Osteogenic hormones acting on the environment of the hematopoietic stem cell niche

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1 Abstract / Zusammenfassung

1.1 Abstract

Hematopoietic stem cells (HSCs) are the most widely studied adult stem cells in vertebrates. Despite this, little is known about the regulation of HSC maintenance in their specialized microenvironment.

The aim of this thesis was to study the influence of osteogenic hormones on the regulation of HSCs. Thereby we could prove that, although estradiol increases osteoblastic cell numbers and bone mass, it does not have any advantageous effects on the endosteal HSC niche. Surprisingly, estradiol displayed alterations in the microenvironment of the vascular niche, by upregulating distinct adhesion molecules and thereby correlating with an increase of HSCs in the vascular niche. Therefore, we suggest an enhanced retention of HSCs in the vascular niche under the influence of estradiol, also proven by a decrease of HSCs in the peripheral blood.

Furthermore, we investigated the effects of long-term growth hormone (GH) administration, which is known to increase bone mineral density and thereby influence the endosteal HSC microenvironment. We clearly showed increased HSC numbers in the vascular and the endosteal niche of wildtype mice after GH administration. Additionally, we proposed a Janus kinases/Signal Transducers and Activators of Transcription (Jak/STAT)-signaling-dependent mechanism of GH in the endosteal niche. To test this hypothesis, we investigated the influences of GH in a conditionally-mutated mouse model ($STAT5^{OB}$), where Jak/STAT signaling is disrupted in osteoblasts by the loss of STAT5. Unexpectedly, these mice showed increased numbers of HSCs in the endosteal niche and displayed strikingly enhanced endosteal HSC numbers after GH treatment compared to wildtype controls. Loss of STAT5 in osteoblasts led to strong activation of STAT3 and, in particular, STAT1, suggesting a compensatory mechanism. We proved that Jak/STAT signaling has an important role in the endosteal HSC niche, particularly for the mediation of GH effects. The strong activation of STAT3 and STAT1 correlated with the increased numbers of HSCs in the endosteal niche.

1.2 Zusammenfassung

Hämatopoetische Stammzellen (HSC) sind die am besten studierten adulten Stammzellen in Wirbeltieren, Trotz dessen ist nur wenig über die Interaktionen mit der Nische und die Mechanismen ihrer Regulierung bekannt.

Ziel dieser Arbeit war es, den Einfluss von osteogenen Hormonen auf die Regulierung der HSCs zu untersuchen. Es konnte gezeigt werden, dass, obwohl Östradiol die Anzahl der Osteoblasten erhöht und damit auch die Knochenmasse, es keinerlei Effekte auf die endosteale HSC-Nische hat. Überraschenderweise zeigt Östradiol einen deutlichen Effekt auf die vaskuläre Nische, was zu einer verstärkten Expression von Adhäsionsmolekülen führt und mit einem Anstieg der HSCs in diesem Teil der Nische verbunden ist. Daher wird vermutet, dass Östradiol die vaskuläre Nische beeinflusst und dies zu einem verstärkten Rückhalt der HSCs in der Nische führt. Diese Schlußfolgerung zeigt sich auch in der geringeren Anzahl von HSCs im peripheren Blut.

Darüber hinaus wurden die Langzeit-Effekte von Wachstumshormon (GH) untersucht. Es ist bereits bekannt, dass GH die Knochendichte erhöht und damit die endosteale HSC-Nische beeinflusst. In diesem Zusammenhang konnte gezeigt werden, dass GH zu einem Anstieg von HSCs in der vaskulären und der endostealen Nische des Knochenmarks führt. Zusätzlich wurde ein Jak/STAT-Signalweg-abhängiger Mechanismus für den Effekt vermutet. Um diese Hypothese zu testen, wurden konditionell mutante Mäuse verwendet ($STAT5^{OB}$), die durch das Fehlen von STAT5 in Osteoblasten eine Unterbrechung des GHR-Signalweges aufweisen. Unerwarteterweise zeigen diese Tiere eine erhöhte Anzahl von HSCs in der endostealen Nische und einen extrem starken Anstieg der endostealen HSCs unter GH-Einfluss im Vergleich zu Wildtyp-Tieren. Der Verlust von STAT5 in Osteoblasten führt zu einer verstärkten Aktivierung von STAT3 und vor allem STAT1, was auf einen Kompensations-Mechanismus hinweist. Es konnte damit bewiesen werden, dass der Jak/STAT-Signalweg eine wichtige Rolle in der endostealen Nische, und vor allem in der Vermittlung von GH-Effekten, spielt. Die starke Aktivierung von STAT3 und, noch deutlicher STAT1 korreliert mit dem Anstieg der HSCs in der endostealen Nische.

2 Introduction

2.1 Adult stem cells in tissue homeostasis

The embryonic stem cell is the only totipotent stem cell able to form a complete multicellular organism with all its different tissue types. However, the maintenance and regeneration of a completely developed organism demand a different mechanism due to the absence of embryonic stem cells. The death of cells, either caused by apoptosis during tissue regeneration or by injury, demands a replenishment of the dying cells. This function is assured by a variety of adult or somatic stem cells found in nearly every tissue. They represent multipotent progenitors able to maintain and to regenerate the tissues in multicellular organisms. These multipotent progenitors are lineage restricted, and therefore only able to give rise to distinct differentiated cells. For example, the microsatellite cells as progenitors of muscle tissue are only capable of forming muscle cells. The main pitfall of this regenerative system is the influence of ageing. DNA damage, and thereby the enhanced incidence of cancer, are consequences of ageing. The depletion of stem and progenitor pools also impairs the regeneration of many tissues in a variety of mouse models, e.g. the telomerase-knockout mouse ($Terc^{-/-}$) loses hematopoietic stem cells (HSCs) with ageing (Fuchs et al. 2004; Ruzankina and Brown 2007). These ageing-related mechanisms have also been suggested to influence the development of cancer because increased tumor suppression in combination with decreased proliferation results in a lower capacity of tissue renewal (Ruzankina and Brown 2007). Therefore, the clarification of the complex regulation of somatic stem cells is necessary to understand molecular mechanisms underlying cancer development and primarily ageing.

2.2 HSCs in hematopoiesis

The multipotent HSC is the most widely studied system for somatic stem cells and for the regulation of their maintenance, particularly their differentiation in vertebrates (Ema et al. 2006). The HSC ensures the maintenance and the development of all cellular blood components, which include the daily formation of about 10^{11}–10^{12} new blood cells in humans. The development from HSCs to differentiated blood cells is achieved via several progenitor stages, which are already lineage restricted including

multipotent progenitor, common lymphoid progenitor, common myeloid progenitor, granulocyte/macrophage lineage-restricted progenitor and megakaryocyte/erythrocyte lineage-restricted progenitor (Akashi et al. 2000). The emerging blood cells are subdivided into three major blood cell lineages, namely the erythroid/megacaryocyte, the lymphoid and the myeloid lineage. The erythroid cells are the oxygen transporters, the megacaryocytes give rise to platelets involved in the clotting response, whereas the lymphoid cells develop into T, B and natural killer cells which are essential in the innate and adaptive immune system. The myeloid lineage comprises mast cells, eosinophils, neutrophils and monocytes, which can develop into macrophages, osteoclasts (OCs) and dendritic cells (Akashi et al. 2000; Lodish et al. 2004, "Molecular Cell Biology").

Hematopoiesis during embryonic development occurs in different locations. The first blood cells are formed in the yolk sac (Moore and Metcalf 1970), then migrate to an area surrounding the dorsal aorta termed the aorta-gonad-mesonephros. During mid-gestation, hematopoiesis occurs in the fetal liver and finally locates in the bone marrow (BM) (Tavian et al. 1996; Labastie et al. 1998; Tavian et al. 1999; Watt and Hogan 2000).

The changeover from one hematopoietic site to another during development takes place due to migration and relocation of HSCs, which are supposed to be regulated by chemokines and adhesion molecules (Nagasawa et al. 1996; Frenette et al. 1998; Vermeulen et al. 1998; Zou et al. 1998; Wright et al. 2002; Yong et al. 2002; Ara et al. 2003; Christensen et al. 2004).

2.2.1 Properties of the HSC

HSCs are a very rare subpopulation of the hematopoietic cells in the BM. Only one cell in about 1×10^4–1.5×10^4 BM cells is a HSC, whereas in the blood stream one cell in about 1×10^5 blood cells is a HSC. HSCs can not be identified according to their morphology because in culture they behave like white blood cells. However, due to extensive studies over several decades, a variety of surface markers for the identification of this small subset of BM cells has been identified. HSCs are discriminated into long-term HSCs (LT-HSCs) and short-term HSCs (ST-HSCs). The ST-HSC is described as already initiated for differentiation. Therefore, only the LT-HSC is capable of several consecutive rounds of transplantation in reconstituting mice (Lerner and Harrison 1990; Ramalho-Santos et al. 2002; Akashi et al. 2003; Uchida et al. 2003; Venezia et al. 2004). In mice, LT-HSCs are characterized by

several surface markers: N-Cadherin^{+}, Tie2^{+}, Endoglin^{+}, CD34$^{low/-}$, Sca1^{+}, Thy1$^{+/low}$, CD38^{+}, CD117 (cKit)$^{+}$ and lin^{-}. Lineage negative (lin^{-}) describes all the cells negative for B, T and granulocyte markers and is, in combination with Sca1 and CD117 (cKit), the most prominent group of HSC markers in fluorescence-activated cell sorting (FACS) analysis (Spangrude et al. 1988; Baum et al. 1992; Morrison and Weissman 1994; Osawa et al. 1996). Kiel et al. showed that the subpopulation of CD244-negative, CD48-negative and CD150-positive cells also includes the competent fraction of HSCs in the BM (Kiel et al. 2005).

Additionally, HSCs possess the P-glycoprotein coded by the multidrug resistance 1 gene, which represents an ATP-binding cassette transmembrane transporter responsible for the detoxification of cells. This ATP-dependent transporter enables HSCs to transport the Hoechst 33342 dye out of the cells after staining. Therefore, Hoechst 33342 is often used as a criterion for the identification of HSCs (Goodell et al. 1996; Goodell et al. 1997; Zhou et al. 2001; Scharenberg et al. 2002; Uchida et al. 2003; Matsuzaki et al. 2004; Takano et al. 2004). Due to the fact that this transporter is also an attribute of tumor cells (Hotta et al. 1999; Scharenberg et al. 2002), the identification of HSCs should always be accompanied by the application of adequate surface markers.

