}$ lin$^-$ cells (see 3.3). This indicates that the loss of Pleiotrophin has no intrinsic effect on the HSCs. In order to confirm this *in vivo*, transplantations were done of Ptn$^{-/-}$, Ptn$^{+/-}$ and Ptn$^{+/+}$ (129xB6)F2 bone marrow cells into (129xLy5.1)F1 mice. The microenvironment of the HSCs is the same after the transplantation. There is only a difference in the HSCs, which are +/+, +/- or -/- for Ptn. The irradiation of the recipients leads to the cell death of most cells of the hematopoietic system, therefore the transplanted HSCs have to get activated and restore these cells. Transplantation experiments therefore display stress-dependent, not steady-state hematopoiesis.

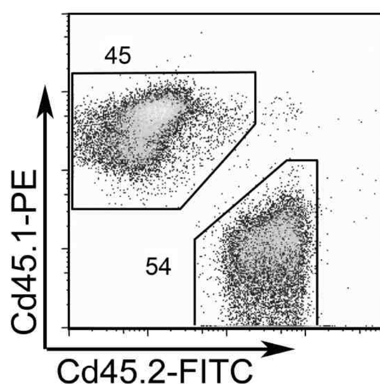


Figure 41. Gating for blood cells differing in Cd45.1 and Cd45.2 expression.

Since the donor and recipient cells differed in the expression of the surface markers Cd45.1 (Ly5.1) and Cd45.2 (Ly5.2), the donor cells could be distinguished from the recipient cells by the means of flow cytometry (Figure 41). The mice were bled 5 and 10 weeks after the transplantation and finally sacrificed after 16 weeks. These transplantation experiments clearly demonstrated there was no difference observed in engraftment levels of wt and Ptn KO marrow cells in any of the examined

organs (bone marrow, spleen, peripheral blood) (Figure 42a). Likewise, the numbers of LT-HSCs, LSKs and MPPs were unchanged in the different groups (Figure 42b, c). The frequencies of lymphoid and myeloid cells over the time of the transplantation did also not differ in dependence of the genotype of the injected HSCs (Figure 42d).

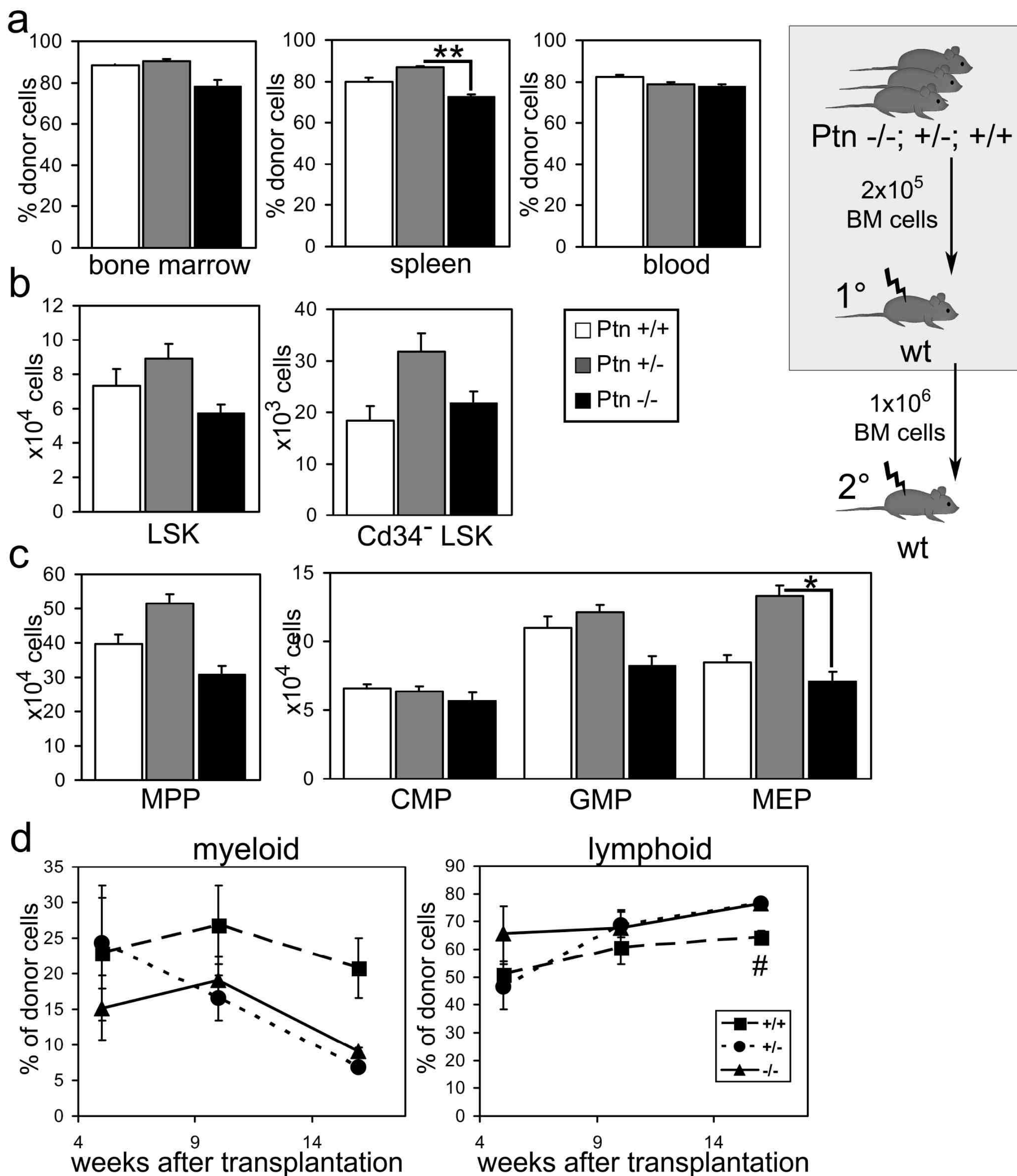


Figure 42. Primary transplantation of $Ptn^{-/-}$, $Ptn^{+/-}$ and $Ptn^{+/+}$ cells into wt recipients. a) Engraftment levels in bone marrow (percentage of donor cells in total cell count of 4 long bones), spleen and blood 16 weeks after transplantation. b) Total number of donor LSK and $Cd34^-$ LSK cells in the bone marrow of recipient mice (per 4 long bones). c) Total number of donor progenitor cells in 4 long bones of the recipients. d) Frequency of myeloid and lymphoid cells in the donor cell population in peripheral blood of the recipient mice. n= 4 (+/+), n=6 (+/-), n=9 (-/-), Mean ± SEM, **p<0.01, #p<0.05 (+/+ vs. +/-).

To find out whether wt and Ptn KO HSC differed in their ability to self-renew, bone marrow cells from the primary recipients were again transplanted into irradiated secondary (129xLy5.1)F1 (Ly5.1xLy5.2 heterozygote) wildtype mice. As in the primary round of transplantation, the mice were bled 5 and 10 weeks after the injection and sacrificed after 16 weeks. In confirmation of the results from the primary transplants, no differences in engraftment levels were detected, neither in bone marrow, spleen nor in the peripheral blood (Figure 43a). The numbers of LT-HSCs, LSKs and MPPs were unchanged as well. Though there was a difference between LT-HSC and LSK numbers between mice which received $Ptn^{-/-}$ and $Ptn^{+/-}$, respectively, the numbers did not differ between mice which received $Ptn^{-/-}$ and wt control ($Ptn^{+/+}$) cells (Figure 43b, c). Comparing the myeloid and lymphoid cells in the peripheral blood, mice injected with $Ptn^{+/+}$ cells seemed to give rise to more myeloid cells in the beginning of the transplantation, but after 16 weeks there was no difference between mice injected with $Ptn^{-/-}$ cells and the controls (Figure 43d).

In summary, it can be stated that the loss of Pleiotrophin expression in the HSC themselves has no effect on the maintenance of the stem cells *in vivo*. Although perhaps some carry over from the Ptn-/- environment of the primary donor cells may have occurred, the engraftment behaviour was completely normal in secondary recipients. This confirms the results of the *in vitro* experiments (see 3.3), and leads to the conclusion that Ptn is not intrinsically involved in HSC maintenance.

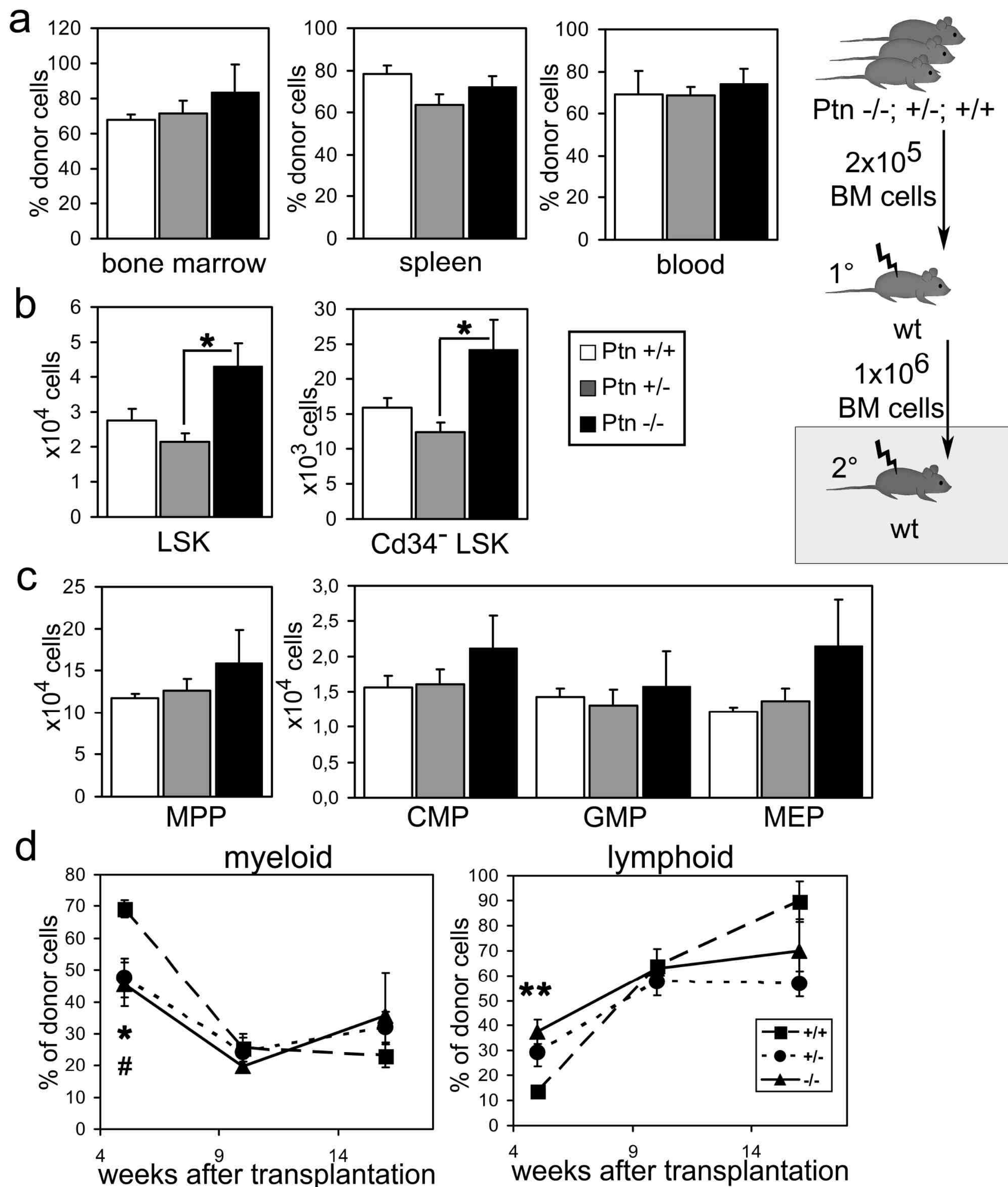


Figure 43. Secondary transplantation of Ptn$^{-/-}$, Ptn$^{+/-}$ and Ptn$^{+/+}$ cells into wt recipients. a) Engraftment levels in bone marrow (percentage of donor cells in total cell count of 4 long bones), spleen and blood 16 weeks after transplantation. b) Total number of donor LSK and Cd34^{-} LSK cells in the bone marrow of recipient mice (per 4 long bones). c) Total number of donor progenitor cells in 4 long bones of the recipients. d) Frequency of myeloid and lymphoid cells in the donor cell population in peripheral blood of the recipient mice. n= 4 (+/+), n=9 (+/-), n=7 (-/-), Mean ± SEM, *p<0.05, **p<0.01, #p<0.05 (+/+ vs. +/-).

3.10. Transplantation into a Ptn-deficient microenvironment

In the *in vitro* experiments the loss of microenvironmental Ptn had severe effects (see 3.3). It is thus important to transplant wt HSCs into Ptn KO mice to observe the effects caused by the loss of microenvironmental Ptn *in vivo*.

Primary transplantation

For this experiment, bone marrow cells of B6 Ly5.1 (Cd45.1) mice were transplanted into irradiated (129xB6)F2 $Ptn^{-/-}$, $Ptn^{+/-}$, and $Ptn^{+/+}$ recipients. The mice were bled after 5 and 10 weeks and sacrificed after 16 weeks. Like in the transplantations before, the analysis of the experiment could be done by flow cytometry because the donor cells expressed Cd45.1 and the recipient cells on the other hand expressed Cd45.2. In both, bone marrow and peripheral blood, the population of donor cells was enhanced in the $Ptn^{-/-}$ mice compared to the $Ptn^{+/+}$ mice (Figure 44a). Furthermore, the numbers of donor MPP cells (more precisely, the numbers of CMPs and GMPs) were higher in the $Ptn^{-/-}$ mice than in the wt controls (Figure 44c). The donor LT-HSC numbers, however, were only significantly elevated in the $Ptn^{-/-}$ mice in comparison with the $Ptn^{+/-}$ mice (Figure 44b). In the frequencies of the donor myeloid and lymphoid cells in the peripheral blood, there were no gross changes. Only after 5 weeks, the population of myeloid cells was enlarged and the lymphoid cells reduced in the blood of $Ptn^{-/-}$ mice compared to wt controls (Figure 44d). The enhanced engraftment in bone marrow and blood points to an enhanced proliferation of HSCs in a Ptn-deficient microenvironment. Such an enhanced proliferation may lead to a diminished HSC self-renewal (see 1.1.4 and (Renstrom *et al.* 2009a)). For that reason, the bone marrow of the primary recipients was injected into secondary irradiated wt recipients. An accelerated HSC exhaustion should lead to a decline in engraftment in the secondary transplantation. If the HSCs, however, proliferate more rapidly without enhanced differentiation, as speculated before (see 3.5), the engraftment of the cells which had been in a Ptn-deficient microenvironment would still be enhanced.