2.2.2 HSC division

The periodical process of differentiation via diverse stages of progenitors leads to the development of mature blood cells. However, HSCs also undergo self-renewal, that is, the capability to go through numerous cycles of cell division while maintaining the undifferentiated stem cell fate. How is the complex decision between the two processes made? The HSC displays unique features of cell division in that it divides symmetrically, resulting in two similar daughter cells, which can be either two committed cells or two completely undifferentiated HSCs. A committed progenitor cell has initiated the differentiation program and thereby loses multipotency. However, the HSC is also capable of performing asymmetric cell division, resulting in one undifferentiated HSC and a committed progenitor (Mayani et al. 1993; Brummendorf et al. 1998; Huang et al. 1999; Giebel et al. 2006). The underlying molecular mechanisms are still being debated. One theory is the equal distribution of specific cell fate determinants, e.g. transcription factors, mRNA or even non-coding RNAs, which would lead to a symmetric cell division. In turn, unequal distribution of these components would lead to asymmetric cell division. This theory has not been proven

for any vertebrate stem cell type, but several *in vitro* studies have indicated that HSCs can undergo asymmetric divisions (Suda et al. 1984; Takano et al. 2004; Ho 2005). Another theory for asymmetric cell division is the influence of the specialized microenvironment. In this case, one daughter cell stays in the original surrounding of the dividing HSC, preserving the undifferentiated HSC type, whereas the other daughter cell leaves this environment and is prone to differentiation (Spradling et al. 2001; Ohlstein et al. 2004).

2.2.3 Intrinsic HSC regulation

HSCs are regulated by cell-intrinsic molecular pathways, but also by extrinsic molecular interactions with environmental cells and the extracellular matrix. The extensive characterization of HSCs in the last few years uncovered a wide diversity of intrinsic factors involved in the maintenance and differentiation pathways.

For instance, it has been proven that the inactivation of phosphatase and tensin homolog (PTEN), a negative regulator of the PI3K-Akt pathway, causes expansion of ST-HSCs. However, $PTEN^{-/-}$ enhances the level of HSC activation and leads to a decline in LT-HSCs. $PTEN^{-/-}$ HSCs engraft in recipient mice with normal efficiency, but can not sustain hematopoietic reconstitution due to a deregulation of the HSC cell cycle and the declining maintenance in the microenvironment (Zhang et al. 2006). Thereby, $PTEN^{-/-}$ HSCs lead to myeloproliferative disease and to transplantable leukemia, since PTEN functions as a tumor suppressor mediated by the mammalian target of rapamycin (Yilmaz et al. 2006).

The cellular oncogene Myc (c-Myc) possesses related functions to PTEN. In $c\text{-}Myc^{-/-}$ BM, LT-HSCs accumulate and are increased about 10-fold by an upregulation of adhesion molecules like N-Cadherin and several adhesion receptors. Conversely, overexpression of c-Myc leads to the opposite effect, a loss of HSCs due to premature differentiation along with a downregulation of adhesion molecules (Wilson et al. 2004). Therefore, c-Myc is an important player in regulating the fate decision between stem cell self-renewal and differentiation.

Cyclin-dependent kinase inhibitors p18 and p21 have also been shown to be involved in the regulation of HSCs. Deletion of p18, an early G1 cyclin-dependent kinase inhibitor, leads to a higher competitive reconstitution potential compared to wildtype HSCs by increased self-renewing divisions in $p18^{-/-}$ HSCs and progenitors (Yuan et al. 2004). In contrast, $p21^{-/-}$ mice show increased HSC numbers under normal homeostatic conditions. However under stress conditions, released by 5-

fluorouracil, HSCs are restricted to enter the cell cycle, resulting in the death of the mice (Cheng et al. 2000). $p18^{-/-}$ is able to compensate for the increased HSC exhaustion in $p21^{-/-}$ mice, permitting the conclusion that p18 acts via a counteracting pathway against cellular senescence of HSCs (Yu et al. 2006).

Another important transcription factor, myeloid elf-1-like factor (MEF/ELF4), has been shown to be involved in the regulation of quiescence of HSCs. MEF or ELF4 belongs to the ETS (E26 transformation-specific**)** family of winged helix-turn-helix transcription factors (Miyazaki et al. 1996; Mao et al. 1999; Miyazaki et al. 2001; Lacorazza and Nimer 2003), and $MEF^{-/-}$ mice show a higher fraction of HSCs. $MEF^{-/-}$ HSCs have been suggested to be more quiescent, although their reconstitution potential is completely normal and they even protect mice against myelotoxic drugs and radiation (Lacorazza et al. 2006).

Early growth response 1 (Egr1) belongs to the immediate early response transcription factor and zinc finger-protein family. In the hematopoietic system, Egr1 is important in lymphoid and myeloid cells, particularly B lymphocytes and thymic precursors (Lee et al. 1996; Bettini et al. 2002; Schnell and Kersh 2005; Schnell et al. 2006). Furthermore, Egr1 is expressed in LT-HSCs, but is strongly reduced after stimulation of proliferation or a pharmacological treatment for mobilization of HSCs. $Egr1^{-/-}$ mice show increased proliferation of HSCs, and thereby mobilization into the peripheral blood, suggesting Egr1 as a retention and quiescence factor of HSCs (Min et al. 2008).

In summary, this list of molecular regulators involved in the fate decision of self-renewal and differentiation of HSCs only represents a brief compendium of influencing molecules because the field of HSC research is constantly evolving.

2.3 The HSC niche

The term ‘niche’ is not only important with respect to HSCs. The niche describes the specialized microenvironment consisting of distinct cell types embedding and regulating the stem cells. Such a complex three-dimensional microenvironment has already been described for several stem cell types including the germinal stem cell in drosophila or the intestinal stem cell, the neuronal stem cell, the stem cell of the skin and the HSC in vertebrates (Fuchs et al. 2004). A specialized niche has also been suggested to exist for tumor stem cells (Favaro et al. 2008).

The niche regulates HSCs by extrinsic molecular mechanisms and is able to express membrane-bound and secreted soluble factors, and to exert influence on the maintenance and migration of HSCs (Schofield 1978; Kiel and Morrison 2008). In the BM, HSCs reside in two different locations. A part of the HSCs is located directly at the inner surface of the bone, named the endosteum, where HSCs are in close contact with bone-forming cells, the osteoblasts (OBs) and osteoclasts (OCs). The other part of the HSCs is located more to the center of the BM, where the microenvironment is formed by various cell types: endothelial, perivascular, reticular cells and sinusoidal blood vessels (Li and Xie 2005; Suda et al. 2005; Adams and Scadden 2006; Sugiyama et al. 2006; Kiel et al. 2007b; Sacchetti et al. 2007).

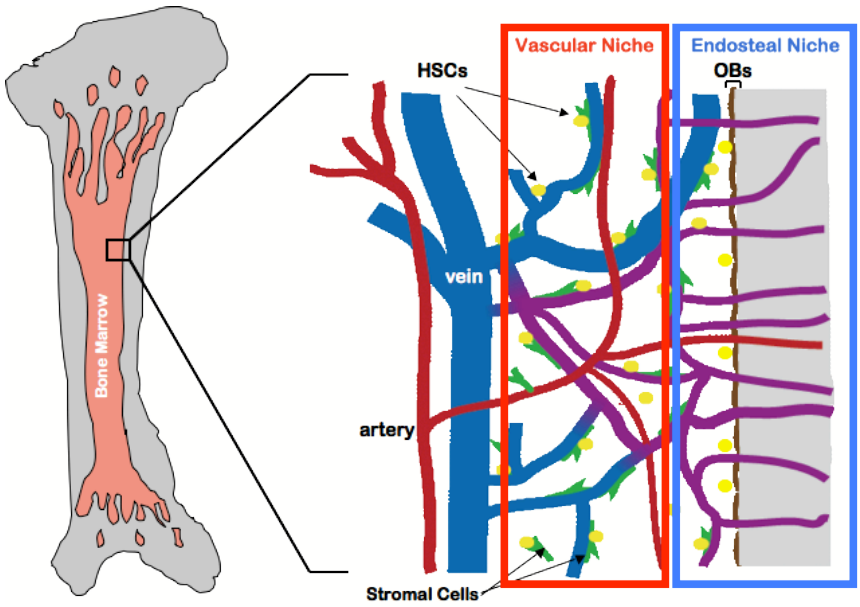


Figure 2.1: **Overview of the endosteal and vascular HSC niche.** Adapted from Kiel and Morrison (Kiel and Morrison 2006).

2.3.1 The endosteal HSC niche

The BM-facing surface of the endosteum is covered by a protective layer of bone-lining cells. These cells are able to differentiate into mature OBs, representing the bone-building compartment. However, the whole compartment is quite heterogeneous in its differentiation state, and at any time-point, only a minority of the cells is actually mature OBs by definition. In addition, OCs, the bone-resorbing cells, are present at the endosteal surface. OBs and OCs form a unity in keeping the balance between bone formation and resorption under steady-state conditions. Furthermore, OBs and OCs are also able to react on external alterations, e.g. during the growth period of an organism, by forming and remodeling bone (Franz-Odendaal et al. 2006; Seeman and Delmas 2006).

In 2003, a revolutionary study showed that HSCs are attached to special spindle-shaped OBs (SNOs) by an asymmetrical distribution of the two adherens junction molecules, β-Catenin and N-Cadherin (Zhang et al. 2003). N-Cadherin is already known to be expressed throughout osteoblastogenesis (Hay et al. 2000), but it is also expressed on LT-HSCs. These data led to the first substantial proof that specialized OBs directly regulate HSCs (Zhang et al. 2003). N-Cadherin, as classic type I cadherin, is a single-chain transmembrane glycoprotein mediating homophilic, calcium-dependent cell-cell adhesion (Takeichi 1991). The newly postulated function

of N-Cadherin in HSC maintenance is highly debated. Kiel et al. were unable to detect the expression of N-Cadherin in isolated HSCs, and showed as proof of principle that N-Cadherin-negative HSCs fully reconstitute lethally-irradiated mice (Kiel et al. 2007b). In the same line of evidence, it was proven that the expression level of N-Cadherin is the critical point for quiescent or primed HSCs. Only the N-Cadherin$^{-/low}$ subpopulation fulfills complete HSC features (Haug et al. 2008). Additionally, mice with reduced numbers of OBs show no defects in hematopoiesis, HSC numbers or functionality (Kiel et al. 2007b).

β-Catenin is also an essential mediator in the Wnt signaling pathway. Although it has been shown that the activation of Wnt in HSCs leads to symmetrical self-renewal and the inhibition of differentiation, the necessity of the canonical Wnt pathway has been doubted due to the dispensability of β-Catenin in the functionality of HSCs. Therefore, one could conclude that Wnt-signaling is perhaps only essential for the expansion and differentiation of progenitor cells and not of LT-HSCs (Reya et al. 2003; Willert et al. 2003; Cobas et al. 2004; Reya and Clevers 2005).

Also important in the regulation of the endosteal HSC niche is angiopoietin-1/Tie2 signaling. Tie2 is a receptor tyrosine kinase expressed on endothelial cells and BM-derived LT-HSCs (Constien et al. 2001; Puri and Bernstein 2003; Arai et al. 2004). Angiopoietin-1 (Ang-1) is expressed by OBs and is able to maintain HSCs *in vitro* (Arai et al. 2002), leading to enhanced adhesion and maintenance of the immature phenotype of the Tie2-expressing HSCs. This adhesion prevents cell division, resulting in the regulation of quiescence and protects HSCs from myelosuppressive stress in the BM niche by the inhibition of apoptosis (Arai et al. 2004).

Osteopontin (OPN) is a multidomain, phosphorylated glycoprotein involved in cell adhesion, tumor metastasis, angiogenesis, apoptosis, in the inflammatory response and in bone homeostasis (Reinholt et al. 1990; Asou et al. 2001; Denhardt et al. 2001). In the BM, the expression of OPN is restricted to OBs. OPN$^{-/-}$ mice lack the localization of HSCs at the endosteum after transplantation. OPN has inhibitory functions in HSC proliferation (Nilsson et al. 2005), since OPN$^{-/-}$ mice display increased primitive hematopoietic cell number correlating with the upregulation of Jagged1 and Ang-1 in the BM stroma (Stier et al. 2005).

With regard to the endosteal niche, the interactions of OBs and HSCs via the Notch signaling pathway have also been extensively discussed. Notch signaling has been suggested to be essential in the endosteal niche, promoting maintenance by the HSC-expressed Notch receptors. Notch and Jagged1 overexpression studies all resulted in enhanced self-renewal and inhibited differentiation of HSC (Varnum-Finney et al. 1998; Carlesso et al. 1999; Varnum-Finney et al. 2000; Karanu et al.

2001; Stier et al. 2002; Calvi et al. 2003; Kunisato et al. 2003; Burns et al. 2005; Suzuki et al. 2006). The combined inactivation of Jagged1 and Notch1 does not influence HSC function (Radtke et al. 1999; Mancini et al. 2005). Compensatory effects of other members of the Notch signaling pathway can not be excluded. The suppression of all canonical Notch signals in adult HSCs does not show any defects *in vivo* (Maillard et al. 2008). Therefore, the suggested essential role for Notch in HSC regulation has not been substantiated.

In summary, due to this constantly expanding field of research, these data represent only an abstract of the signaling involved in the interaction of HSCs and their endosteal niche compartments.

2.3.2 The vascular HSC niche

Since the complex three-dimensional structure of the HSC environment has become clearer, the question arose whether there are relations between the regulation of the endosteal and the vascular niche. To date, there are only hints for such relations. For instance, the endosteum is intensively vascularized, suggesting a possible role for vascular cells in the regulation of HSCs at the endosteum (De Bruyn et al. 1970). It was recently shown that specialized reticular cells, expressing high levels of CXC chemokine ligand 12 (CXCL12), are important in both niche types. These CXCL12-abundant reticular (CAR) cells have been found to be in direct contact with HSCs, and they either surround sinusoidal endothelial cells in the vascular niche or they are located quite close to the endosteum (Sugiyama et al. 2006). CXCL12 has been shown to be expressed by OBs regulating migration and localization of HSCs within the BM (Peled et al. 1999; Petit et al. 2002). The receptor CXCR4 is widely expressed throughout the immune and central nervous systems (Jazin et al. 1997; Moepps et al. 1997). $CXCR4^{-/-}$ mice show an important role for the G-protein-coupled chemokine receptor in both cerebellar development and hematopoiesis (Zou et al. 1998). Furthermore, the ablation of the adrenergic neurotransmitter norepinephrine leads to granulocyte colony-stimulating factor (G-CSF)-mediated inhibition of OBs, resulting in the mobilization of progenitor cells and the downregulation of CXCL12. This suggests the involvement of the sympathetic nervous system in HSC mobilization (Katayama et al. 2006). In addition to cytokines, effects of other hormones have been discovered. Growth hormone (GH)-treated and bGH-transgenic mice have higher numbers of HSCs due to mobilization of the cells. Administration of GH leads to an upregulation of suppressor of cytokine signaling (SOCS) 1 and 3,

which in turn blocks the CXCL12/CXCR4 signaling, leading to a distribution of HSCs in the peripheral blood. Thus, the Janus kinases/Signal Transducers and Activators of Transcription (Jak/STAT) signaling pathway participates in HSC regulation (Pello et al. 2006).

Ju et al. (2007) proved an influence of DNA damage signaling pathways on the function of the HSC niche. DNA damage is known to be closely related to stem cell exhaustion and ageing (Lieber and Karanjawala 2004). The activation of DNA damage signaling pathways can be caused by the loss of the capping functions of telomeres at the chromosome ends, resulting in widespread damages in cell and tissue function (Vaziri and Benchimol 1996; d'Adda di Fagagna et al. 2003). Stromal cells from telomerase-knockout mice ($Terc^{-/-}$) have a decreased potential to maintain HSCs and their early progeny. A role for the stromal cells is further substantiated by the fact that the non-endothelial stromal cells, the vascular and endosteal cells of the BM, decrease in numbers with ageing. This indicates that both regulatory compartments, vascular and endosteal, are essential for the maintenance of HSCs (Ju et al. 2007).

The variety of signaling pathways shown to be involved in the maintenance of HSCs and their distribution between the two niches raise the question what is the difference between the niches and is there a necessity for the existence of two niches? One current hypothesis argues for the need of two niches with a dormant HSC. Dormant HSCs are directly attached to the SNOs (specialized spindle-shaped N-Cadherin-expressing OBs) at the endosteal surface. The SNOs are, in turn, in contact with the previously mentioned CAR cells, which are more frequently found at sinusoids in the vascular niche. It is suggested that in this surrounding, the CAR cells together with OBs, stromal fibroblasts and possibly other cell types, create a microenvironment with only low oxygen levels and a dense extracellular matrix. This environment keeps the HSCs dormant, and the activation of dormant HSCs leads to translocation to the CAR cells into the vascular niche next to sinusoids. For the maintenance of the dormant stem cell pool, the HSCs undergo asymmetric cell division forming one dormant HSC and a committed progenitor (Wilson et al. 2007).

Several arguments against this hypothesis exist. One argument is that HSCs isolated from the vascular niche can also establish long-term reconstitution over several generations of mice (Liang et al. 2007). Secondly, subendothelial stromal cells on the sinusoidal wall have also been shown to express Ang-1, which was initially thought to be specifically important in the endosteal HSC niche (Sacchetti et al. 2007). Furthermore, N-Cadherin, described as essential for HSCs, has been recently found as dispensable for LT-HSCs (Kiel et al. 2007b). This could also be the case for other

factors shown to be important due to their specialized expression by OBs, because to date none of these factors has been conditionally deleted only in OBs. Thus, OBs may not be the main source for these factors as already proven for Ang-1 (Li et al. 2001; Sacchetti et al. 2007). Another argument for the dispensability of the endosteal microenvironment is that some vertebrate species do not have any hematopoiesis associated with the bone, e.g. the zebrafish (Murayama et al. 2006). In mammals, extramedullary hematopoiesis can be observed under different circumstances in liver and spleen, representing at least a partially redundant role for the endosteum (Wright et al. 2001). The occurrence of extramedullary hematopoiesis reflects the high motility of HSCs, where HSCs exit and re-enter the BM stem cell niche via the vascularization. The mobilization and homing of HSCs are facilitated by regulatory alterations of adhesive connections, formed by membrane-bound stem cell factor, vascular cell adhesion molecules and integrins (Lapidot and Petit 2002; Papayannopoulou 2003; Cancelas et al. 2005; Lapidot et al. 2005).

In conclusion, the vascular and the endosteal HSC niche form a highly specialized microenvironment for HSCs. Although HSCs are the most widely studied adult stem cells, many questions remain open and further studies on the cell fate decisions in HSCs are required.

2.4 Osteogenic hormones presumably affecting the endosteal HSC niche

2.4.1 Estrogen in bone metabolism

17-β-Estradiol (17-β-E_2) is a steroid hormone preferably involved in the development of primary and secondary sexual organs and the further development of the oocytes in females, whereas in males estrogen levels are rather low although not at all dispensable. An important phase when even males essentially need estrogens is the onset of bone formation, the skeletal growth throughout puberty and for the regulation of gonadotropin (Bhatnagar et al. 1992; Bulun 1996; Carani et al. 1997; Bilezikian et al. 1998; Rochira et al. 2000).

Most of the effects of 17-β-E_2 are mediated via the two known estrogen receptors, ERα and ERβ (Kuiper et al. 1996; Couse et al. 1997; Kuiper et al. 1997). Both receptors belong to the steroid/thyroid hormone superfamily of nuclear receptors, possessing similar structural characteristics (Evans 1988; Giguere et al. 1988; Tsai and O'Malley 1994; Gronemeyer and Laudet 1995; Mangelsdorf et al. 1995; Katzenellenbogen and Katzenellenbogen 1996). The receptors act as transcription factors, they consist of independent but interacting domains, named A/B, C, D, E, F. A/B at the N-terminus of the protein is the AF-1 domain, which exerts ligand-independent activation function important for protein-protein interactions and transcriptional activation of target gene expression. The AF-1 domain is much more active in ERα than in ERβ, as tested with estrogen responsive element (ERE)-reporter constructs (McDonnell et al. 1995; McInerney et al. 1998).

C represents the DNA-binding domain, which is well conserved between ERα and ERβ. It contains two zinc-finger structures, which are essential for receptor dimerization and the binding to specific DNA sequences, the EREs. The P-box is necessary for the recognition and the specificity of the target DNA (Beato 1989; Umesono and Evans 1989; Hard et al. 1990; Schwabe et al. 1993; Eriksson et al. 1995; Enmark et al. 1997; Vanacker et al. 1999). ERs can also affect gene expression without binding to the DNA. For instance, ERα is able to bind to NFκB, thereby inhibiting the induction of IL-6 by NFκB. ERα is able to physically interact with Sp1. The DNA-Sp1 binding is hormone independent. Both ERs are also able to interact with the fos/jun transcription factor complex at AP1 sites. This leads to gene expression, but only in the presence of estrogens (Klein-Hitpass et al. 1986; Ray et

al. 1994; Webb et al. 1995; Batistuzzo de Medeiros et al. 1997; Galien and Garcia 1997; Paech et al. 1997; Sun et al. 1998; Duan et al. 1999; Qin et al. 1999; Webb et al. 1999; Zou et al. 1999).