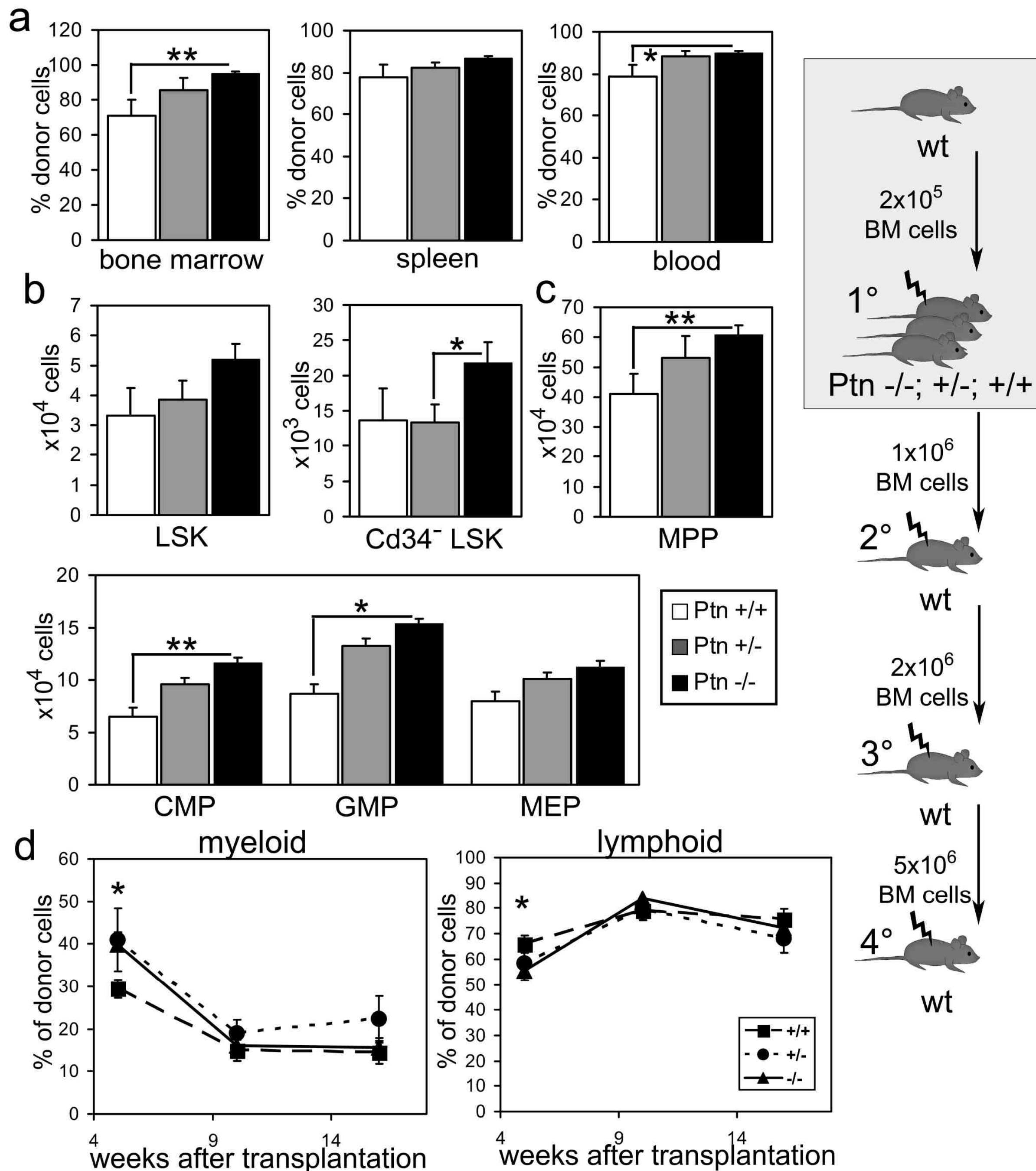


Figure 44. Primary transplantation of wt cells into Ptn$^{-/-}$, Ptn$^{+/-}$ and Ptn$^{+/+}$ recipients. a) Engraftment levels in bone marrow (percentage of donor cells in total cell count of 4 long bones), spleen and blood 16 weeks after transplantation. b) Total number of donor LSK and Cd34^{-} LSK cells in the bone marrow of recipient mice (per 4 long bones). c) Total number of donor progenitor cells in 4 long bones of the recipients. d) Frequency of myeloid and lymphoid cells in the donor cell population in peripheral blood of the recipient mice. n= 5 (+/+), n=10 (+/-, -/-), Mean ± SEM, *p<0.05, **p<0.01.

Secondary transplantation

After transplantation of the bone marrow of the previously transplanted mice, the recipients were treated analogously to the primary recipients: The mice were bled after 5 and 10 weeks and sacrificed after 16 weeks. The engraftment of cells that had been in a Ptn-deficient microenvironment was still enhanced in the peripheral blood compared to wt controls (Figure 45a). There were no other significant differences in engraftment or donor HSC and progenitor cell numbers (Figure 45b, c). In the first weeks of the transplantation, similarly to the primary transplantation, an enlarged population of myeloid donor cells and on the other hand, reduced lymphoid cell numbers were found in the peripheral blood of mice which received cells from a Ptn-deficient microenvironment (Figure 45d). This enhanced engraftment in the peripheral blood indicates that the loss of Ptn secretion by the microenvironment may indeed lead to an enhanced HSC self-renewal activity.

To confirm this further, the bone marrow of the secondary recipients was used for a tertiary transplantation into wt recipients. This experiment should also show whether the effect of the enhanced HSC self-renewal is reversible, since the wt donor cells were then for the second passage back in a wt microenvironment and the effects of the primary Ptn-/- environment should have diluted at this stage.

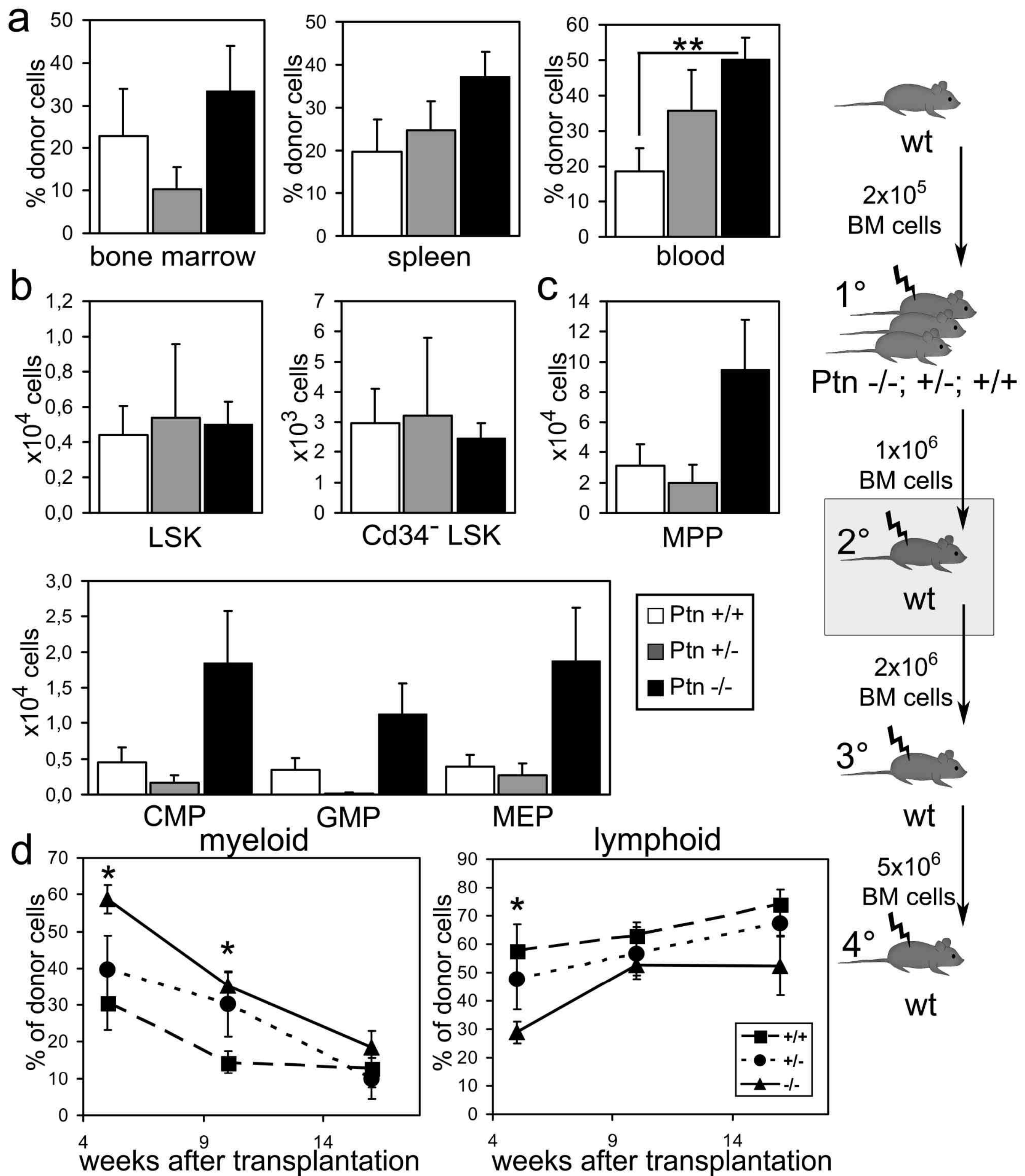


Figure 45. Secondary transplantation of wt cells into Ptn$^{-/-}$, Ptn$^{+/-}$ and Ptn$^{+/+}$ recipients. a) Engraftment levels in bone marrow (percentage of donor cells in total cell count of 4 long bones), spleen and blood 16 weeks after transplantation. b) Total number of donor LSK and Cd34^{-} LSK cells in the bone marrow of recipient mice (per 4 long bones). c) Total number of donor progenitor cells in 4 long bones of the recipients. d) Frequency of myeloid and lymphoid cells in the donor cell population in peripheral blood of the recipient mice. n= 10 (+/+), n=6 (+/-), n=8 (-/-), Mean ± SEM, *p<0.05, **p<0.01.

Tertiary transplantation

For the tertiary transplantation, only the cells which had been in a $Ptn^{-/-}$ and in a $Ptn^{+/+}$ microenvironment were used. The mice were treated in the same manner as in the transplantations before. The analysis of the mice after 16 weeks revealed a strongly enhanced engraftment in the bone marrow of mice which had received cells that once had been in a Ptn-deficient microenvironment (Figure 46a). The numbers of the donor LT-HSCs which lacked microenvironmental Ptn before were increased 10.3 fold compared to the controls (Figure 46b). Likewise, the numbers of all progenitor populations were elevated in mice receiving cells which had been in $Ptn^{-/-}$ mice two passages before (Figure 46c). Similar to the preceding transplantations, the cells that had once been in the primary Ptn-deficient environment (Figure 44) regenerated higher levels of myeloid but less lymphoid cells in the peripheral blood (Figure 46d).

This transplantation again supports the hypothesis that the Ptn-deficient microenvironment enhances the self-renewal capability of the HSCs. Furthermore, the experiment suggests that the effect is not reversible by the reoccurrence of microenvironmental Ptn in secondary mice.

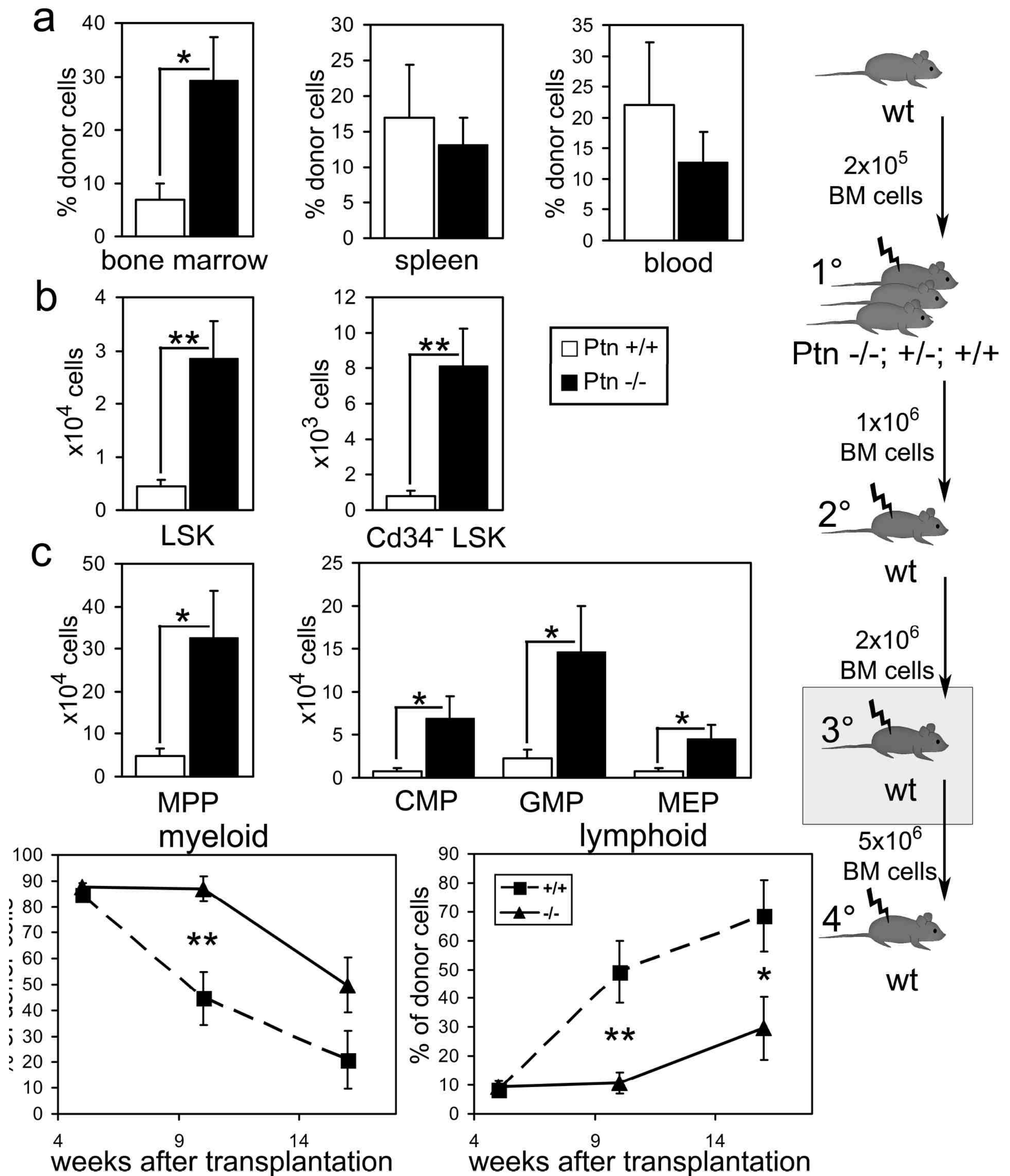


Figure 46. Tertiary transplantation of wt cells into Ptn$^{-/-}$, Ptn$^{+/-}$ and Ptn$^{+/+}$ recipients. a) Engraftment levels in bone marrow (percentage of donor cells in total cell count of 4 long bones), spleen and blood 16 weeks after transplantation. b) Total number of donor LSK and Cd34^{-} LSK cells in the bone marrow of recipient mice (per 4 long bones). c) Total number of donor progenitor cells in 4 long bones of the recipients. d) Frequency of myeloid and lymphoid cells in the donor cell population in peripheral blood of the recipient mice. n= 7 (+/+), n=5 (-/-), Mean ± SEM, *p<0.05, **p<0.01.

Quaternary transplantation

The bone marrow of the tertiary recipients was used for another round of transplantation into irradiated quaternary wildtype (129xB6)F1 recipient mice. In the quartenary transplantation, similar effects were observed as in the tertiary transplantation. But the differences between the groups were even more outspoken than in the previous, the tertiary transplantation. The engraftment in the bone marrow by the cells which had been in the primary Ptn-deficient microenvironment was 12.5 fold higher than the control cells (Figure 47a). The number of the donor LT-HSCs was even 26.3 fold higher (Figure 47b). Likewise, the numbers of the progenitor cells were strongly enhanced in mice which received cells that had suffered from Ptn-deficiency three transplantation passages before (Figure 47c). Again, the cells which had been in a $Ptn^{-/-}$ microenvironment showed a higher frequency of myeloid and lower frequency of lymphoid donor cells in the peripheral blood (Figure 47d).

In summary, the serial transplantation of wt bone marrow cells first in $Ptn^{-/-}$, $Ptn^{+/-}$ and $Ptn^{+/+}$ (129xB6)F2 mice and then three times into wt recipients has shown that the loss of Pleiotrophin in the primary microenvironment even over a short period leads to an enhanced HSC self-renewal. This effect is not reversible, even if Ptn reappears in the microenvironment in later transplantations. Pleiotrophin therefore seems to play an important role in the regulation of HSC self-renewal in the stress-dependent hematopoiesis.