The domains D, E and F together are folded into 12 helices. They represent the ligand-binding domain located at the C-terminus. Helices 3 to 12 are directly involved in ligand binding. The ligand-binding domain harbors the so-called AF-2 domain overtaking an activation function under ligand binding. AF-2 is formed by amino acids of the helices 3, 4, 5 and 12. Helix 12 undergoes a positional change upon ligand binding. Helix 12 is essential and therefore often described as cap for the ligand-binding pocket, changing its position depending on the ligand (Danielian et al. 1992; Denton et al. 1992; Wurtz et al. 1996; Brzozowski et al. 1997; Henttu et al. 1997; Feng et al. 1998; Shiau et al. 1998; Mak et al. 1999).

All steps in the activation of gene expression, ligand binding, dimerization of the receptor, binding to the DNA and the interaction with co-factors, are phosphorylation dependent (Migliaccio et al. 1989; Denton et al. 1992; Arnold et al. 1994; Chen et al. 1999; Endoh et al. 1999). Also, ligand-independent effects of ERs require phosphorylation of the receptors. Phosphorylation sites have been extensively studied in ERα. These sites are distributed in all domains of the protein. All serine residue phosphorylation sites are conserved between the two receptors. ERα can be phosphorylated in the absence of estrogens, but phosphorylation is enhanced under physiological levels of 17-β-E_2 (Denton et al. 1992; Ali et al. 1993; Aronica and Katzenellenbogen 1993; Arnold et al. 1994; Lahooti et al. 1994; Le Goff et al. 1994; Weigel 1996; Weigel and Zhang 1998; Shao and Lazar 1999).

In bone architecture, a lack of estrogen leads to a destabilization of the bones due to osteoporosis. The high turnover of the trabecular bone in post-menopausal women leads to a loss of volume, density, strength and structural integrity. These symptoms culminate in the elevated risk of bone fractures (Eriksen et al. 1990; Hernandez et al. 2006; Genant et al. 2007; Sornay-Rendu et al. 2007; Tsangari et al. 2007; Bigley et al. 2008). Conversely, it has been shown that long-term 17-β-E_2 administration leads to an increase of bone mass by increased activity of OBs and induced apoptosis of OCs (Liu and Howard 1991; Zecchi-Orlandini et al. 1999; Ramalho et al. 2002; Seeman and Delmas 2006). A variety of factors is involved in the underlying regulatory mechanisms. In OBs, 17-β-E_2 stimulates the synthesis and secretion of insulin-like growth factor (IGF)-1, and in turn inhibits cytokines involved in bone resorption. Also, osteoprotegrin (OPG), which is responsible for the functional inhibition of OCs, is activated by 17-β-E_2 (Ernst and Rodan 1991; Ishii et al. 1993;

Roodman 1996; Hofbauer et al. 1999). Receptor activator for nuclear factor kappa B ligand (RANKL) is expressed on the surface of OBs and stromal cells, leading to the differentiation and activation of OCs upon receptor-ligand interaction. In this context, OPG functions as a decoy receptor, binding to RANKL and thereby inhibiting the completion of the OC development (Simonet et al. 1997; Bucay et al. 1998). Both ERs can be detected in OBs and osteocytes *in situ* and in chondrocytes of the epiphyseal growth plate, whereas only few reports exist on the expression of ERs in OCs. The effects on OCs are thought to be rather indirect via regulatory molecules, e.g. OPG (Komm et al. 1988; Pensler et al. 1990; Hoyland et al. 1997; Onoe et al. 1997; Huang et al. 1998; Nilsson et al. 1999; Oreffo et al. 1999). To study the putative roles for either ERα or ERβ, knockout mice have been created. Both ERKO (ERα knockout) and BERKO (ERβ knockout) do not show any bone phenotype before puberty. However, in the adult mouse, both receptors are important for the maintenance of normal bone. ERKO mice show decreased longitudinal and radial limb growth and cortical osteopenia in both sexes. In contrast, BERKO mice show a mild phenotype: the females have increased limb length and increased bone mineral density, but do not show any signs of osteopenia (Vidal et al. 1999; Windahl et al. 1999; Windahl et al. 2000). The ERα and ERβ double-knockout mice (DERKO) display a similar phenotype to the ERKO mice, resulting in decreased longitudinal and radial skeletal growth associated with lower IGF-1 serum levels (Vidal et al. 2000). In conclusion, ERα is obviously the main mediator of the growth-promoting effects of 17-β-E_2 and the maintenance of the trabecular bone.

Several studies in the last five years have proved that OBs are able to regulate the endosteal HSCs (Calvi et al. 2003; Zhang et al. 2003; Sacchetti et al. 2007; Wilson et al. 2007). Currently, there are no data available regarding whether 17-β-E_2 can also play a role in the regulation of HSCs in their specialized microenvironment. In summary, although the roles of estrogens are well defined in the reproductive tract, sexual development and the formation and maintenance of bone, the role of estrogens in the HSC niche still has to be defined.

2.4.2 GH in bone metabolism

Although GH has been shown to have a wide range of indirect effects mediated via IGF-1, there is also evidence for a variety of direct effects (Denko and Bergenstal 1955; Murphy et al. 1956; Salmon and Daughaday 1957; Daughaday and Reeder 1966; Garland et al. 1972). Direct actions of GH on longitudinal bone growth in rats,

in particular on cartilage tissue (Isaksson et al. 1982), have been further substantiated by increased growth of the epiphyseal plate in the hindlimb of rats under GH treatment (Russell and Spencer 1985; Schlechter et al. 1986). These bipartite effects are termed 'dual effector theory'. For the effects on bone, it could be proven that GH acts directly on growth plate germinal zone cells, leading to increased proliferation. The growth plate germinal zones are clearly expanded in mice lacking IGF-1, due to elevated endogenous GH levels (Ohlsson et al. 1992; Hunziker et al. 1994; Wang et al. 1999). Whether IGF-2 can play a role in this system remains unclear. Despite the direct effects of GH on the bone, IGF-1 has essential roles in bone development and maintenance. The absence of IGF-1 leads to dwarfism in mice and short stature in humans. The possible mechanism is that IGF-1 regulates chondrocytes. However, in the absence of IGF-1, the zone of hypertrophic chondrocytes is enlarged (Liu et al. 1993; Powell-Braxton et al. 1993; Woods et al. 1996). Thereby, IGF-1 is an important regulator for chondrocytes, culminating in increased hypertrophic chondrocytes in the absence of IGF-1 (Wang et al. 1999).

IGF-1 and IGF-2 are part of a system which includes several components: six binding proteins (IGFBP-1 to -6) and the essential cell surface receptors, IGF-1 receptor, insulin receptor, plus the IGF-2 mannose-6-phosphate receptor (Nissley and Lopaczynski 1991; Jones and Clemmons 1995; LeRoith et al. 1995). Both receptors consist of α- and β-subunits; the α-subunit is extracellularly localized and the β-subunit spans throughout the membrane and is partially localized intracellularly (Steele-Perkins et al. 1988). The α-subunit mediates the ligand binding and forms the binding pocket. The intracellular part of the β-subunit carriers the tyrosine kinase, which acts on tyrosine residues upon receptor activation (Sasaki et al. 1985). Phosphorylation leads to the recruitment of various endogenous substrates, which can activate several signaling pathways including the PI3-kinase pathway and the MAP-kinase pathway (Sasaoka et al. 1994; Ricketts et al. 1996; D'Mello et al. 1997).

The anterior pituitary, more precisely the somatotroph cells, produces and stores GH, which is a cytokine peptide and mediates its effect via the GH receptor (GHR). The GHR is a ubiquitously expressed transmembrane receptor, which can be modified at post-transcriptional and post-translational levels. The most important modification results in the soluble GH binding protein, consisting of the extracellular ligand-binding domain and serving as stabilization factor for GH in plasma. The mechanisms for the production of GH binding protein vary among species from alternative splicing to proteolytic cleavage (Leung et al. 1987; Spencer et al. 1988; Baumbach et al. 1989; Baumann 1995b; Baumann 1995a; Barnard and Waters 1997; Ross 1999). The GHR

uses Jak/STAT signaling as its main signaling pathway. Dimerization of the receptor, upon ligand binding, results in the activation of the Jak2. Jak2 are tyrosine kinases able to cross-phosphorylate each other after activation by the GHR. The kinases phosphorylate then the mediators, STAT, which translocate to the nucleus and activate the target genes at special DNA sequences (γ-interferon-activation sites) (Leonard and O'Shea 1998; Davey et al. 1999; Takeda and Akira 2000). One important mediator in the Jak/STAT signaling pathway involved in hematopoiesis is STAT5. This has been shown by the creation of STAT5-knockout mice ($STAT5^{-/-}$), which die after birth due to hematopoietic failure (Cui et al. 2004).

In addition to the direct GH effects on bone, GH has been shown to increase the number of HSCs in transgenic mice (bGH transgenic), as well as in GH-treated wildtype mice. Furthermore, $CD34^{+}$ cell numbers in humans are elevated upon GH injection, suggesting a mobilization effect. This effect is mediated by upregulated SOCS1 and SOCS3, which inhibit the important CXCL12/CXCR4 axis between HSCs and their microenvironment (Dorshkind and Horseman 2000; Pello et al. 2006; van der Klaauw et al. 2008).

These data raise the question as to whether STAT5 in the GH/GHR signaling pathway is important for the regulation of the HSC niche? STAT5 could play a role, especially in the endosteal niche, due to the known effects of GH on OBs.

2.5 Aims of this study

17-β-E_2 and GH are osteogenic hormones. Both hormones influence OBs and are therefore an important part of the endosteal HSC niche. This study should clarify whether 17-β-E_2 and GH also affect the HSCs in their microenvironment.

To address the effects of 17-β-E_2, HSCs were investigated in the vascular and endosteal niche by FACS analysis. To estimate the ability to reconstitute lethally-irradiated mice, HSCs from 17-β-E_2-treated mice were isolated and analyzed in competitive repopulation assays. This study should also address whether 17-β-E_2 influences HSCs directly or rather the specialized microenvironment. Therefore, we tested the maintenance of wildtype HSCs in a 17-β-E_2-treated surrounding *in vitro* and *in vivo*. The molecular mechanisms that mediate the effects of 17-β-E_2 were investigated with different knockout models and by microarray analysis.

To investigate whether long-term GH treatment increases the numbers of HSCs from the vascular and endosteal niche, we again used FACS analysis. Western blot analysis was used to test which pathway mediates the effects of GH in OBs. Furthermore, a conditional knockout for STAT5 in OBs should clarify whether this molecule is essential for the mediation of GH-effects on HSCs.

3 *Results*

3.1 Effects of estrogens on the HSC niche

3.1.1 Long-term treatment of mice with 17-β-E_2 increases the bone mass but not the bone-adhered HSCs

Interactions between HSCs and special spindle-shaped OBs of the endosteum (Calvi et al. 2003; Zhang et al. 2003) have been demonstrated to be enhanced upon treatment with the osteogenic hormone parathyroid hormone. Estrogens increase bone mass and might therefore also affect the endosteal niche of HSCs, which should result in increased HSC numbers. To investigate if the ERα in OBs is the essential mediator of the effects of 17-β-E_2 on the bone mass, we analyzed $ER\alpha^{Runx2cre}$ mice under 17-β-E_2 treatment. The $ER\alpha^{Runx2cre}$ mice lack the ERα only in their OBs. This mouse was created using the Cre/loxP system by crossing $ER\alpha^{loxP}$ mice with mice expressing the Cre recombinase under the control of the OB-specific Runx2 promoter. Thereby the Cre recombinase, only active in OBs, is able to recognize the loxP sites, special short DNA sequences, flanking the ERα in the $ER\alpha^{loxP}$ mice. The offspring are deleted for the ERα only in OBs (Wintermantel et al. unpublished). These mice showed no increase in bone mass under the influence of 17-β-E_2 compared to $ER\alpha^{loxP}$ mice, which reacted to 17-β-E_2 treatment with a clear increase in bone mass similar to wildtype animals. These results are represented by Fig. 3.1 showing a van Kossa staining, indicating the calcium content of the bone (black areas), of the vertebrae and tibia of $ER\alpha^{loxP}$ and $ER\alpha^{Runx2cre}$ mice.

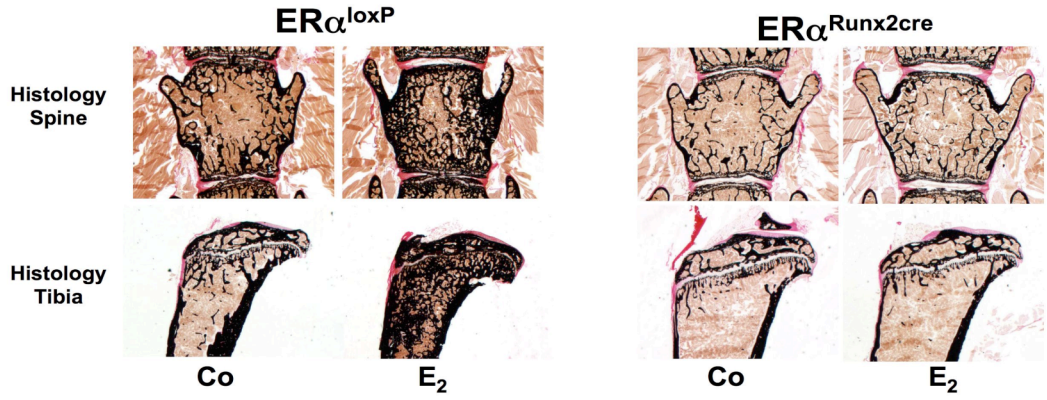


Figure 3.1: **17-β-E_2 treatment increases bone mass in wildtype but not in ERα^{Runx2cre} mice.** Van Kossa staining for the calcium content of bones (black areas) of vertebrae and tibia from ERα^{loxP} mice and ERα^{Runx2cre} mice (conditional ERα knockout in OBs) treated with (E_2) and without (Co) 17-β-E_2 .

These data clearly showed that the ERα in OBs is the essential molecular mediator of the effects of 17-β-E_2 on the bone mass. Furthermore, mainly the OBs were affected. The increase of the number of OBs under 17-β-E_2 treatment resembled that by parathyroid hormone. Therefore, we asked if 17-β-E_2 also influences the HSCs in the endosteal niche. To address the effects of 17-β-E_2 on HSCs, wildtype mice received in all *in vitro* experiments a slow-release pellet under the skin for 30 days releasing 17-β-E_2 (6 μg 17-β-E_2 per day per mouse). To isolate the hematopoietic cells of the endosteal niche, the upper arms and the legs were prepared and all muscles were removed. The BM was flushed with a medium-filled syringe. The harvested cell suspension represented the vascular niche. The empty bones were crushed and the bone pieces digested with a collagenase/dispase mix. The cells from the digestion represented the hematopoietic fraction of the endosteal niche.

Addressing first of all the progenitor cells in the endosteal niche, we analyzed by flow cytometry the fraction of undifferentiated lineage-negative (lin^-) cells in the endosteal niche of control and 17-β-E_2-treated mice. No differences in this cell population could be detected (Fig. 3.2).

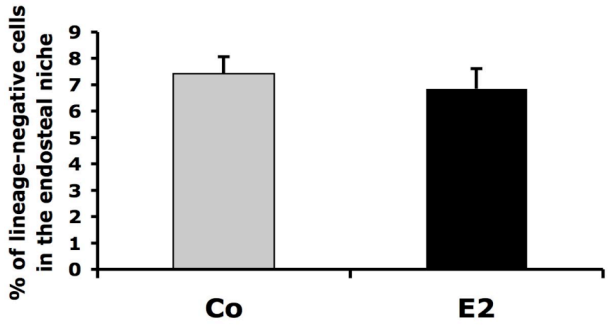


Figure 3.2: Undifferentiated, lineage$^-$ cells in the BM of the endosteal niche are not altered by 17-β-E_2 treatment. FACS analysis of hematopoietic cells from the endosteal niche for undifferentiated cells (B220$^-$, CD3$^-$, Gr-1$^-$, CD11b$^-$, Ter-119$^-$), (n=5).

To determine the influence of 17-β-E_2 on the frequency of HSCs, we determined the fraction of cells expressing the surface marker CD150 but being negative for CD48 (Kiel et al. 2005) (Fig. 3.3). The percentage of CD150$^+$/CD48$^-$ cells was not altered in 17-β-E_2-treated and control animals.

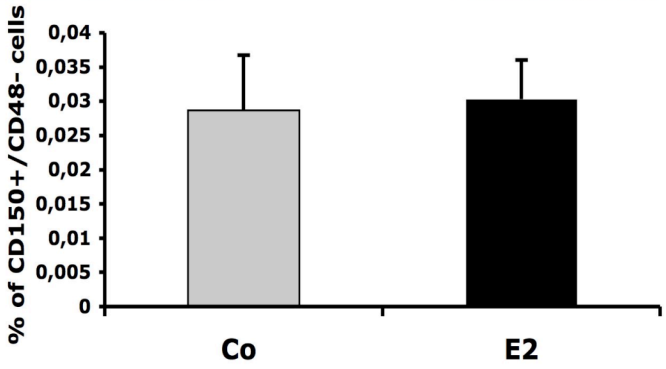


Figure 3.3: The percentage of CD150$^+$/CD48$^-$ cells, representing HSCs, in the endosteal niche of the BM is not altered by 17-β-E_2 treatment. FACS analysis of HSCs from the endosteal niche, analyzed with SLAM markers (n=5).

To confirm the data obtained by flow cytometry, we determined the HSC fraction functionally *in vitro* by a co-culture assay, the cobblestone area-forming cell (CAFC) assay. The assay was performed using a BM-derived feeder cell line, the fat bone marrow derived 1 (FBMD1) cells. BM was seeded in limited dilutions on confluent cell layers of the stromal cell line FBMD1. After five weeks of co-culture, the occurrence of 'cobblestone colonies' strongly correlated with the fraction of most primitive HSCs in the tested BM (Ploemacher 1994, "Hematopoietic Stem Cell Protocols").

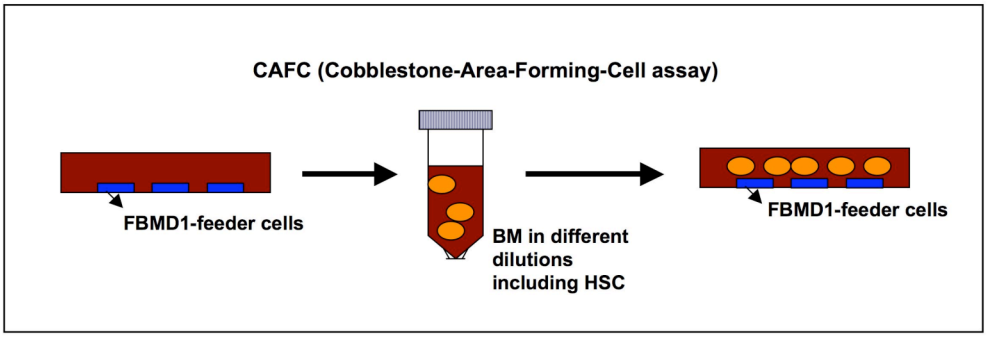


Figure 3.4: Overview of the CAFC assay. FBMD1 cells were used as confluent feeders. The BM was seeded in six dilutions (the following dilution was three-fold apart), and cobblestone colonies were counted after five weeks of culture.

In accordance with the flow cytometry analysis, the frequency of functional HSCs in the endosteal niche was not changed under the influence of 17-β-E_2 (Fig. 3.5).

Thus, although bone mass was dramatically changed after estrogen treatment, the frequency of HSCs was not influenced.