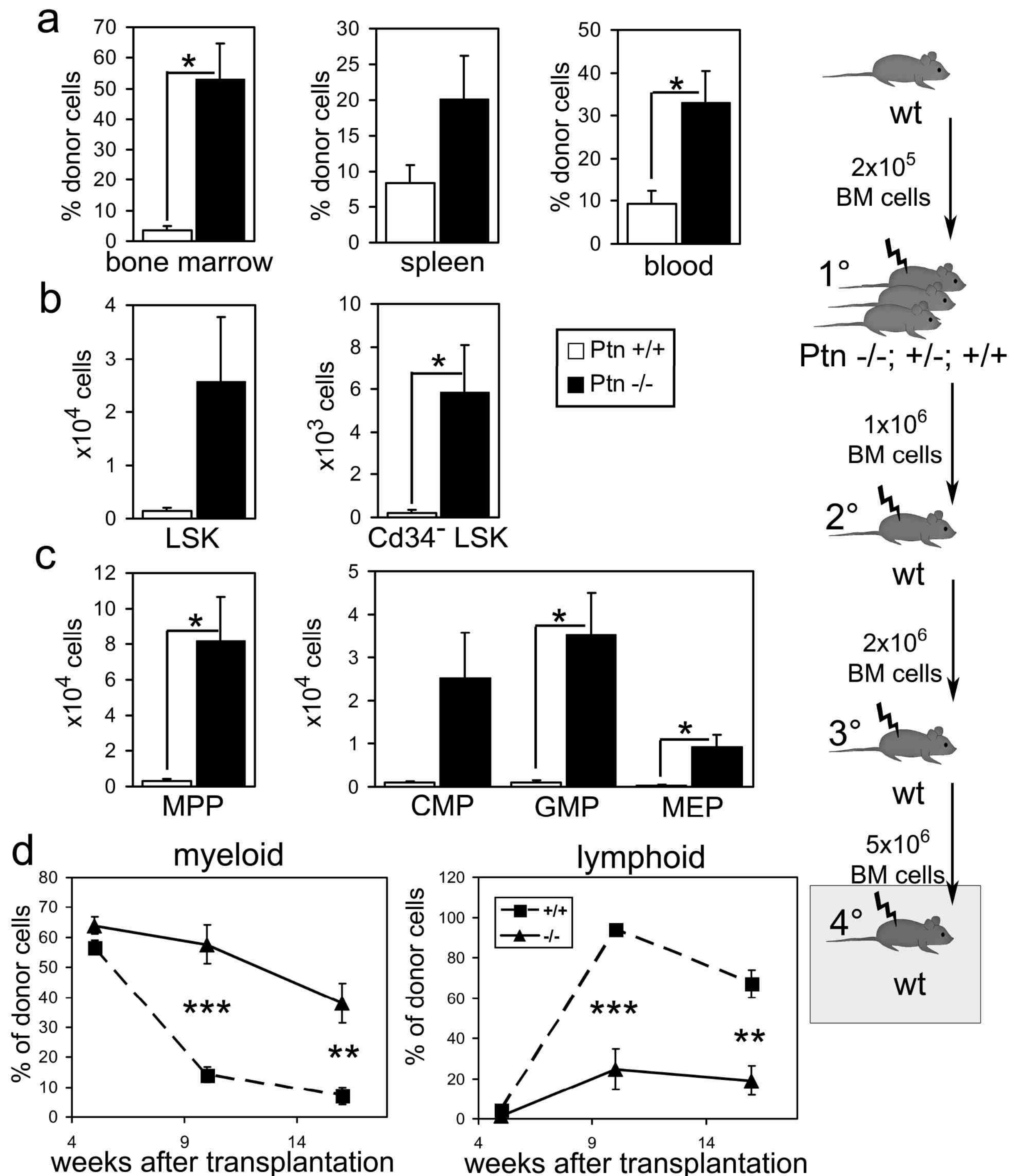


Figure 47. Quaternary transplantation of wt cells into Ptn$^{-/-}$, Ptn$^{+/-}$ and Ptn$^{+/+}$ recipients. a) Engraftment levels in bone marrow (percentage of donor cells in total cell count of 4 long bones), spleen and blood 16 weeks after transplantation. b) Total number of donor LSK and Cd34^{-} LSK cells in the bone marrow of recipient mice (per 4 long bones). c) Total number of donor progenitor cells in 4 long bones of the recipients. d) Frequency of myeloid and lymphoid cells in the donor cell population in peripheral blood of the recipient mice. n= 5 (+/+), n=6 (-/-), Mean ± SEM, *p<0.05, **p<0.01, ***p<0.001.

3.11. Transplantation of *in vitro* co-cultures

It has been shown that Pleiotrophin regulates HSC self-renewal during HSC engraftment *in vivo*. Engraftment can be regarded as a stress situation in which, in a short period of time the HSC is activated to regenerate the hematopoietic tissue in a myeloablated environment. The co-culturing of lin⁻ with stromal cells also represents a stress situation, where HSC are taken out of their microenvironment and inoculated in an artificial environment, low on hematopoietic cells. If the situation *in vitro* resembles the *in vivo* situation, HSCs may also be better maintained in co-cultures with shPtn UG26-1B6. Therefore, we revisited the co-culture experiments described earlier. For these experiments, wt lin⁻ cells were co-cultured with shPtn and pLKO.1 UG26-1B6 for 3 weeks and subsequently transplanted into irradiated wt mice. Like with the previous transplantations, the mice were bled after 5 and 10 weeks and sacrificed after 16 weeks. After 16 weeks, all mice were counted as positively engrafted that showed at least 1 % engraftment in the peripheral blood. Within these donor cells at least 1 % had to be myeloid and lymphoid cells, respectively. The analysis showed that only 2 out of 13 mice were positive of those which had received lin- cells co-cultured on pLKO.1 UG26-1B6. On the other hand, of the mice transplanted with cells cultured on shPtn UG26-1B6, 8 out of 12 mice were positive (Figure 48). Subsequent analysis with the statistic program L-Calc estimated that co-cultures with shPtn UG26-1B6 contained significantly more HSCs than co-cultures with pLKO.1 UG26-1B6. The difference was about 6 to 7 fold (Figure 48). The numbers of donor LT-HSCs and

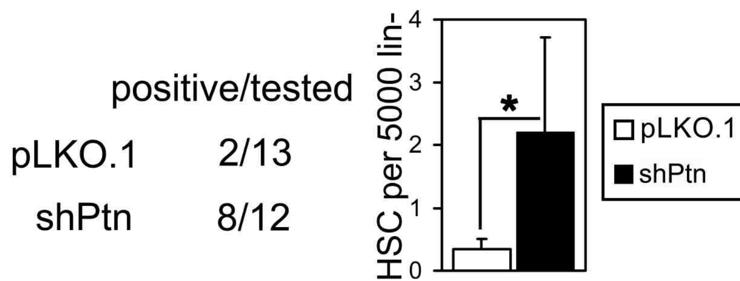


Figure 48. Transplantation of co-cultures of wt lin⁻ with shPtn and pLKO.1 UG26-1B6. Shown are results from peripheral blood 16 weeks after transplantation. Animals were counted positive with at least 1% engraftment in blood, and donor cells containing at least 1% myeloid and lymphoid cells, respectively. HSC frequency after 3 week coculture was calculated with L-calc software (Stem Cell Technologies), *$p<0.05$.

progenitors in the bone marrow of positive engrafted mice did not differ between the different groups (Figure 49b, c).

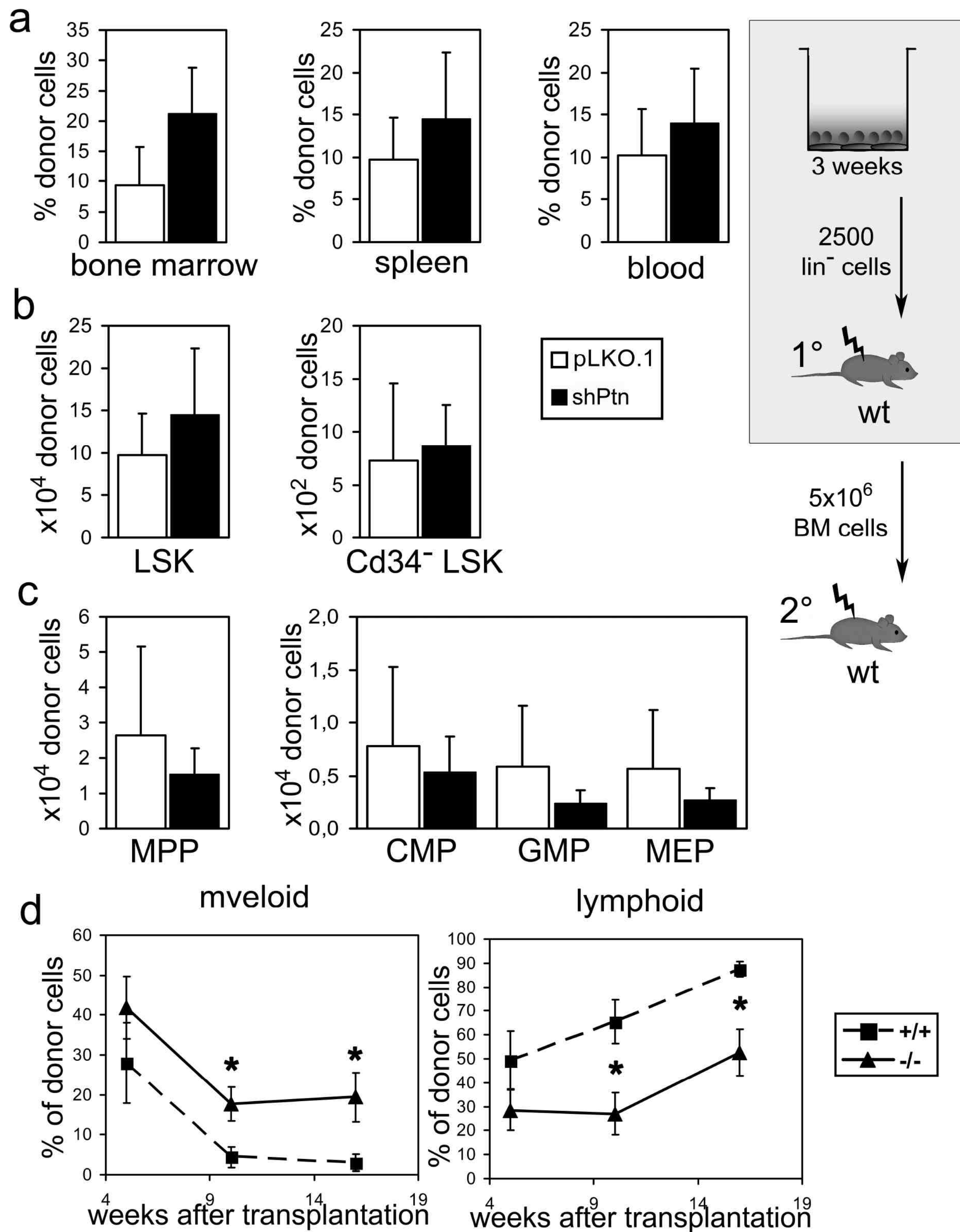


Figure 49. Primary transplantation of co-cultures of wt lin⁻ with shPtn and pLKO.1 UG26-1B6. a) Engraftment levels in bone marrow (percentage of donor cells in total cell count of 4 long bones), spleen and blood 16 weeks after transplantation. b) Total number of donor LSK and CD34⁻ LSK cells in the bone marrow of recipient mice (per 4 long bones). c) Total number of donor progenitor cells in 4 long bones of the recipients. d) Frequency of myeloid and lymphoid cells in the donor cell population in peripheral blood of the recipient mice. Shown are all mice with at least 1% engraftment in peripheral blood. n=6 (pLKO.1), n=8 (shPtn), Mean ± SEM, *p<0.05.

It is, however, possible that the cells causing this rise in engraftment are not LT-HSC, but ST-HSC, which are only capable of restoring the hematopoietic system over a short time. For addressing this question, the bone marrow of the transplanted mice was injected again into irradiated recipients. ST-HSCs would probably not be able to restore the hematopoietic system over such a long time and therefore the engraftment would be decrease. However, the analysis 16 weeks after injection showed a notably increased engraftment of cells cultured on shPtn UG26-1B6 in the bone marrow (Figure 50a). The cell numbers of LT-HSCs in this group were 2.2 fold higher compared to the controls (Figure 50b).

These experiments thus confirmed the results obtained *in vivo*. In summary, it was shown that the loss of microenvironmental Ptn leads to a higher self-renewal of LT-HSCs not only *in vivo*, but also *in vitro* during culture-associated stress.

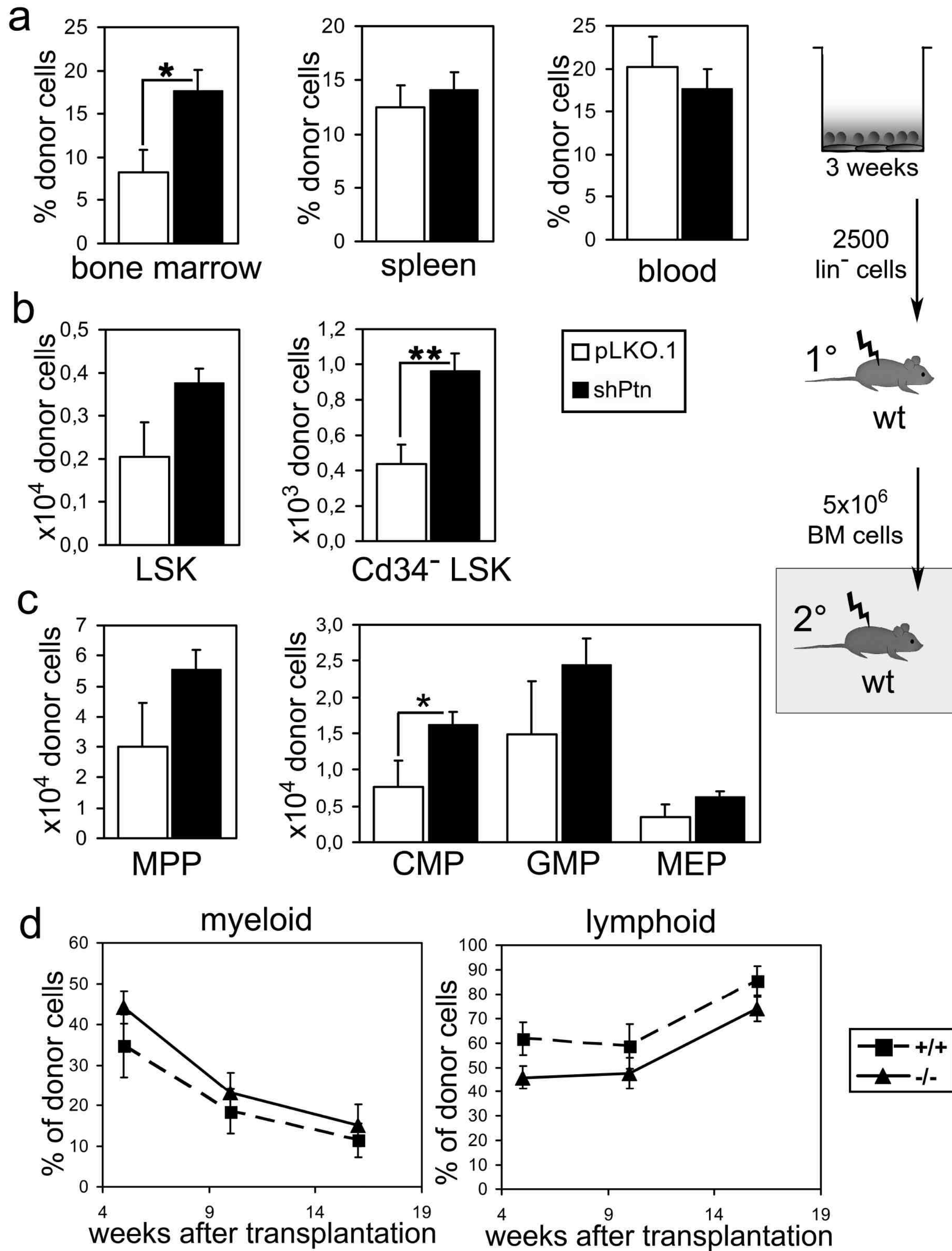


Figure 50. Secondary transplantation of co-cultures of wt lin⁻ with shPtn and pLKO.1 UG26-1B6. a) Engraftment levels in bone marrow (percentage of donor cells in total cell count of 4 long bones), spleen and blood 16 weeks after transplantation. b) Total number of donor LSK and CD34⁻ LSK cells in the bone marrow of recipient mice (per 4 long bones). c) Total number of donor progenitor cells in 4 long bones of the recipients. d) Frequency of myeloid and lymphoid cells in the donor cell population in peripheral blood of the recipient mice. n=9. Mean ± SEM, *p<0.05, **p<0.01.

3.12. Analysis of 2 weeks co-cultures

In a first attempt to understand the mechanisms behind the enhanced HSC self-renewal due to the loss of microenvironmental Ptn, co-cultures of wt lin$^-$ cells with shPtn and pLKO.1 UG26-1B6 were analysed more closely than before. First, co-cultures were analysed by flow cytometry after 2 weeks. It was found that in the cultures with shPtn UG26-1B6 there were notably more hematopoietic cells than in the control cultures (Figure 51). Both the populations of granulocytes and of MPPs were significantly increased in the cultures with shPtn UG26-1B6 (Figure 52). Meanwhile, the numbers of LSK cells were not significantly altered.

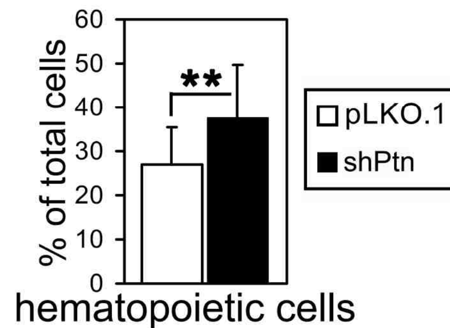


Figure 51. Flow cytometry analysis after 2 weeks co-culture. Hematopoietic cells were separated from stromal cells as non-autofluorescent cells. Statistics were done with a paired Student's t-test, n=5. Mean ± SEM, **p<0.01.