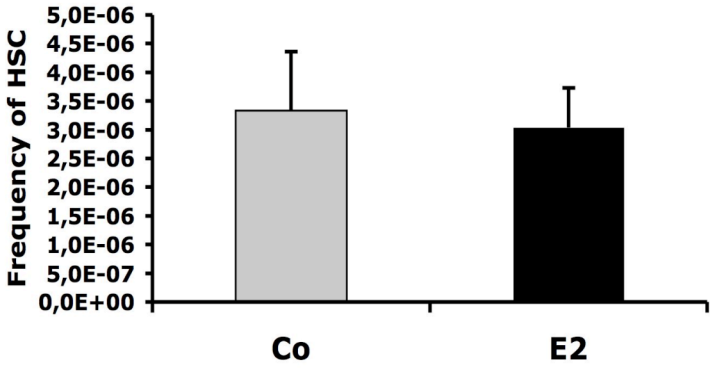
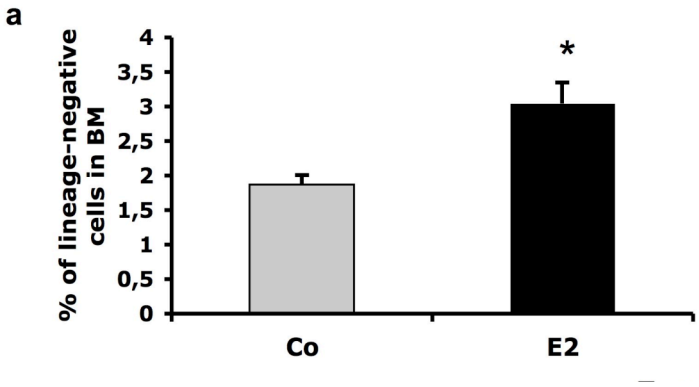
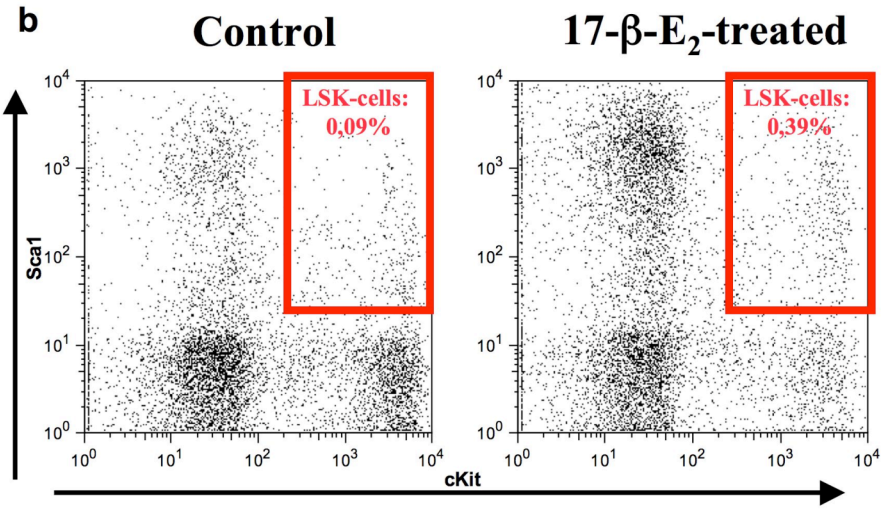


Figure 3.5: The frequency of HSCs of the BM of the endosteal niche is not altered by 17-β-E_2 treatment. CAFC assay of the endosteal hematopoietic cells after 17-β-E_2 treatment of mice (n=3).

3.1.2 Long-term treatment of mice with 17-β-E_2 leads to an increase in vascular HSCs

Since the endosteal niche is not the only location for HSCs in the BM, we also tested the effects of 17-β-E_2 on HSC frequency located at the vascular niche (Kiel and Morrison 2006). The hematopoietic cells of the vascular niche were analyzed by flushing the prepared bones with a medium-filled syringe. First, we analyzed, via FACS, the population of progenitor cells in the flushed BM. This subpopulation was clearly increased by 17-β-E_2 treatment. The frequency of lin^-, $Sca1^+$, $cKit^+$ (LSK) (Spangrude et al. 1988; Morrison and Weissman 1994; Osawa et al. 1996) cells, a fraction in which HSCs are enriched, in the vascular niche of control and 17-β-E_2-treated mice was also highly increased in 17-β-E_2-treated mice (Fig. 3.6b and c). Also, the absolute number of LSK cells was increased (Fig. 3.6d) upon 17-β-E_2 treatment.





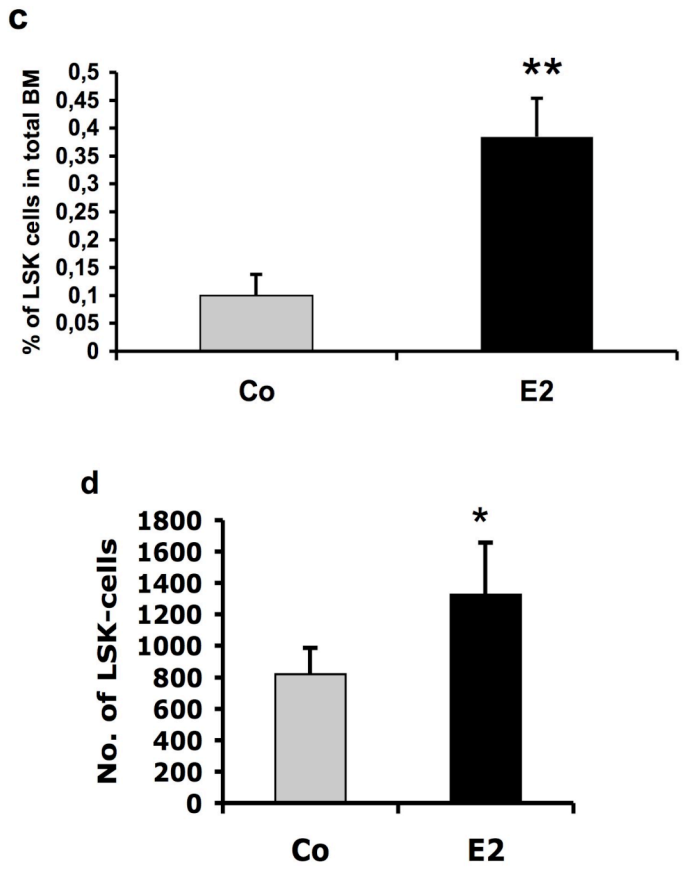


Figure 3.6: The percentage of the Sca1$^+$ and cKit$^+$ fraction of lin$^-$ BM cells is increased in the vascular niche of 17-β-E_2-treated mice. a) Percentage of lin$^-$ cells in the vascular niche of the BM of control and 17-β-E_2-treated mice analyzed by flow cytometry (B220$^-$, CD3$^-$, Gr-1$^-$, CD11b$^-$, Ter-119$^-$), (n=5, $p<0.05$). **b)** Representative dot plots of LSK cells in the vascular niche of control and 17-β-E_2-treated mice analyzed by flow cytometry. **c)** Summarized data of LSK cells in the vascular niche of control and 17-β-E_2-treated mice analyzed by flow cytometry (n=5, $p<0.01$). **d)** Absolute numbers of LSK cells per hindlimb in the vascular niche from control and 17-β-E_2-treated mice analyzed by flow cytometry (n=5, $p<0.05$).

Taken together, long-term 17-β-E_2 treatment increased the number of LSK cells in the vascular niche of the BM.

To confirm an increase of HSCs indicated by the elevated LSK cell numbers, we determined the fraction of CD150$^+$/CD48$^-$ cells (Fig. 3.7) as another set of surface markers. Like LSK cells, the CD150$^+$/CD48$^-$ subpopulation was increased under long-term 17-β-E_2 treatment.

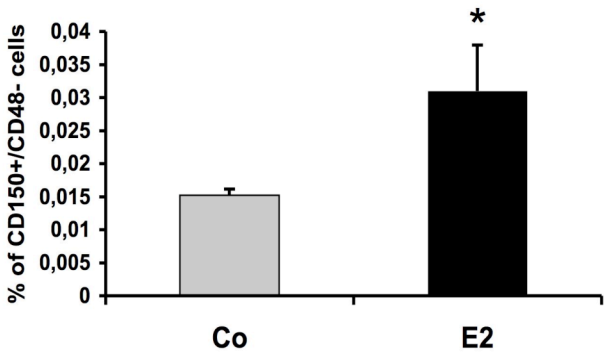


Figure 3.7: $CD150^+/CD48^-$ cells, also representing HSCs, of the BM from the vascular niche are increased by 17-β-E_2 treatment. Flow cytometry analysis of BM of the vascular niche for SLAM markers (n=5, $p<0.05$).

Next, we wondered if 17-β-E_2 treatment also changes the numbers of cells that are able to form cobblestone areas in long-term co-cultures, indicative of primitive stemness of hematopoietic cells. As described above, we seeded BM cells of the vascular niche from control and 17-β-E_2-treated mice in limited dilutions on top of FBMD1 cells, and analyzed colony formation after five weeks. Fig. 3.8 shows the calculated frequencies of HSCs in the BM of control and 17-β-E_2-treated mice. 17-β-E_2 treatment led to an increased frequency of HSCs in the BM based on the capacity for cobblestone area formation. Therefore, we concluded that 17-β-E_2 increased HSC numbers, as shown by FACS analysis and the capacity to form cobblestone colonies.

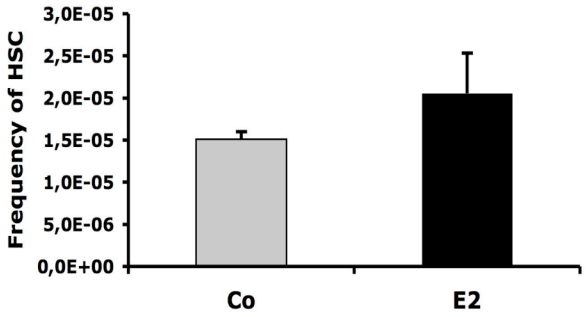


Figure 3.8: Frequency of HSCs from the vascular niche is increased by 17-β-E_2 treatment. The frequency of HSCs from control and 17-β-E_2-treated mice was determined by the CAFC assay in long-term culture conditions for five weeks (n=3).

3.1.3 17-β-E_2 increases the multipotent long-term repopulating HSCs

One characteristic of HSCs is their quiescent state, mainly retaining them in G0/G1 phase of the cell cycle, which is in contrast to the rapidly amplifying progenitors. Since we observed an increase of the number of LSK cells and $CD150^+/CD48^-$ cells, cell populations enriched for HSCs, we would expect an increase of slow-cycling cells in the BM of 17-β-E_2-treated mice. Therefore, we decided to perform a label-retention assay (Zhang et al. 2003; Arai et al. 2004). Wildtype mice were treated with 17-β-E_2 for 30 days as previously described. Eighteen days after the beginning of estradiol treatment, we started to supply bromodeoxyuridine (BrdU) in the drinking water for 10 days. In a latent phase without BrdU application, the rapid amplifying cells lost the BrdU label of their DNA. Therefore, we analyzed the BM of the vascular niche for label-retaining cells by FACS analysis 70 days later (Fig. 3.9). In agreement with the previous data, slow-amplifying cells were also increased in their number by 17-β-E_2 treatment.

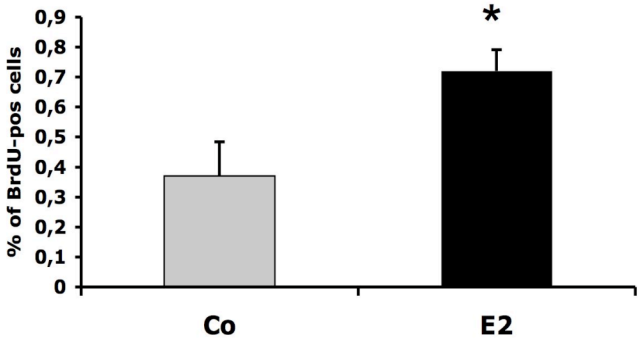
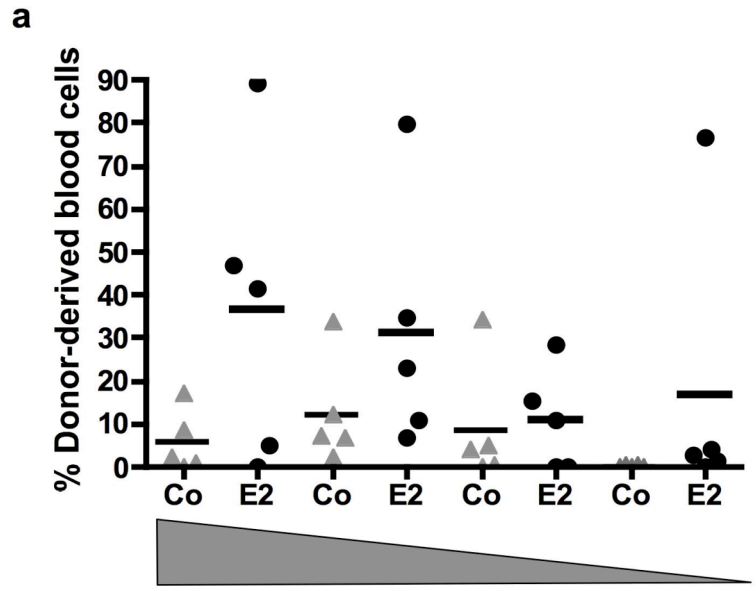


Figure 3.9: The percentage of $BrdU^+$ cells in the BM of the vascular niche is increased under 17-β-E_2 treatment. Flow cytometry analysis for $BrdU^+$ cells in the vascular niche of 17-β-E_2-treated mice after BrdU application (n=5, $p<0.01$).

So far we could show that 17-β-E_2 treatment elevated the HSC fraction, based on surface marker expression (i.e. LSK cells and $CD150^+/CD48^-$ cells), their capacity for cobblestone area formation and the determination of label retention in slow-cycling cells. However, the gold standard for the characterization of the frequencies of HSCs in the BM is the determination of their capacity for long-term repopulation *in vivo*. We therefore performed a limiting dilution analysis (LDA) of BM cells competitively transplanted into lethally-irradiated mice (competitive repopulation assay) (Szilvassy et al. 1990). We transplanted 5.4×10^5, 1.8×10^5, 6.0×10^4 and 2.0×10^4 BM cells derived from control and 17-β-E_2-treated mice into untreated lethally-irradiated CD45.1 mice. After 16 weeks, blood cells of donor-derived origin (CD45.2 staining) were analyzed by flow cytometry (Fig. 3.10a). The mice receiving BM of the vascular niche from 17-β-E_2-

treated mice displayed more donor-derived HSCs than the mice transplanted with BM from control mice (Fig. 3.10b). The regression analysis using the maximal likelihood from the transplantation efficiency values of the different dilutions led to a calculation of the frequency of long-term repopulating cells in the donor BM. This analysis also revealed a strong increase of long-term repopulating HSCs in BM of 17-β-E_2-treated mice.



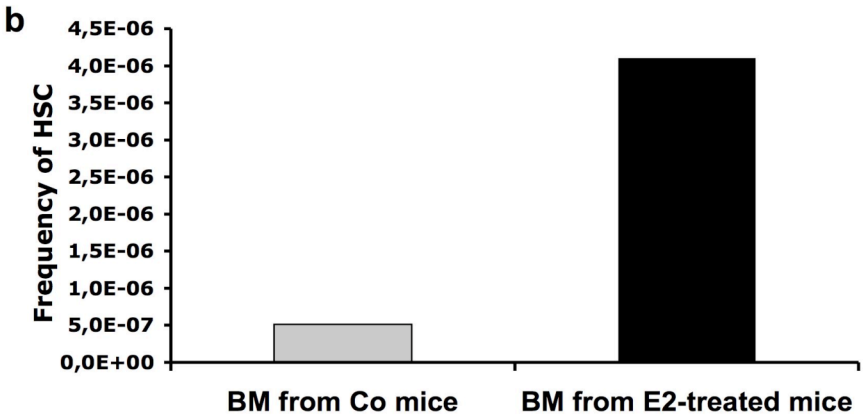


Figure 3.10: The frequency of donor-derived HSCs in CD45.1 mice four months post-transplantation is increased after reconstitution with BM from 17-β-E_2-treated mice. a) Reconstitution analysis of lethally-irradiated mice after transplantation with BM from control or 17-β-E_2-treated mice. Controls and E_2 represent the four dilutions of transplanted BM cells (5.4×10^5, 1.8×10^5, 6.0×10^4, 2.0×10^4 BM cells, cell numbers are decreasing from left to right) in relation to the percentage of donor-derived blood cells in the recipient. Each data point represents one mouse. **b)** Calculated frequency of donor-derived HSCs in CD45.1 mice four months post-transplantation with BM from control or 17-β-E_2-treated mice (n=20).

In summary, we could demonstrate that 17-β-E_2 treatment increased the frequency of HSCs by several lines of evidence: i) on the basis of surface marker expression (LSK and $CD150^+/CD48^-$ cells); ii) the presence of label-retention cells in the BM; iii) the capacity for cobblestone area formation and iv) by the increase of the fraction of cells able to repopulate long-term *in vivo*.

3.1.4 The role of ERα is dispensable for the 17-β-E_2-induced increase of HSC numbers

Having established that long-term treatment of mice with 17-β-E_2 increases the immature undifferentiated fraction of HSCs in the BM, we asked whether this effect is mediated by the estrogen receptors.

To analyze whether the ERα contributes to the effect of 17-β-E_2 on HSCs, we treated ERα-knockout mice with 17-β-E_2 and analyzed them for the frequency of functional long-term repopulating stem cells in the BM. For this purpose, we used the competitive repopulation assay again, where lethally-irradiated CD45.1 mice were transplanted with BM from 17-β-E_2-treated wildtype or ERα-knockout mice (Fig. 3.11).

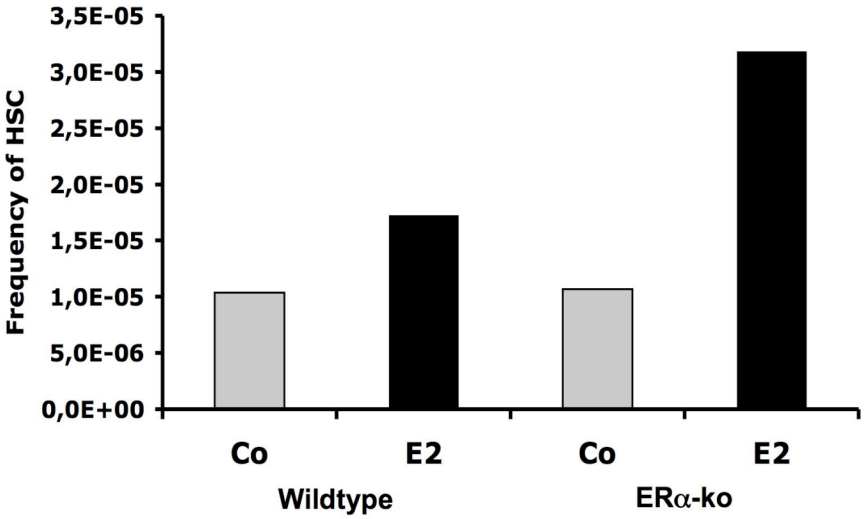


Figure 3.11: The frequency of donor-derived HSCs in CD45.1 mice four months post-transplantation is increased after reconstitution with BM from 17-β-E_2-treated wildtype and ERα-knockout mice. Competitive repopulation analysis for the frequency of HSCs in the vascular BM from 17-β-E_2-treated wildtype or ERα-knockout mice (n=20).

The percentage of donor-derived blood cells was analyzed four months post-transplantation. As expected, lethally-irradiated mice were better reconstituted after transplantation with BM from 17-β-E_2-treated wildtype animals compared to mice receiving BM from control wildtype animals (Fig. 3.11). Therefore, they showed a higher frequency of donor-derived HSCs. However, the effect of 17-β-E_2 on HSC number was still maintained in ERα-knockout mice, since lethally-irradiated mice reconstituted with BM from 17-β-E_2-treated ERα-knockout mice were more efficiently reconstituted compared to a reconstitution with BM from sham-operated ERα-knockout mice.

In summary, also in the absence of ERα, 17-β-E_2 treatment led to an increase of the number of HSCs. This could also be confirmed by the measurement of overall BM cells under 17-β-E_2 treatment (Fig. 3.12). The increase of bone mass by 17-β-E_2 treatment led to decreased space in the BM cavity, resulting in fewer hematopoietic cells in the vascular niche. Hence, one could suggest a shift in BM populations if the hematopoietic cells are reduced but HSC numbers are stable. However, the data from the ERα-knockout mice refuted this hypothesis. HSCs were increased in the ERα-knockout mice, but the cell numbers from the vascular niche were not changed by 17-β-E_2 treatment. We concluded that the increase of HSCs by 17-β-E_2 treatment was not due to a shift of hematopoietic populations in the BM after the strong increase of bone mass.