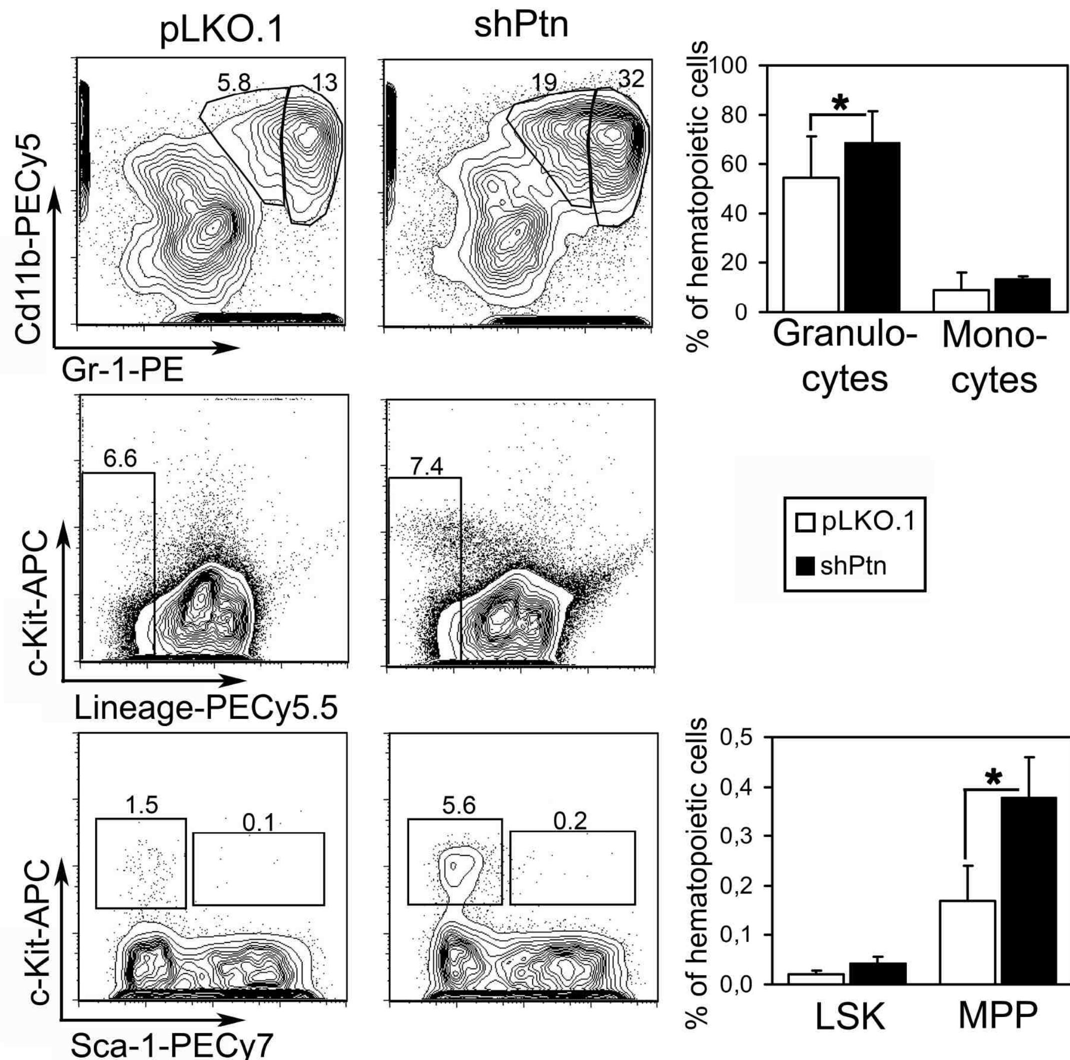


Figure 52. Flow cytometry analysis after 2 weeks co-culture of wt lin⁻ on shPtn and pLKO.1 UG26-1B6. Statistics were done with a paired Student's t-test, n=5. Mean ± SEM, *p<0.05.

The increase in hematopoietic cell numbers indicates that the cells in the cultures with low Ptn expression might proliferate faster than cells in control cultures.

3.13. Analysis of 3 days cultures

To address the hypotheses that the rise in hematopoietic cell numbers in the co-cultures with shPtn UG26-1B6 may be a consequence of an enhanced proliferation, wt lin⁻ cells were cultured in supernatant of shPtn and pLKO.1 UG26-1B6 and analysed after 3 days. It was found that after 3 days, the frequency of LSK cells were increased when cultured in supernatant of shPtn UG26-1B6 (Figure 53).

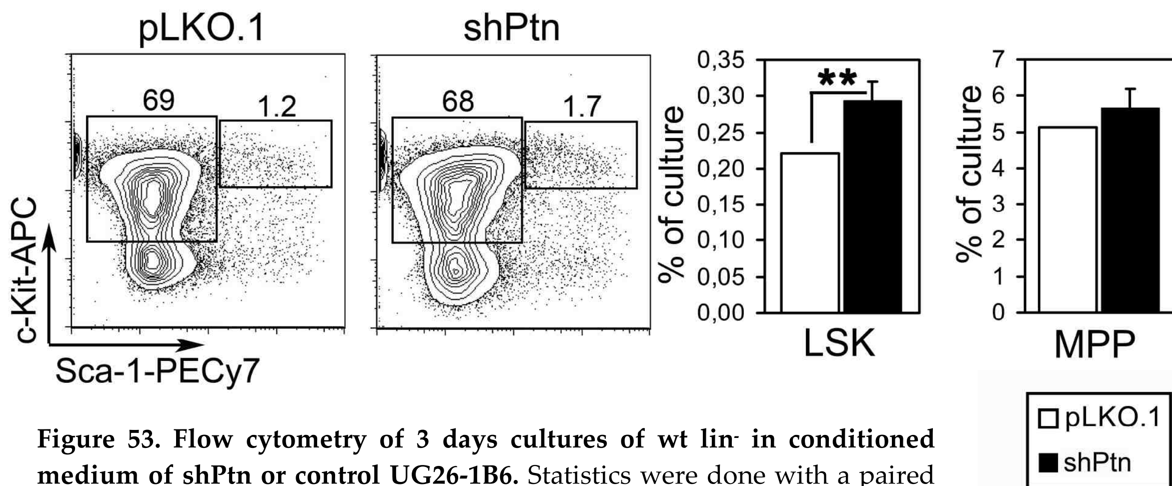


Figure 53. Flow cytometry of 3 days cultures of wt lin⁻ in conditioned medium of shPtn or control UG26-1B6. Statistics were done with a paired Student's t-test, n=6. Mean ± SEM, **p<0.01.

To investigate the proliferation rate of the cells, the lin⁻ cells were stained with carboxyfluorescein succinimidyl ester (CFSE) shortly before starting the culture. The cell-permeable dye enters the cytoplasm and there covalently binds to cytoplasmic proteins, turning the cells highly fluorescent. During cell division the dye is evenly distributed between the two daughter cells, resulting in two cells each carrying half of the amount of the dye. In this manner, the intensity of the CFSE-staining thus decreases with each division and it is therefore possible to track the proliferation of cells. An analysis of the CFSE staining of the cells cultured in stromal cell supernatants revealed that the proportion of dividing cells with the LSK phenotype was higher in supernatant of shPtn UG26-1B6 than in control supernatant (Figure 54). The proliferation rate of the MPPs, on the other hand, was not altered.

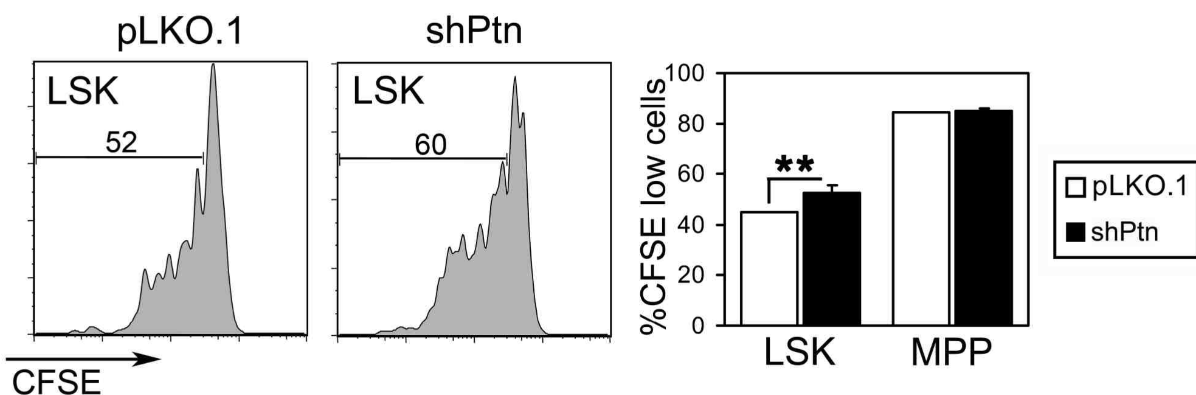


Figure 54. CFSE analysis of 3 days cultures of wt lin⁻ in conditioned medium of shPtn or control UG26-1B6. Statistics were done with a paired Student's t-test, n=9. Mean ±SEM, **p<0.01.

It therefore seems likely that the effect of the enhanced HSC self-renewal due to the loss of microenvironmental Ptn is mainly caused by an enhanced proliferation rate of these cells.

3.14. Gene expression analysis of 3 days co-cultures

For further insights into the mechanisms behind the increased self-renewal of HSCs in the absence of Pleiotrophin, the gene expression in the cells co-cultured for 3 days with shPtn or pLKO.1 stromal cell lines (UG26-1B6 and EL08-1D2) was analysed. For this, LSKs, CMPs and GMPs were sorted out of the co-cultures after 3 days. After that, real time PCR analysis was performed on the sorted cell populations. Various genes were measured, amongst them genes which are known to play important roles in HSC regulation and different cell cycle regulators. The cells cultured on UG26-1B6 or EL08-1D2 showed the same results, therefore the results were summarized.

Since the proliferation of the LSK cells on shPtn UG26-1B6 is altered, the gene expression of cell cycle regulators was checked at first. The expression of members of the Cdkn1 family, Cdkn1a, Cdkn1b (p27) and Cdkn1c (p57) was not changed in the LSKs and GMPs cultured on shPtn UG26-1B6 or EL08-1D2 instead of control stromal cells, even though Cdkn1a and Cdkn1c expressions were slightly, but significantly reduced in CMPs (Figure 55).

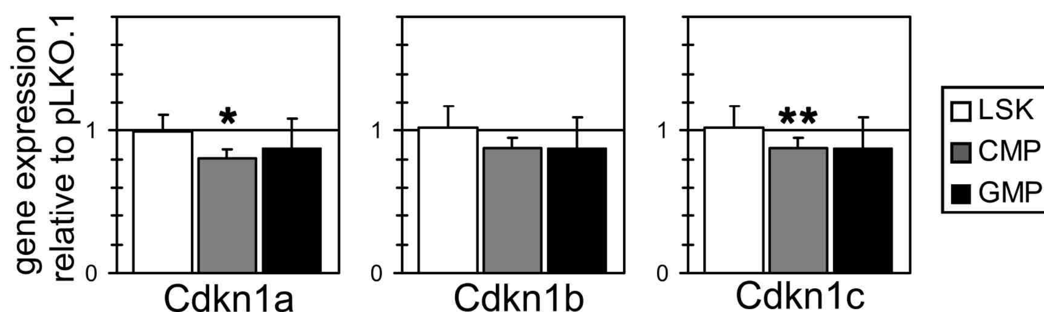


Figure 55. Gene expression analysis of 3 days co-cultures of wt lin⁻ with shPtn and pLKO.1 UG26-1B6 or EL08-1D2. Results of 6 independent experiments with 2 housekeeping genes (Gorasp2, Nme3) each. Shown are expression levels of LSK, CMP and GMP cells cultured on shPtn stroma cells compared to control cells, n=12. Mean ± SEM, *$p<0.05$, **$p<0.01$.

Similar results were obtained for the Cdkn2 family. None of the genes examined (Cdkn2a ($p16^{Ink4a}$/$p19^{Arf}$), Cdkn2b ($p15^{Ink4b}$), Cdkn2c ($p18^{Ink4c}$) and Cdkn2d ($p19^{Ink4d}$)) showed altered expression levels in either LSKs or GMPs, but the expression of both Cdkn2a and Cdkn2b was reduced in CMPs cultured on shPtn stromal cells compared to controls (Figure 56).

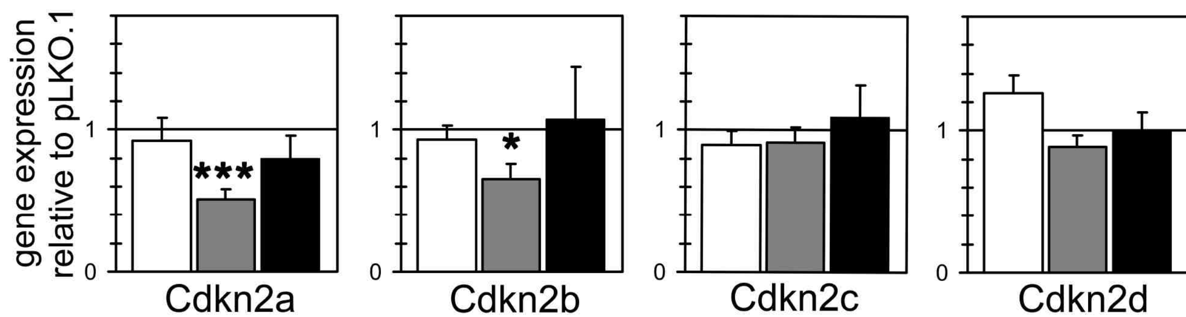


Figure 56. Gene expression analysis of 3 days co-cultures of wt lin⁻ with shPtn and pLKO.1 UG26-1B6 or EL08-1D2. Results of 6 independent experiments with 2 housekeeping genes (Gorasp2, Nme3) each. Shown are expression levels of LSK, CMP and GMP cells cultured on shPtn stroma cells compared to control cells, n=12. Mean ± SEM, *p<0.05, ***p<0.001.

Similar to that, the expression levels of Ki67 (Mki67) and Geminin were reduced in CMPs, but unaltered in the LSKs and GMPs (Figure 57).

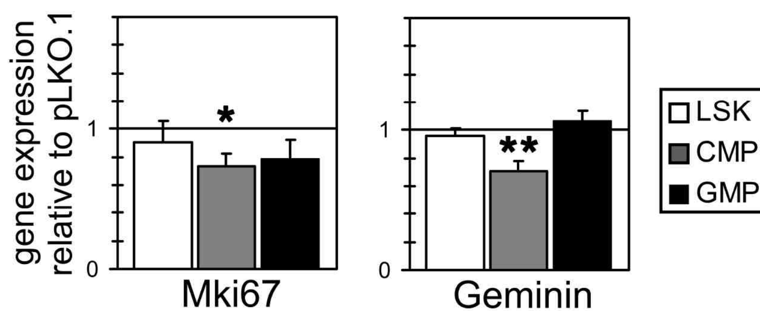


Figure 57. Gene expression analysis of 3 days co-cultures of wt lin⁻ with shPtn and pLKO.1 UG26-1B6 or EL08-1D2. Results of 6 independent experiments with 2 housekeeping genes (Gorasp2, Nme3) each. Shown are expression levels of LSK, CMP and GMP cells cultured on shPtn stroma cells compared to control cells, n=12. Mean ± SEM, *p<0.05, **p<0.01.

Eventually, the expression of several cyclins was investigated. While Cyclin D2 was reduced in CMPs, but otherwise unchanged and Cyclin E1 was unaltered in all populations, the expression of Cyclin D1 was enhanced in LSK cells cultured on shPtn UG26-1B6or EL08-1D2, unchanged in CMPs and finally reduced in the GMP population (Figure 58).

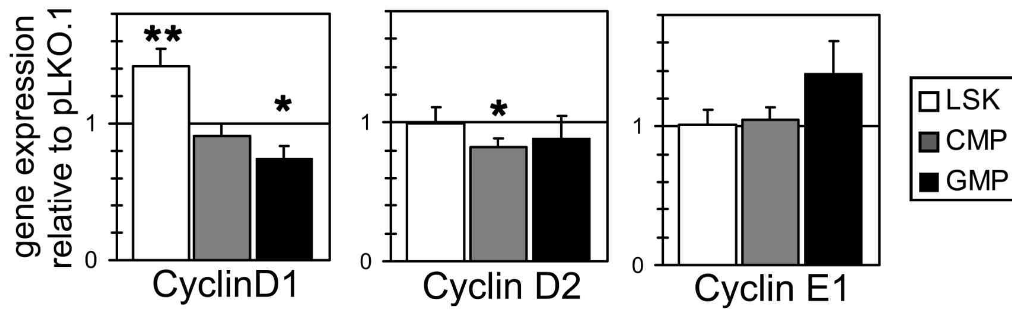


Figure 58. Gene expression analysis of 3 days co-cultures of wt lin⁻ with shPtn and pLKO.1 UG26-1B6 or EL08-1D2. Results of 6 independent experiments with 2 housekeeping genes (Gorasp2, Nme3) each. Shown are expression levels of LSK, CMP and GMP cells cultured on shPtn stroma cells compared to control cells, n=12. Mean ± SEM, *p<0.05, **p<0.01.

Amongst the genes known to play important roles in early hematopoiesis are PU.1 (Sfpi), Runx1, Gata1, Gata2 and Cebpa. While the expression levels of PU.1, Runx1, Gata1 and Gata2 are unchanged in all populations, the expression of Cebpa was enhanced in LSK cells, unchanged in CMPs and finally reduced in GMPs cultured on shPtn stromal cell lines (Figure 59).

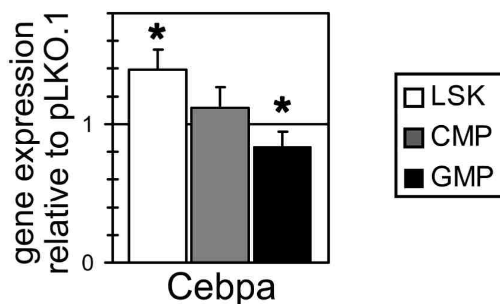
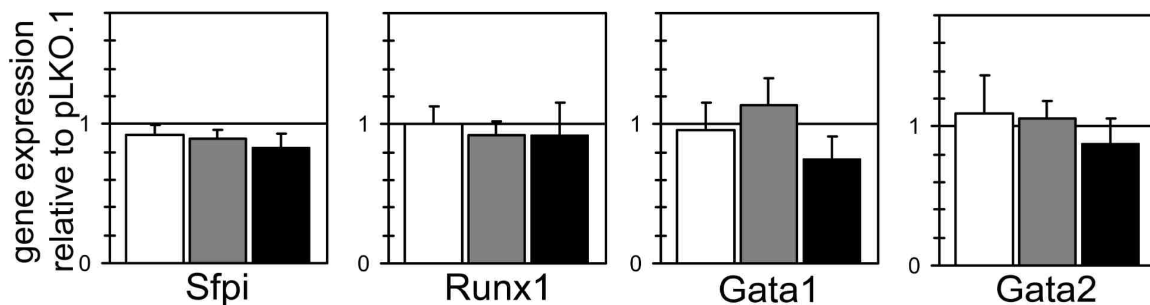


Figure 59. Gene expression analysis of 3 days co-cultures of wt lin⁻ with shPtn and pLKO.1 UG26-1B6 or EL08-1D2. Results of 6 independent experiments with 2 housekeeping genes (Gorasp2, Nme3) each. Shown are expression levels of LSK, CMP and GMP cells cultured on shPtn stroma cells compared to control cells, n=12. Mean ± SEM, *p<0.05.



Since Ptn is known to often signal via the central canonical Wnt mediator ◎-catenin (Ctnnb1)(Weng *et al.* 2009), the expression of this gene and of Pparg, a target of the non-canonical Wnt signalling, was analyzed. Expression of both genes was found to be differentially expressed between wt and Sfrp1-/- mice (Renstrom *et al.*

2009a). In the current study, it was found that the expression of Ctnnb1 was unchanged in all populations, on the other hand the expression of Pparg was strongly reduced in all cells cultured on shPtn UG26-1B6 or EL08-1D2 (Figure 60).

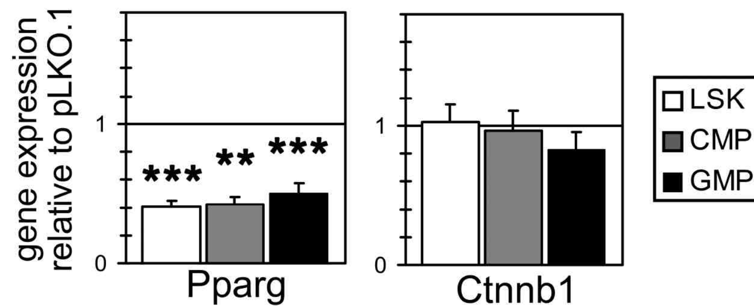


Figure 60. Gene expression analysis of 3 days co-cultures of wt lin$^-$ with shPtn and pLKO.1 UG26-1B6 or EL08-1D2. Results of 6 independent experiments with 2 housekeeping genes (Gorasp2, Nme3) each. Shown are expression levels of LSK, CMP and GMP cells cultured on shPtn stroma cells compared to control cells, n=12. Mean ± SEM, **p<0.01, ***p<0.001.

Additional to Wnt signalling, targets of the Notch pathway, Hes1 and Dlk1, were studied. Both genes were not differentially expressed in the LSK population, but the expression was reduced in the GMPs cultured on shPtn stromal cell lines (Figure 61).

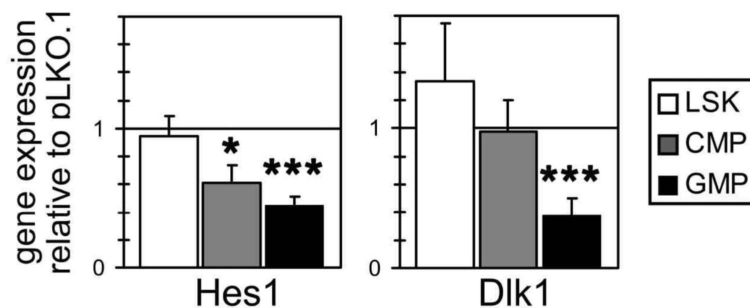


Figure 61. Gene expression analysis of 3 days co-cultures of wt lin$^-$ with shPtn and pLKO.1 UG26-1B6 or EL08-1D2. Results of 6 independent experiments with 2 housekeeping genes (Gorasp2, Nme3) each. Shown are expression levels of LSK, CMP and GMP cells cultured on shPtn stroma cells compared to control cells, n=12. Mean ± SEM, *p<0.05, ***p<0.001.

The expression levels of p53 (Trp53) and Pten were not changed in any of the populations. The polycomp gene Ezh2 was also not differentially expressed in LSKs and GMPs, but its expression was reduced in CMPs cultured on shPtn stromal cells (Figure 62).

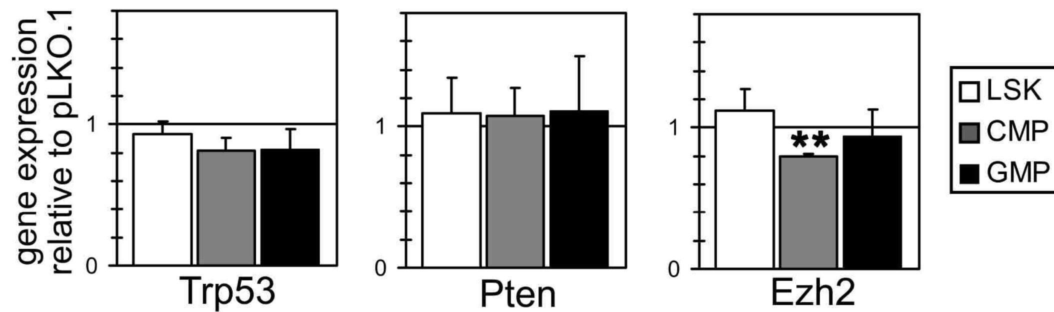


Figure 62. Gene expression analysis of 3 days co-cultures of wt lin⁻ with shPtn and pLKO.1 UG26-1B6 or EL08-1D2. Results of 6 independent experiments with 2 housekeeping genes (Gorasp2, Nme3) each. Shown are expression levels of LSK, CMP and GMP cells cultured on shPtn stroma cells compared to control cells, n=12. Mean ± SEM, **p<0.01.

The transplantation results demonstrate that the lack of Ptn in primary recipients or HSC cultured on shPtn stromal cells leads to an increase in $Cd34^{-}$LSK cells and a myeloid-skewed engraftment. Similar observations were reported in the literature when "old" (i.e. from mice older than 20 months of age) HSCs were transplanted (Mayack *et al.* 2010; Rossi *et al.* 2005), or when a myeloid-biased subset of HSCs was transplanted (Challen *et al.* 2010; Dykstra *et al.* 2007). It was thus investigated whether the lack of Ptn activated some "old" or "myeloid-bias" program in the HSCs. To study this, the expression of a genes that were expressed more in "young" HSCs (for instance Hmga2 (Noda *et al.* 2009)) or in "old" HSCs (such as Rorb (Rossi *et al.* 2005; Noda *et al.* 2009)) was explored. The analyses showed that the expression of Hmga2 was unchanged in LSK cells recovered from normal or shPtn stromal cell co-cultures, but that Rorb was induced in the LSK cells co-cultured with shPtn stromal cells (the expression of Rorb was almost not detectable in LSK cells cultured on control stromal cells) (Figure 63).

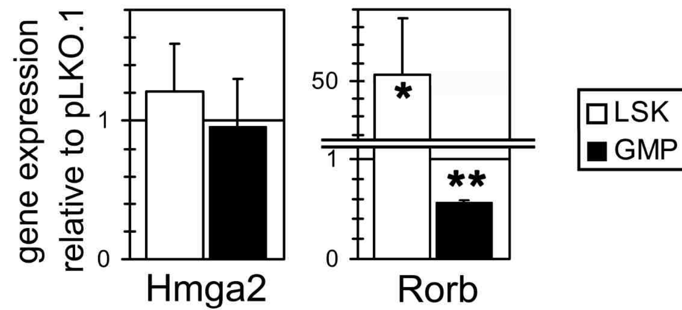


Figure 63. Gene expression analysis of 3 days co-cultures of wt lin⁻ with shPtn and pLKO.1 UG26-1B6 or EL08-1D2. Results of 3 independent experiments with 2 housekeeping genes (Gorasp2, Nme3) each. Shown are expression levels of LSK, CMP and GMP cells cultured on shPtn stroma cells compared to control cells, n=6. Mean ± SEM, *p<0.05, **p<0.01.

In summary, it can be stated that the only genes whose expression was altered in the LSK cells which were cultured on shPtn stromal cells compared to controls were Cyclin D1, Cebpa, Rorb and Pparg. While CyclinD1, Cebpa and Rorb expression levels were increased in LSK cells, but unchanged or decreased with ongoing differentiation, Pparg expression levels were low in all populations. These genes might play important roles in delivering Ptn signals, but therefore it is important to know if they are also increased in protein levels. In addition, we observed many smaller changes in gene expression of molecules involved in the cell cycle as well as chromatin remodelling in the CMP population suggesting that environmental Ptn may also affect the transition from LSK to GMP.

3.15. Analysis of protein levels after 3 days co-culture

It is known that Ptn often signals through Ctnnb1, which is thereby translocalized to the nucleus (Weng *et al.* 2009). Nevertheless, no changes were found in the transcript levels of Ctnnb1 in LSKs and progenitors cultured on shPtn or control stromal cell. However, since the protein level of Ctnnb1 is mainly regulated through the proteasome, Ptn may affect the stability of Ctnnb1. To address this question, Ctnnb1 was stained in LSKs and GMPs cultured on shPtn or pLKO.1 UG26-1B6 (single cell assay). These experiments demonstrated that the lack of Ptn in stromal cells did not affect either transcript or protein levels of Ctnnb1 in sorted LSK or GMP (Figure 64, Figure 65). In addition, no alterations were found in the ratio of nuclear to

cytoplasmic localization between cells recovered from co-cultures on Ptn-deficient or control stromal cells.

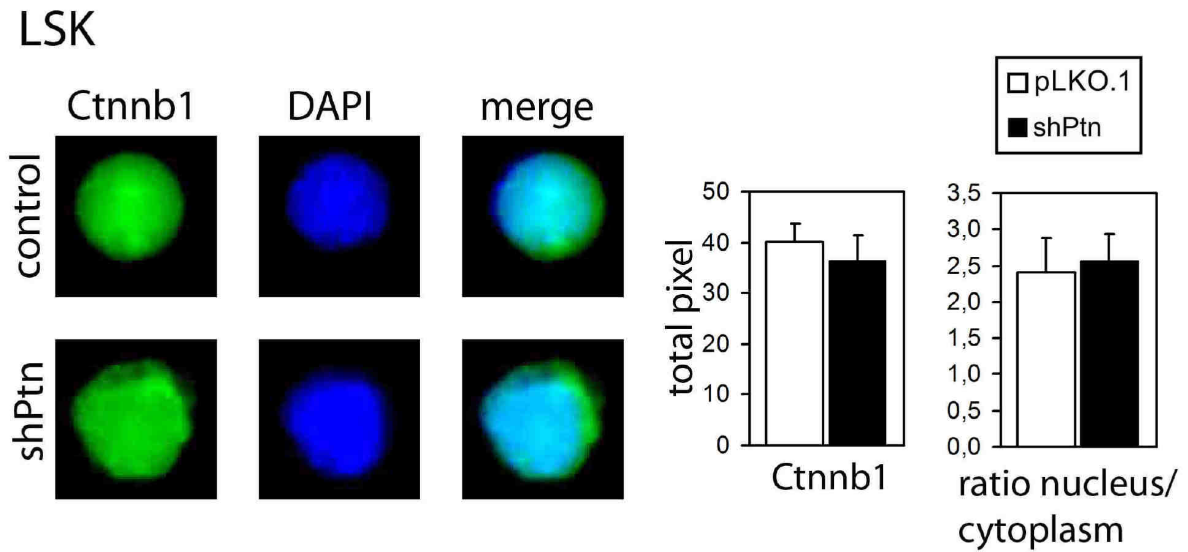


Figure 64. Ctnnb1 protein levels in LSK cells cultured on shPtn or pLKO.1 UG26-1B6. Single cell staining assay and quantification using ImageJ. n= 7 (shPtn), n=10 (pLKO.1). Mean ± SEM.

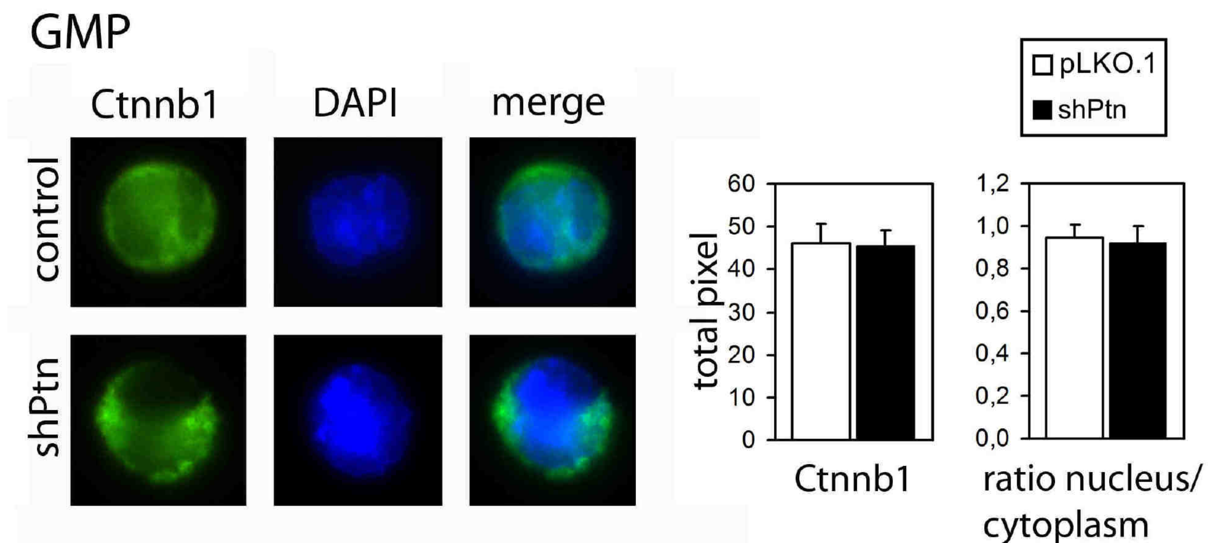


Figure 65. Ctnnb1 protein levels in GMP cells cultured on shPtn or pLKO.1 UG26-1B6. Single cell staining assay and quantification using ImageJ. n= 6 (shPtn), n=7 (pLKO.1). Mean ± SEM.

Therefore, it can be said that Ctnnb1 is not involved in the enhanced HSC maintenance observed after the loss of microenvironmental Ptn.

The protein levels of Cyclin D1 were analyzed by intracellular flow cytometry analysis. As a result, the frequencies of cells that express Cyclin D1 were measured. We found that all LSK cells expressed cyclin D1. However, it was found that in the

cultures with shPtn UG26-1B6 a larger fraction of LSK cells could be detected which expressed a high level of Cyclin D1 as compared to LSK cells recovered from co-cultures on control pLKO.1 UG26-1B6 (Figure 66).

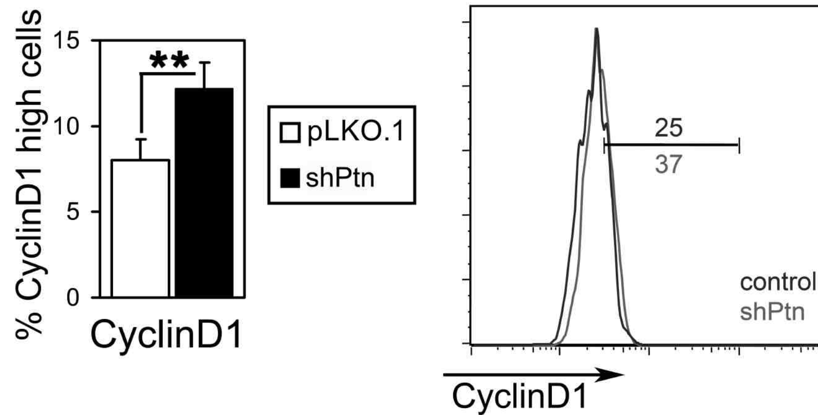


Figure 66. Ccnd1 protein levels in LSK cells cultured 3 days in contact with shPtn or pLKO.1 UG26-1B6. Obtained by flow cytometry in 3 independent experiments, shown are percentages of Ccnd1^{high} cells. Mean ± SEM, **p<0.01.

In the above experiments it was shown that Cebpa transcript levels were increased in LSK cells recovered from co-cultures on shPtn.UG26-1B6. Hence, protein levels and localisation of this transcription factor in sorted LSK cells recovered from the co-cultures were assessed. These experiments showed that LSKs cultured on shPtn UG26-1B6 contain more Cebpa protein than control cells (Figure 67).

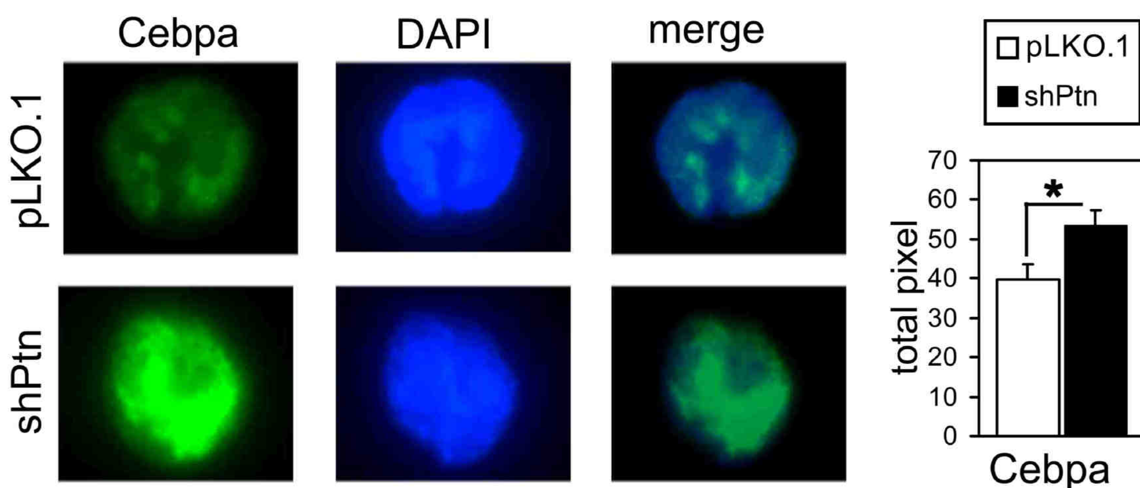


Figure 67. Cebpa protein levels in LSK cells cultured on shPtn or pLKO.1 UG26-1B6. Single cell staining assay and quantification using ImageJ. n=10. Mean ± SEM, *p<0.05.

The analysis of Ctnnb1, Ccnd1 (cyclin D1) and Cepba protein levels therefore parallels the mRNA expression data. Furthermore, these experiments show a clear association of upregulated expression of Ccnd1 and Cebpa with increased HSC self-renewal in a Ptn-deficient environment.

3.16. Analysis of apoptosis in cells lacking microenvironmental Ptn

In the course of evaluating possible mechanisms for the enhanced HSC maintenance due to the loss of stromal Ptn, the possibility of altered apoptosis was examined in different primitive and mature hematopoietic cell subpopulations. For testing this, wt bone marrow cells were cultured in supernatant of shPtn and pLKO.1 UG26-1B6, respectively. Apoptosis had been induced by the addition of cycloheximide to the supernatant, which inhibits the protein translation. The different cell populations were distinguished by flow cytometry, apoptotic cells were thereby identified by the presence of annexin-V as a surface marker.

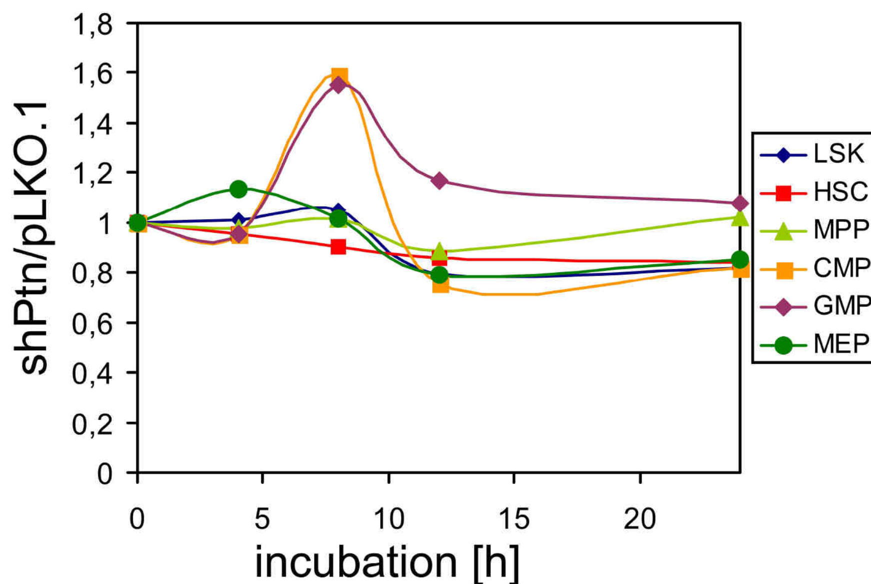


Figure 68. Apoptosis of progenitor populations. Wt bone marrow cells were cultured in supernatant from shPtn and pLKO.1 UG26-1B6 with 50 μg/ml cycloheximide. The percentages of living cells (Annexin$^-$PI$^-$) from total cells was measured. Shown is the quotient of these frequencies obtained from cultures with shPtn and pLKO.1 supernatant.

In these experiments, none of the early and late progenitor populations showed gross changes in apoptosis rates depending on the supernatant they were cultured in (Figure 68). However, mature granulocytes (Gr-1^{high}Cd11b$^+$) which were cultured in supernatant of shPtn UG26-1B6 died much more quickly than granulocytes cultured in control supernatant (Figure 69). After 12 h of culturing, there were 4.1 fold more

granulocytes alive in control supernatant than in supernatant lacking Ptn. On the other hand, no difference was found in monocytes (Figure 69).

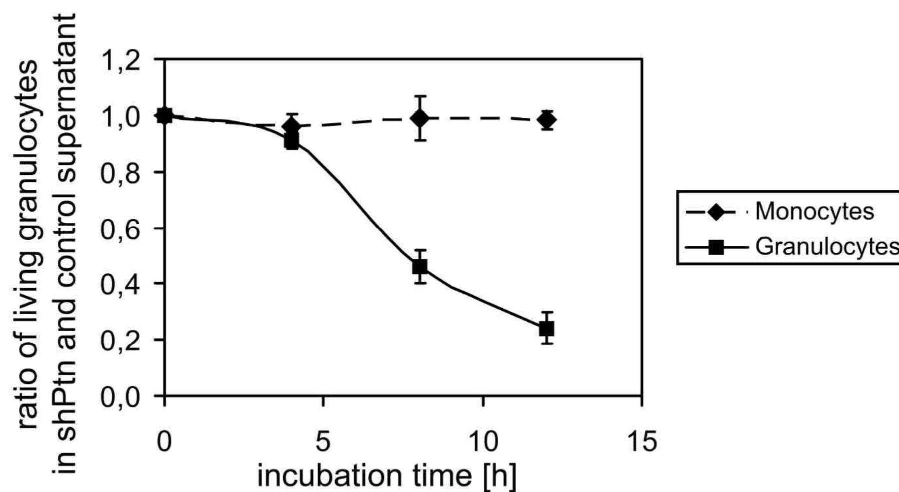


Figure 69. Apoptosis of myeloid populations. Wt bone marrow cells were cultured in supernatant from shPtn and pLKO.1 UG26-1B6 with 50 µg/ml cycloheximide. The percentages of living cells (Annexin$^-$PI$^-$) from total cells was measured. Shown is the quotient of these frequencies obtained from cultures with shPtn and pLKO.1 supernatant. Mean ± SEM.

For a further study of this effect, the same assay was done with other inducers of apoptosis than cycloheximide. For this purpose, 5-FU, MG-132 and Gossypol were used for initiating apoptosis. MG-132 is a potent proteasome inhibitor; Gossypol works pro-apoptotic due to the regulation of Bax and Bcl2. All of these additives led to the apoptosis of granulocytes in the assay, but no difference was detected between cells cultured in supernatants of shPtn or pLKO.1 UG26-1B6 (Figure 70).

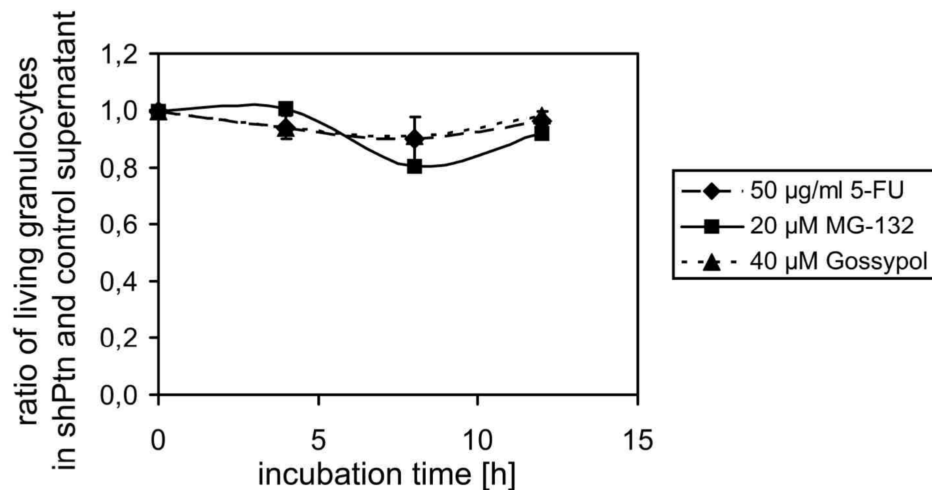


Figure 70. Apoptosis of granulocytes. Wt bone marrow cells were cultured in supernatant from shPtn and pLKO.1 UG26-1B6 with additives as indicated. Granulocytes were identified by flow cytometry as Gr-1^{high}Cd11b^{+} cells. The percentage of living granulocytes (Annexin^{-}PI^{-}) from total cells was measured. Shown is the quotient of these frequencies obtained from cultures with shPtn and pLKO.1 supernatant. Mean ± SEM.

To determine whether the apoptosis-inducing effect of cycloheximide and its modulation in the absence of Ptn depended on interference with increased protein degradation, the assay was performed with cycloheximide and the proteasome inhibitor MG-132 added together. This combination led to a loss of the effect (Figure 71).

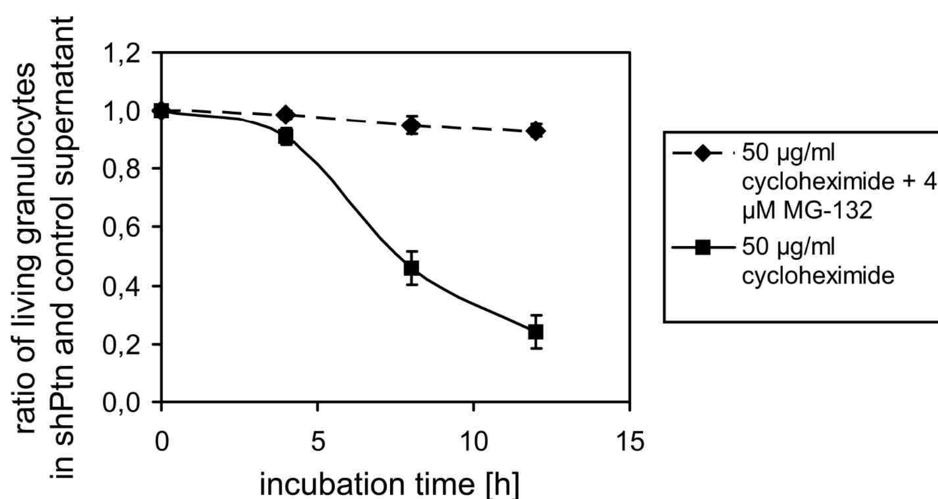


Figure 71. Apoptosis of granulocytes. Wt bone marrow cells were cultured in supernatant from shPtn and pLKO.1 UG26-1B6 with additives as indicated. Granulocytes were identified by flow cytometry as Gr-1^{high}CD11b^{+} cells. The percentages of living cells (Annexin^{-}PI^{-}) from total cells was measured. Shown is the quotient of these frequencies obtained from cultures with shPtn and pLKO.1 supernatant. Mean ± SEM.

These experiments indicate that one or several anti-apoptotic proteins in the granulocytes are degraded more rapidly when the cells lack Ptn in the supernatant. When cycloheximide is added, the protein translation stops and this molecule/these molecules are not produced any further. The enhanced degradation leads to a drop in the levels of this protein/these proteins and therefore initiates or advances the apoptosis of the cell. After addition of MG-132, protein degradation is stopped as well and the effect therefore vanishes.

In the attempt to gain further insights into the mechanism of this effect, inhibitors for several other pro-apoptotic pathways were tested. Amongst the tested substances were Wortmannin and LY294002, both known inhibitors of phosphatidylinositol-3 kinase (PI3K). Another inhibitor tested was Nutlin-3, which inhibits the interaction between MDM2 and p53. Caspase-3-inhibitor II was used as well. None of these inhibitors, however, did notably alter the effect of cycloheximide on granulocytes cultured in shPtn and pLKO.1 UG26, respectively (Figure 72).

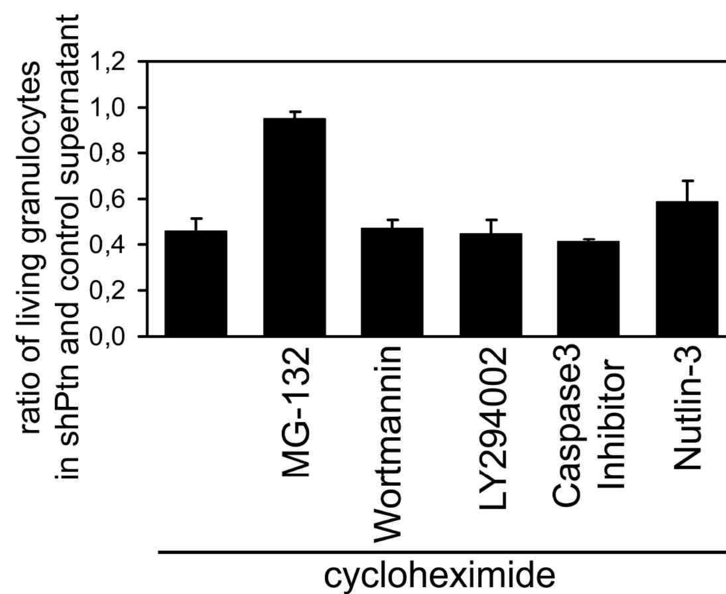


Figure 72. Apoptosis of granulocytes. Wt bone marrow cells were cultured in supernatant from shPtn and pLKO.1 UG26-1B6 with additives as indicated (50 µg/ml cycloheximide, 4 µM MG-132, 100 nM Wortmannin, 50 µM LY294002, 50µM Caspase3-InhibitorII, 0.25µg/ml Nutlin-3). Granulocytes were identified by flow cytometry as Gr-1^{high}Cd11b^{+} cells. The percentages of living granulocytes (Annexin^{-}PI^{-}) from total cells was measured. Shown is the quotient of these frequencies obtained from cultures with shPtn and pLKO.1 supernatant after 8 h incubation. Mean ± SEM.

In summary, the lack of secreted Pleiotrophin in the culture may lead to an enhanced apoptosis of granulocytes after the addition of cycloheximide. Under these circumstances, increased apoptosis is caused by an enhanced degradation of one or several anti-apoptotic proteins. The above results further show that neither PI3K-, MDM2/p53- nor Caspase-3-mediated apoptotic pathways play significant roles in this effect.

4. Discussion

In adult life, hematopoietic stem cells (HSC) mainly reside within the bone marrow. It is assumed that the marrow microenvironment is a critical regulator of HSC maintenance and regeneration. The precise mechanisms involved, however, have not been established so far. As models of the hematopoietic miocroenvironment, several adherent cell lines were established which were able to maintain both murine and human HSCs *in vitro*, like the murine fetal liver-derived AFT024 (Moore *et al.* 1997; Nolta *et al.* 2002), the day 11 embryo-derived cells EL08-1D2 and UG26-1B6 (Kusadasi *et al.* 2002; Oostendorp *et al.* 2002), and human brain endothelial cells (Chute *et al.* 2005). Interestingly, a common feature of these HSC-supportive cells is the overexpression of the secreted factor pleiotrophin (Himburg *et al.* 2010; Hackney and Moore 2005; Oostendorp *et al.* 2005). The secreted 17 kDa cytokine Pleiotrophin (Ptn) is known to be involved in diverse cellular functions in various cell types (Deuel *et al.* 2002). Amongst the known features of Ptn is its expression by osteoblasts (Tezuka *et al.* 1990; Petersen and Rafii 2001), which most probably form part of the HSC microenvironment (Taichman and Emerson 1994; Calvi *et al.* 2003; Zhang *et al.* 2003a). It is also known that Ptn is involved in the regulation of functions like proliferation and differentiation of human embryonic stem cells, murine germ cells and neural stem cells (see 1.2.3) (Zhang *et al.* 1999; Hienola *et al.* 2004; Jung *et al.* 2004; Soh *et al.* 2007). Nevertheless, so far, the role of Pleiotrophin in the regulation of early adult hematopoiesis has never been explored.

The aim of this project was therefore to examine the role of Ptn in the regulation of hematopoiesis and especially in the regulation of HSC function. It is shown in this thesis, that Ptn secreted by the microenvironment is involved in HSC maintenance after hematopoietic injury by regulating Cyclin D1 (Ccnd1), C/EBPα (Cebpa), and Pparg in HSC. More importantly, HSC regulation could entirely be attributed through extrinsic signals *in vivo* and *in vitro*. Furthermore, the regeneration of myeloid lineages is dominant over the lymphoid lineages in the cases when Ptn secretion by the microenvironment is lost. In addition to the regulation of HSC, Ptn

also plays a role in the regulation of transcription and apoptosis in mature granulocytes.

4.1. Functions of Pleiotrophin in steady-state hematopoiesis

To investigate the role of Ptn in hematopoiesis, we used mice deficient for Ptn expression (Amet *et al.* 2001). Ptn KO mice on different genetic backgrounds were investigated in the process of this project. First, Ptn KO mice on the original 129 background 129P2x129S6 (Amet *et al.* 2001) were examined. These mice showed clear alterations in both primitive and mature hematopoietic populations due to the loss of Ptn expression. To enable transplantation experiments using the Ly5.1/Ly5.2 Cd45 isotype system, these mice were crossed with C57Bl/6.J (B6). Surprisingly, the 129xB6 mice did not show the changes in hematopoieic subpopulations noted in the "pure" 129 mice, but seemed to have lost the hematopoietic phenotype. Finally, after the rederivation of the 129 Ptn KO mice, these Ptn KO mice did not differ from the control mice in any population examined, suggesting that Ptn does not play a significant role in steady-state hematopoiesis under pathogen-free conditions.

One possible explanation for these observations might be that the phenotype of the Ptn knockout mice is dependent on the background of the mice, similar as in the Cdkn1a-deficient mice. Kept on a 129 background, the proliferation of HSCs in these mice is increased, which leads to HSC exhaustion which then is followed by hematopoietic failure (Cheng *et al.* 2000). On a C57Bl/6 background, however, the loss of Cdkn1a does not effect steady-state hematopoiesis and engraftment after serial transplantations (van Os *et al.* 2007). Thus, on a B6 background, the Cdkn1a remain healthy. Cdkn1a is known to act as a proliferation repressor (Harper *et al.* 1993; Xiong *et al.* 1993). It has also long been known that Ptn acts as a cell cycle regulator for various cell types (Fang *et al.* 1992; Hienola *et al.* 2004), and it has also been shown in this thesis that Ptn is involved in cell cycle regulation of hematopoietic cells. It might therefore not be too surprising if the phenotype of the Ptn KO mice depends on their background. This might also explain the loss of the phenotype after rederivation. The family of 129 mice consists of different substrains

(Simpson *et al.* 1997). While the Ptn KO mice before the rederivation were a mixture of the 129S6 and 129Ola (129P2) strains (Amet *et al.* 2001), the mice were crossed with 129S2 during the rederivation because the original strains were not available for breeding. The rederived mice therefore were a mixture of the three strains 129S6, 129Ola and 129S2. It might be that this slight change in background caused the loss of the phenotype.

Another explanation for this observation might be that the unrederived 129 Ptn KO mice were kept stressful housing conditions in the mouse facility. Prior to rederivation, the mice were kept in "dirty" rooms, whereas after rederivation, the mice could be moved to a specific pathogen-free facility. This stress might for example be exerted by an infection, sound level, or a frequent change of animal caretakers. It was shown before that Ptn is important for the reaction to stress like injuries, for example after nerve injury or incisional wounds (Takeda *et al.* 1995; Deuel *et al.* 2002; Blondet *et al.* 2005). Likewise, it became evident in this project that Ptn functions mainly in hematopoiesis in situations following stress, like in transplantations or in co-cultures which also resemble stress-situations. The enhanced expression of Cd34 in the LSK cells might also point to the conclusion, that the phenotype was evoked by stress. This enhanced Cd34 expression might indicate an increased activation of LSKs and perhaps premature ageing of the HSC under these conditions which is often the consequence of stress because these cells have to restore the lost hematopoietic cells.

Therefore, the possible explanations for the changes in the phenotype of the Ptn KO mice might be a dependence on background or stress. Naturally, it might also be that both factors work together. The stress-dependency of the Ptn KO phenotype would not become evident on all backgrounds. To summarize that, except for maybe some special backgrounds, Pleiotrophin most probably is not required for normal steady-state hematopoiesis.

4.2. Phenotype of 129 Ptn KO mice

Independent from the question of the source of the phenotype which was observed in the 129 Ptn KO mice, the changes found in early hematopoiesis in the original 129P2x129S6 Ptn KO mice are remarkably similar to the observations made in the transplantation experiments. For example, the myeloid populations are clearly enlarged in the blood of the 129 Ptn KO mice, similarly to the enhanced myeloid populations seen in transplantation and co-culture experiments. As mentioned before, the enhanced expression of Cd34 in the LSK cells of 129 Ptn KO mice might indicate an activation of the LSK cells in these mice. This would support the hypothesis that the enhanced engraftment in transplantations is based on an increased population of actively proliferating HSCs in the Ptn KO microenvironment compared to controls. Since the engraftment of these cells did not decline in serial transplantations, this would mean that Ptn belongs to the few known molecules from which it is known that their knockout increases HSC proliferation without exhaustion of HSCs (for example Cdkn2c, see 1.1.4). This was confirmed by the analysis of the old 129 Ptn KO mice as well as with the results from the quaternary transplantations of cells from primary Ptn KO recipients. These mice show a similar phenotype like the young mice. Therefore, the enhanced activation of LSK cells does not lead to their exhaustion over time.

4.3. Intrinsic functions of Pleiotrophin

Intrinsic functions of Pleiotrophin in HSC and progenitor populations were investigated *in vivo* and *in vitro*. One of the possible receptors for Ptn is nucleolin (Take *et al.* 1994), a molecule that could possibly shuttle extracellular molecules from the cell surface to the nucleus, where nucleolin acts as a histone chaperone in chromatin remodelling (Mongelard and Bouvet 2007). Thus it is conceivable that endogenously produced Ptn is kept within the cell and may have intrinsic regulatory properties on chromatin remodelling. It was thus examined if the loss of Ptn expression in these cells leads to an alteration even if microenvironmental Ptn is still available. In *in vitro* co-cultures of wt and $Ptn^{-/-}$ lin^{-} cells with shPtn and pLKO.1

UG26-1B6, no difference could be detected between the different lin$^-$ cells. In transplantation of Ptn deficient HSCs into wt recipients, the engraftment in the primary recipient mice was indistinguishable from recipients transplanted with Ptn$^{+/+}$ littermate control cells. Only in the secondary transplantation of these cells were slight alterations observed in short-term engraftment (5 weeks after transplantation) and the number of donor-derived LSK and Cd34$^-$ LSK cells in mice which received Ptn$^{-/-}$ cells compared to recipients with Ptn$^{+/-}$ cells. It seems likely that this is caused by the loss of Ptn expression in the microenvironment of the primary donors, and as it was seen in the serial transplantations after the transplantation into a Ptn-deficient microenvironment, the effects of the microenvironmental Ptn deficiency carry on in the following transplantations. Therefore, Pleiotropin does not seem to have any intrinsic effects on HSC maintenance or early hematopoiesis.

4.4. Extrinsic functions of Pleiotrophin

Co-cultures with stromal cells in which Ptn was stably knocked down showed increased numbers of HSCs and hematopoietic progenitors compared to control cultures. Similarly, transplantation of wt bone marrow cells into a Ptn-deficient microenvironment led to an enhanced maintenance and self-renewal of HSCs. In following serial transplantations into wt recipients, this increased maintenance and self-renewal of HSCs was found up to quaternary recipients. This was accompanied by an increase in HSC numbers and an increasing dominance of myeloid cells in the donor-derived populations in the peripheral blood of the recipients. Notably, the increased self-renewal rate of the wt HSCs which had been only for the first transplantation in a Ptn-deficient microenvironment did not fade in the following transplantations into wt recipients. This shows that even if microenvironmental Ptn is again available after one transplantation, this could not make up for the effects imprinted on the HSCs due to the lack of Ptn secretion by the microenvironment.

The secondary transplantation after the transplantation of co-cultures and the tertiary transplantation after the transplantation of wt bone marrow cells into a Ptn-deficient microenvironment show strong similarities. In both cases, an enhanced

engraftment, particularly myeloid engraftment in the bone marrow is observed alongside increased numbers of Cd34⁻ LSK cells. The difference between the two experiments was that in the first case, wt lin⁻ cells were cultured on shPtn and pLKO.1 stromal cell lines, whereas in the other experiments, wt HSCs were transplanted into a Ptn-deficient microenvironment. This leads to the conclusion that the effect on HSC function was mainly produced by the loss of Ptn expression by the stromal cells in the HSCs' microenvironment.

There are several possible models for explaining the effects observed after the loss of microenvironmental Ptn. The first model would assign Ptn a role in the cell cycle regulation of HSCs, namely in the transition from the G0 state. HSCs are mostly quiescent, meaning that they remain in the G0/G1 state at most times (Cheshier *et al.* 1999). There are several genetic models known in which this quiescence is disturbed and in most of these models HSCs are progressively lost over time due to this enhanced proliferation (see 1.1.4) (reviewed by Orford and Scadden 2008). Nevertheless, there are also some genetic models known in which the enhanced proliferation of HSCs did not lead to their exhaustion, like in mice deficient for the cell cycle regulator Cdkn2c (also known as $p18^{INK4c}$)(Yuan *et al.* 2004; Yu *et al.* 2006) or the transcription factor Snai2 (also known as Slug) (Sun *et al.* 2010). The Ptn KO mice might be included in this list of models, with the exception that in this case not a defect in the HSCs themselves but an alteration in the niche is responsible for the enhanced HSC proliferation and self-renewal. This view is supported by the transplantation experiments, and additional support comes from CFSE analyses of wt lin⁻ cultured in conditioned medium of shPtn and control stromal cells. In these stroma-free cultures, there were more dividing cells retaining the LSK phenotype in the supernatant of shPtn compared to controls. The increased myeloid populations in the peripheral blood of recipients after transplantation might also be a consequence of this, similar to the effects which have been described for the Cdkn2c KO mice. This model might also explain the irreversibility of the effect of a Ptn-deficient microenvironment on wt HSCs. The HSCs which have left the quiescent state in the

Ptn-deficient microenvironment would not have the possibility to become quiescent anymore, even if Ptn is again present in the microenvironment.

Considering the finding that the loss of Ptn has no gross effect on hematopoiesis under steady-state condition, it can be hypothesized that Ptn expressed by the niche cells controls the transition step between the quiescent state and the actively proliferating state of HSCs. If Ptn expression is lost, then the threshold for the HSC to enter the proliferating state is lowered. During steady-state hematopoiesis, there is no urge for the HSCs to leave their quiescent state, but if stress is put upon the cells, HSCs are forced to leave this state and start proliferating. Because of the lowered threshold in a Ptn-deficient microenvironment a notably higher percentage of HSCs would leave the G0 phase and would start to proliferate and self-renew (Figure 73).

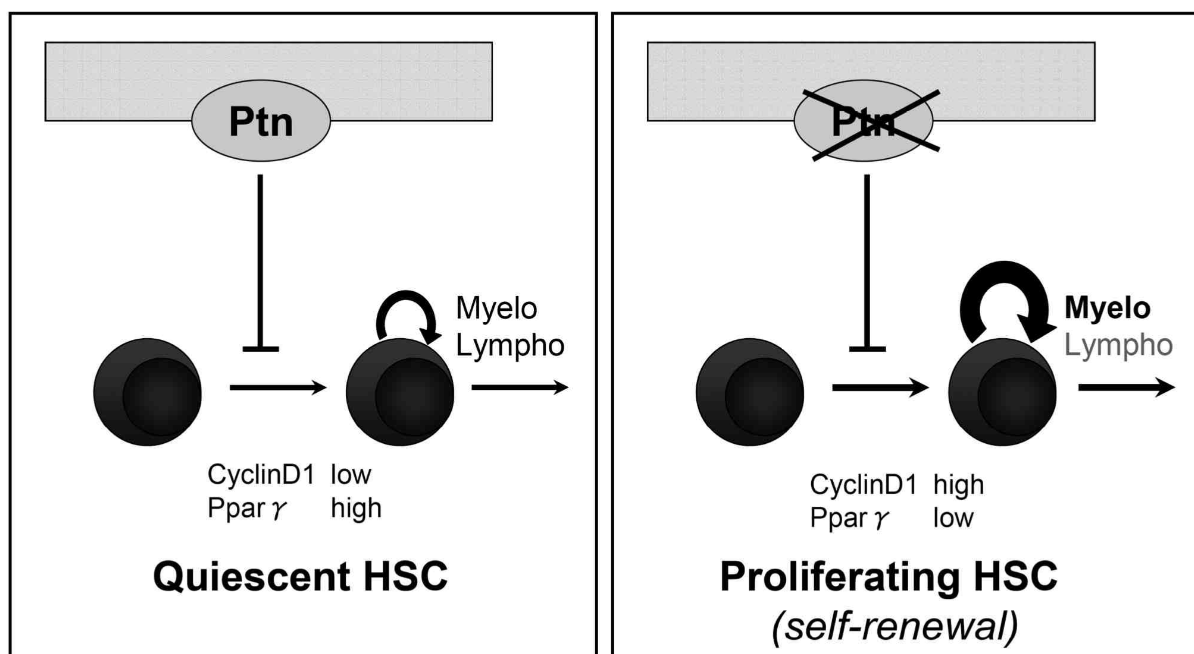


Figure 73. Model 1: Ptn represses the transition of HSCs from quiescence to the proliferating state. If microenvironmental Ptn is absent, HSCs can easily enter the cell cycle. This is associated with an increased self-renewal rate.

Another possibility is that the loss of Ptn expression by the microenvironment leads to the premature aging of the HSCs. This model is mainly supported by the increased donor-derived myeloid population found in the blood and an increase in $Cd34^-$ LSK cells in the bone marrow of wt recipients in serial transplantations of bone marrow or co-cultures. The same observations were described in aged mice and

transplantation experiments using ageing HSCs (Rossi *et al.* 2005; Chambers and Goodell 2007). On the other hand, ageing HSCs are also known to show a functional impairment of engraftment (Rossi *et al.* 2005; Chambers and Goodell 2007; Noda *et al.* 2009). Thus, if the model of premature ageing due to the loss of microenvironmental Ptn were true, this would mean that this defect in engraftment would not necessarily be part of HSC ageing. This hypothesis is supported by recent findings, which show that the functional engraftment defect can be restored in contact with a young niche (Mayack *et al.* 2010). On the other hand, in most published models competitive transplantations were performed, so that aged HSCs would compete with young HSCs, both derived from the bone marrow. In the experiments done during this study, however, there was no competition since the spleen "helper" cells that were used did not contain a sufficient number of HSCs to compete with the transplanted bone marrow HSC. Thus, in this non-competetive setting, every transplanted HSC would be able to engraft, and, if there is be a competitive disadvantage of these HSC, we would not be able to detect it using noncompetitive transplantation experiments.

The increased self-renewal abilities of HSCs and the increased donor-derived myeloid populations in the blood of recipient mice can also be explained by a third model. From clonal transplantation experiments, it was found that the HSC compartment is heterogeneous, and that it can roughly be subdivided into myeloid-biased and lymphoid-biased stem cells (Muller-Sieburg *et al.* 2002). Myeloid-biased stem cells, which preferentially differentiate to myeloid cells, have been reported to show improved engraftment and self-renewal capacities compared to lymphoid-biased stem cells (Dykstra *et al.* 2007). It was further shown that the Hoechst exclusion (also known as "side population") phenotype of myeloid-biased and lymphoid-biased HSC may be stable over time even in serial transplantations and therefore might represent two distinct classes of HSCs (Challen *et al.* 2010; Muller-Sieburg *et al.* 2002). Since it was shown that TGF-β1 differentially affects the two HSC subclasses (Challen *et al.* 2010), it would be proposed that the loss of microenvironmental Ptn will not affect these subclasses in an equal manner:

Myeloid-biased stem cells would be preferred over lymphoid-biased cells due to the loss of microenvironmental Ptn. This model would also explain the increased self-renewal capacity and more myeloid than lymphoid differentiation we observed in our experiments (Figure 74).

In this model, the altered ageing processes proposed for the second model might be integrated, since it was proposed that during ageing, lymphoid-biased HSCs are being exhausted, while myeloid-biased HSCs are maintained, leading to an enrichment of myeloid-biased HSCs (Muller-Sieburg and Sieburg 2008).

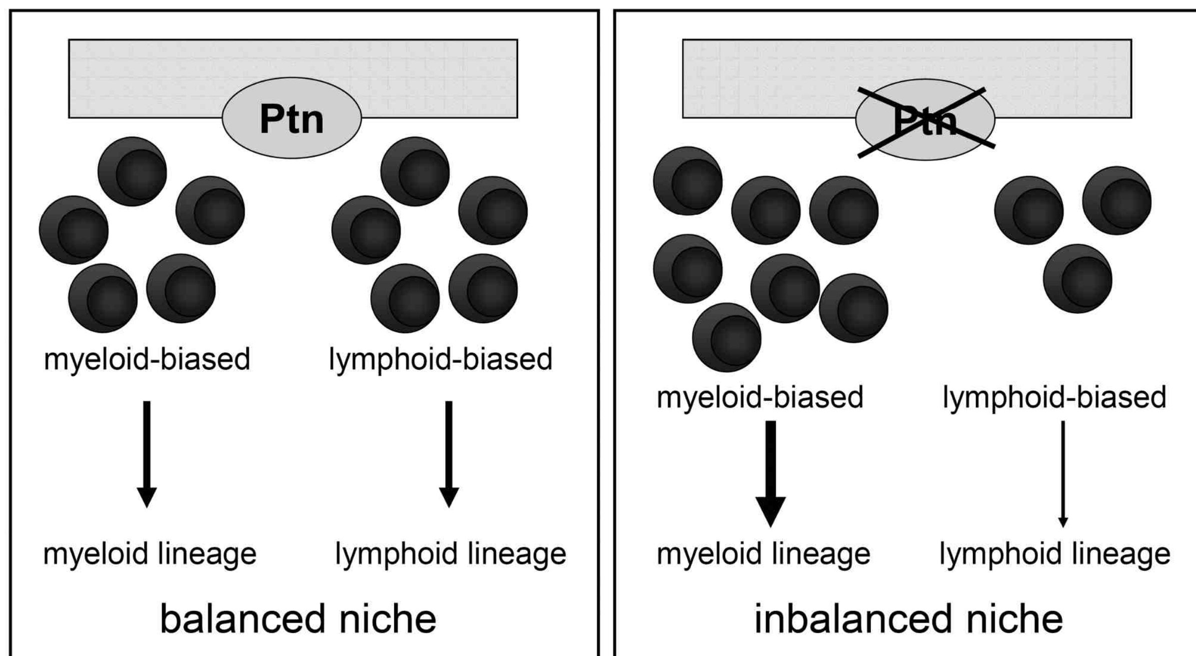


Figure 74. Model 3: The loss of microenvironmental Ptn leads to a dysregulation of maintenance between myeloid-biased and lymphoid-biased HSCs.

With the current results, it is not possible to distinguish between the three models for the action of Ptn. The study of G0 stem cells is severely hampered by the fact that at present it is unclear what determines G0. Whereas the cell cycle and the molecules involved in the transition steps in the cell cycle are well described, the molecules determining the G0 state of the cell cycle are mostly unkown. Both other models can be studied more easily. For instance, whether the loss of Ptn causes premature HSC ageing will have to be determined in competitive transplantation experiments to

show whether these cells have a functional engraftment defect. This aspect of HSC behaviour may have been overlooked in the noncompetitive experiments performed during this study. It was only in the weeks before submission of this thesis that a method became available to study the third hypothesis, namely that Ptn may differentially affect myeloid- and lymphoid-biased HSC subclasses. In future experiments, primary donor HSC could be sorted on the bases of Hoechst exclusion (side population) (Challen *et al.* 2010) and then both classes of cells could be transplanted separately. The third hypothesis would also predict a decay in lymphoid function in the absence of Ptn, an aspect we didn't address either. Thus, future experiments should be able to determine which model for the functional mode of action for the loss of Ptn is correct.

4.5. Mechanistical insights into the molecular basis of Ptn functions

To determine which molecular mechanisms form the basis of the effects seen after transplantation of HSC which were activated in a Ptn-deficient environment, analyses of gene expression and protein levels were performed to reveal some of the possible mechanisms behind the increased self-renewal of HSCs after the loss of microenvironmental Ptn. LSK cells co-cultured with stromal cells in which Ptn was stably knocked down showed an increased expression of Ccnd1 and Cebpa as well as a reduced expression of Pparg. In the myeloid progenitor populations (CMP and GMP), further genes were differentially expressed after co-culture on shPtn stromal cells compared to controls, suggesting that Ptn has additional effects on myeloid committed cells. The expression of Notch signalling targets Dlk1 and Hes1 was reduced. Additionally, the expression of several cell cycle regulators was altered. On the other hand, survival-associated genes like Trp53 and Pten or genes associated with lineage fate decisions like Runx1, Gata-1 and Gata-2 were unchanged in all the populations. Mice deficient for Cdkn2c show a phenotype very similar to the one observed here (Yuan *et al.* 2004; Yu *et al.* 2006), but the expression of Cdkn2c was not altered in LSK cells cultured on shPtn stromal cells compared to controls.

The increased Ccnd1 levels in LSK cells cultured on shPtn stromal cells in comparison with controls may contribute to the increased proliferation observed in these cells. It has been hypothesized that HSCs which are able to proliferate more rapidly than normal without exhaustion might achieve that by a direct transition from G0 to the late G1 phase of the cell cycle (Orford and Scadden 2008). This transition might be allowed by an increased Ccnd1 activation in these HSCs, since Ccnd1-Cdk complexes regulate the transition from early to late G1 phase (Orford and Scadden 2008).

We chose to assess Cebpa expression, since a knockout of this molecule enhanced stem cell maintenance in much the same way as loss of Ptn (but in embryonic stem cells) (Zhang *et al.* 2004). Thus, it was expected to find that Cebpa would be down-regulated by the loss of Ptn in the environment. Instead, an upregulation of Cebpa was observed, consistent with the ability of Cebpa to promote granulocytic, while suppressing erythroid differentiation (Suh *et al.* 2006). Thus, our results suggest that in the absence of Ptn, Cebpa appears to act mainly on myeloid differentiation and less on HSC self-renewal.

Pleiotrophin is known to bind to the receptor tyrosine phosphatase RPTP β/ζ, forcing a dimerization and therefore inactivation of the phosphatase (Meng *et al.* 2000). This inactivation leads to an increasing phosphorylation status of various substrates of this phosphatase, including Ctnnb1 (Meng *et al.* 2000). It was shown in different cell types that Ptn signals through Ctnnb1 (Gu *et al.* 2007; Yanagisawa *et al.* 2009). Additionally, Ctnnb1 is known to play an important part in the regulation of hematopoietic stem cells (Kirstetter *et al.* 2006; Scheller *et al.* 2006). However, neither in gene expression, in protein levels nor in the distribution between cytoplasm and nucleus was there a difference observed between LSK, CMP or GMP cultured on Ptn knockdown or control stromal cells. Since Ctnnb1 is a key target of the canonical Wnt-signalling it seems that Ctnnb1-dependent Wnt signalling is not affected by the loss of microenvironmental Ptn. However, Ccnd1 is a classical target of canonical

Wnt-signalling. This suggests that in the absence of microenvironmental Ptn Ccnd1 is upregulated independently of Ctnnb1 regulation.

Beside the canonical Wnt-signalling, the non-canonical pathway has also been shown to be involved in HSC regulation. It was found that the stimulation of this pathway in HSCs led to an enhanced self-renewal (Murdoch *et al.* 2003; Nemeth *et al.* 2007). The stimulation of non-canonical Wnt signalling leads to the transcriptional repression of Pparg, and Pparg was found to be expressed in much lower levels in HSCs and progenitors cultured on shPtn stromal cells than in controls, meaning that non-canonical Wnt signalling is probably activated in these cells. Pparg is also known to induce cell cycle withdrawal (Altiok *et al.* 1997), which would provide a possible explanation for the enhanced proliferation rate observed for HSCs and progenitors. In addition to that the suppressed Pparg transcription might also contribute to the enhanced CyclinD1 levels, as Pparg is known to negatively regulate CyclinD1 expression in an Ctnnb1-independent way (Sharma *et al.* 2004).

Our results show that in the absence of stromal Ptn, the expression of Hes1 and Dlk1, two important Notch signalling targets are downregulated in mature cells (CMP and GMP). Ptn has already been found to be related to Notch signalling, another important pathway for regulation of HSC (Fukazawa *et al.* 2008; Weng *et al.* 2009). Overexpression of Hes1 is known to enhance HSC maintenance (Kunisato *et al.* 2003), thus Notch signalling could in theory contribute to the observed enhanced HSC maintenance. In LSK, however, expression levels of neither Hes1 nor Dlk1 are altered by the absence of Ptn, so it seems unlikely that these molecules cause the effect of enhanced stem cell maintenance in the absence of microenvironmental Ptn.

In the experiments to find out whether in the absence of stromal Ptn, some kind of “ageing” or “myeloid-bias” transcriptional program was activated, it was found that expression of the RAR-related orphan receptor beta Rorb was induced in LSK cells co-cultured with shPtn stromal cells. Increased expression of Rorb is associated with aged cells (Rossi *et al.* 2005; Noda *et al.* 2009), which also show myeloid skewed engraftment and an increase in the $Cd34^-$ LSK subpopulatiuon after engraftment.

Rorb knockout mice show postnatal retinal degeneration and alterations in circadian rhythm (Andre *et al.* 1998). However, hematopoiesis was not specifically investigated in these mice. Since engraftment of cells co-cultured on shPtn stromal cells was not impaired, as would have been expected when dealing with "aged" HSC, the results suggest that Rorb induction may be associated with myeloid skewing. One could speculate that the molecules that are normally associated with "young" HSC and good engraftment (for instance Hmga2 (Noda *et al.* 2009)), but that are unchanged in LSKs co-cultured with shPtn stromal cells, are associated with the poor functional capacity of "old" HSC.

In conclusion, several slight changes of gene expression were observed in the first days of culture lacking microenvironmental Ptn (Ccnd1: 1.4-fold up, Cebpa: 1.4-fold up, Pparg: 0,4-fold down) in addition to the induction of Rorb. Still, our experiments demonstrate that such subtle changes may lead to profound late-acting effects on HSC maintenance and self-renewal, which can be detected even in quaternary recipients. Thus, our results show that some of the more subtle effects the microenvironment imposes on HSC can only be delineated by a combination of detailed analyses of both molecular changes as well as serial transplantations.

4.6. Functions of Ptn in granulocytes

Granulocytes are short-lived hematopoietic cells which are subject to a constant and fast turn-over. The effects of the absence of microenvironmental Ptn on the myeloid lineage prompted us to study its effects on mature populations of myeloid cells more closely. When mature and immature cells were treated with cycloheximide, monocytes or any other more primitive progenitor population examined did not seem to be affected by the absence of Ptn. However, the experiments described in this thesis clearly demonstrate that the loss of secreted Ptn in the medium leads to an enhanced rate of apoptosis in granulocytes when translation was inhibited by cycloheximide. Other triggers of apoptosis did not appear to be affected by the absence of Ptn, suggesting that protein turnover is the main target of Ptn in granulocyte apoptosis.

PI-3-Kinase has repeatedly been found to be involved in granulocyte apoptosis (for review: Lindemans and Coffer 2004). Likewise, Bcl2 and Caspase3 play important roles in the programmed cell death of these cells (for review: Simon 2001) Nevertheless, PI-3-Kinase, Bcl2 and Caspase3 do not seem to be involved in the enhanced apoptosis observed due to the absence of secreted Ptn. The only mediator which could inhibit the effects of absence in stroma was the rather general proteasome inhibitor MG132. However, although this result clearly indicates that loss of Ptn affects degradation of a protein by the MG132-sensitive proteasome, this protein target could not be identified in the experiments performed during this study.

The experiments do not confirm that Ptn directly affects granulocyte apoptosis. It is very well possible, that the absence of secreted Ptn leads to an alteration in the gene expression of the stromal cells and subsequent secretion of soluble factors, one of which affects granulocytes. Further experiments will be necessary to find out whether Ptn itself is this factor, or whether another factor, which can be regulated by Ptn regulates granulocyte apoptosis.

4.7. Conclusion

In this project, the functions of the cytokine Pleiotrophin in hematopoiesis and especially in the regulation of HSCs were investigated. Ptn was found to be dispensable for steady-state hematopoiesis, and no intrinsic defects in HSCs were detected due to the loss of Ptn. However, the loss of microenvironmental Ptn leads to an enhanced maintenance and self-renewal of HSCs *in vivo* and *in vitro*, which is accompanied by early changes in Ccnd1, Cebpa and Pparg expression and an induction of Rorb expression. Several models are available that might display the function of Ptn, but further experiments would be necessary to clarify the exact mode of action of Ptn. In addition to that, Ptn was found to be involved in granulocyte apoptosis and/or protein turnover. Alltogether, it was shown in this thesis that soluble factors like the cytokine Pleiotrophin can be important for HSC maintenance and tissue homeostasis.

5. Abbreviations

5-FU	5-Fluorouracile
AP	Alkaline phosphatase
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumine
CFA	Colony forming assays
CFSE	Carboxyfluorescein succinimidyl ester
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
DAPI	4,6-diamino-2-phenylindole dihydrochloride
DMEM	Dulbecco's modified Eagle's medium
DNA	Desoxyribonucleic acid
DTT	Dithiothreitole
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal Calf Serum
GMP	Granulocyte-monocyte-progenitor
HBSS	Hank's buffered salt solution
HCl	Hydrochloride
HE	Heterozygous
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horse radish peroxidase
HSC	Hematopoietic stem cells
Ig	Immunoglobulin
IL	Interleukin
KCl	Potassium chloride
KO	Knockout

LT-HSC	Long-term repopulating hematopoietic stem cell
MEP	Megakaryocyte-erythrocyte progenitor
MPP	Multipotent progenitor
mRNA	Messenger ribonucleic acid
NaF	Sodium fluoride
NP40	Tergitol-type NP-40
PBS	Dulbecco’s phosphate buffered saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI	Propidiumiodide
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidene difluoride
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
shRNA	Short hairpin ribonucleic acid
ST-HSC	Short-term repopulating hematopoietic stem cells
Tris	Tris(hydroxymethyl)aminomethane
Wt	Wildtype

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7. Publications

Renström, J., Istvanffy, R., Gauthier, K., Shimono, A., Mages, J., Jardon-Alvarez, A., **Kröger, M.**, Schiemann, M., Busch, DH., Esposito, I., Lang, R., Peschel, C., Oostendorp, RA. (2009). "Secreted frizzled-related protein 1 extrinsically regulates cycling activity and maintenance of hematopoietic stem cells." Cell Stem Cell **5**(2): 157-67.

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Kröger, M., Istvanffy, R., Graf, S., Schiemann, M., Peschel, C., Oostendorp, RA. "Stromal Pleiotrophin Extrinsically Regulates Hematopoietic Stem Cell Engraftment". Manuscript in preparation.

Graf, S., Platz, U., Hölzlwimmer, G., Bassermann, F., **Kröger, M.**, Peschel, C., Quintanilla-Fend, L., Keller, U. B. “Cks1 promotes S-phase progression in a $p27^{Kip1}$-independent manner”. Manuscript in preparation.

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