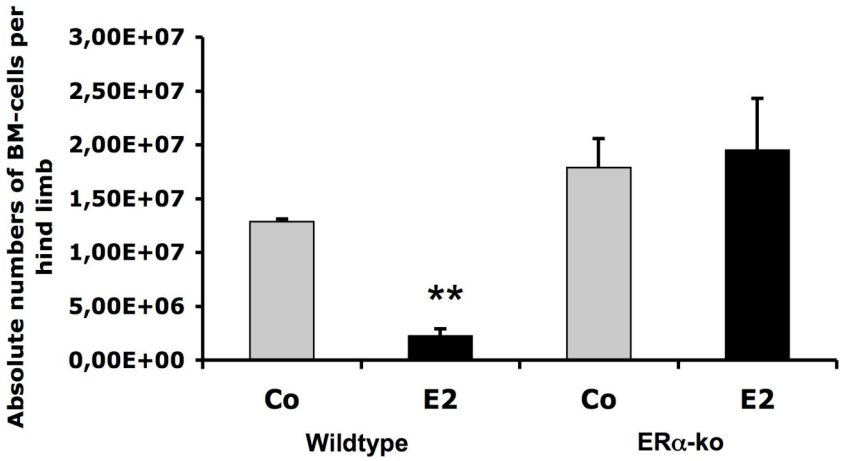


Figure 3.12: The absolute numbers of cells per hindlimb in the vascular HSC niche are not influenced by 17-β-E_2 in ERα-knockout mice. Absolute cell numbers per hindlimb from the vascular BM niche were measured with the Casy Cell Counter (n=5, $p<0.01$).

3.1.5 The role of ERβ is dispensable for the 17-β-E_2-induced increase of HSC numbers

To answer the question whether the ERβ could be the possible mediator for the effect of 17-β-E_2 on HSC numbers, we tested the numbers of HSCs in ERβ-knockout mice with long-term 17-β-E_2 treatment. Fig. 3.13 shows the absolute percentage of LSK cells in the BM of ERβ-knockout mice. In the absence of ERβ, LSK cells were increased after four weeks of 17-β-E_2 treatment. The repetition in wildtype mice (shown in Fig. 3.13) could thereby confirm the previously obtained results.

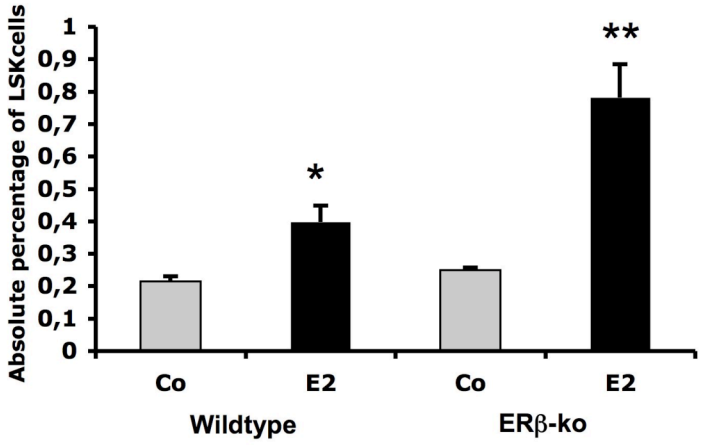


Figure 3.13: The percentages of lin$^-$/Sca1$^+$/cKit$^+$ cells in the BM of the vascular niche are increased with 17-β-E_2 treatment in wildtype and ERβ-knockout mice. LSK cells in 17-β-E_2-treated wildtype and ERβ-knockout mice were measured by flow cytometry (n=5, *p<0.05, **p<0.01).

Furthermore, we investigated for the ERβ-knockout mice the increase of bone mass, which correlated with a decrease of hematopoietic cells in the vascular niche due to limited space. ERβ-knockout mice behaved like wildtype mice under 17-β-E_2 treatment, as measured by counting the numbers of cells in the flushed BM (Fig. 3.14). Although wildtype and ERβ-knockout mice showed constricted space in the BM cavity, we are confident, due to the data of the ERα-knockout mice, that this effect did not lead to a shift of BM populations and thereby to an apparent increase of HSCs only.

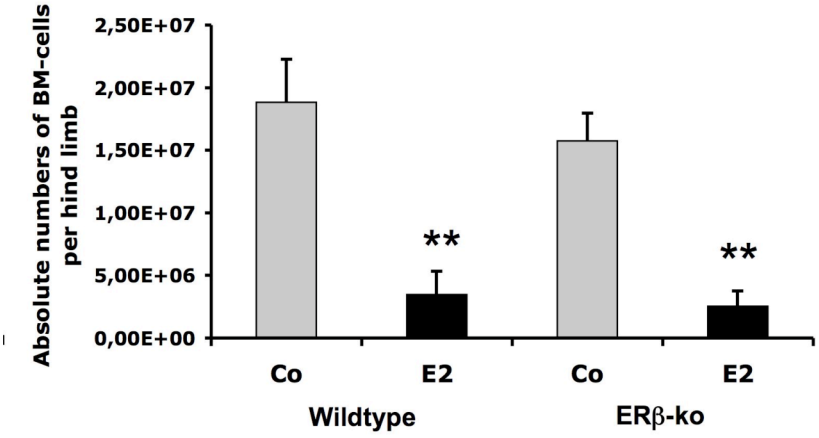


Figure 3.14: The absolute numbers of cells per hindlimb in the vascular HSC niche are decreased by application of 17-β-E_2 in wildtype and ERβ-knockout mice. Absolute numbers of hematopoietic cells from the vascular BM niche were counted by the Casy Cell Counter (n=5, p<0.01).

3.1.6 17-β-E_2 treatment affects the niche cells and not the HSCs directly

The effects of estradiol on the HSC numbers in the vascular niche were not affected by the absence of ERα nor ERβ. Therefore, the use of the single ER-knockout mice was counterproductive in determining the effects of 17-β-E_2 in the HSC niche.

To circumvent this drawback, we simulated the HSC niche *in vitro* by modifying the CAFC assay. We used the FBMD1-feeder cell line as a model for the surrounding cells of the HSC niche, and pre-treated these feeders with 17-β-E_2 for two weeks. Afterwards, we removed the 17-β-E_2 and seeded wildtype BM cells onto the pre-treated feeders. Five weeks later we analyzed the assay for cobblestone-forming areas (Fig. 3.15).

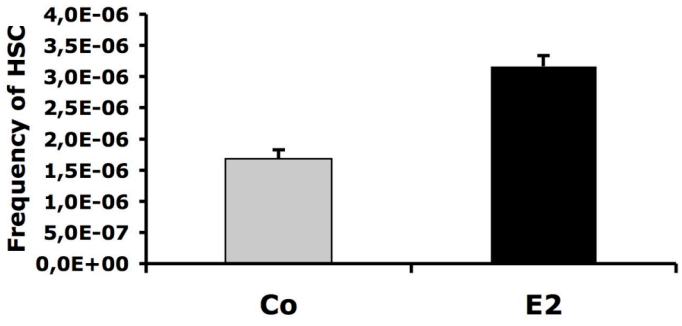


Figure 3.15: The frequency of HSCs in wildtype BM after pre-treatment of FBMD1 feeder cells with 10^{-6}M 17-β-E_2 is increased. Frequency of HSCs in wildtype BM under support of 17-β-E_2-treated FBMD1 cells measured by the CAFC assay (n=3, $p<0.05$).

17-β-E_2-pre-treated feeders were more efficient in the support of undifferentiated HSC numbers as reflected by the calculation of the frequency of HSCs.

To answer the question whether 17-β-E_2 treatment improves the HSC niche and thereby the environment for HSCs *in vivo*, we performed a 'stem cell homing assay'. Therefore, we treated mice with 17-β-E_2 for four weeks, sublethally irradiated these mice with 8 Gy, and four days post-irradiation we injected 1×10^6 carboxyfluorescein succinimidyl ester (CFSE)-labeled lin^- BM cells from untreated mice into the tail vein. Twelve hours after injection we analyzed the mice for $CFSE^+$ cells in the vascular niche of the BM. Fig. 3.16 illustrates one representative histogram plot for $CFSE^+$ cells in the flushed BM of either 17-β-E_2-treated (blue) or control (red) mice. More labeled cells were found in 17-β-E_2-treated animals. Four of these experiments were evaluated and compiled in Fig. 3.16b.

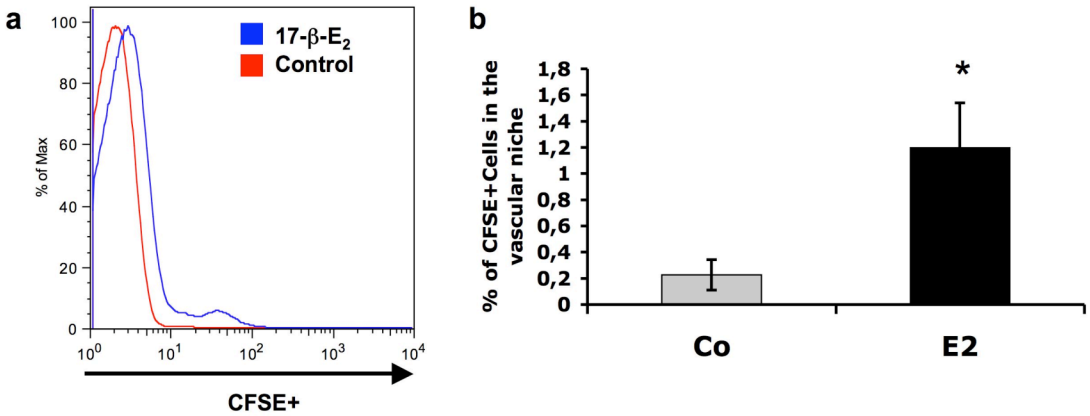


Figure 3.16: $CFSE^+$ cells in the vascular HSC niche are increased upon pre-treatment of recipients with 17-β-E_2. a) Representative histogram plot of $CFSE^+$ cells in the vascular HSC niche of control (red line) and 17-β-E_2-treated (blue line) mice. **b)** Summarized analysis of $CFSE^+$ cells in the vascular HSC niche 12 hours after injection in control and 17-β-E_2-treated mice, analyzed by flow cytometry (n=5, $p<0.05$).

The content of $CFSE^+$ cells in the BM of 17-β-E_2-treated mice was greatly increased, indicating that long-term effects of estrogens affect the environment of HSCs, enabling efficient homing of these cells to the BM cavity. Thus, estradiol affected at least in part the environment of HSCs, such that it enhanced HSC abundance in the vascular niche of the BM (Fig. 3.16).

3.1.7 17-β-E_2 leads to lower numbers of HSCs in the peripheral blood

We have observed higher numbers of progenitors and HSCs in the BM of the vascular niche, determined by investigation of surface markers, the potential to form cobblestone areas, the label retention in slow-cycling BM cells and the reconstitution of lethally-irradiated mice. Furthermore, *in vivo* and *in vitro* data indicated that the microenvironmental cells of the vascular niche are influenced by 17-β-E_2. To investigate a possible mobilization effect in the vascular niche by 17-β-E_2, we tested via surface markers the numbers of progenitors (lin^- cells) and HSCs (LSK cells) in the peripheral blood.

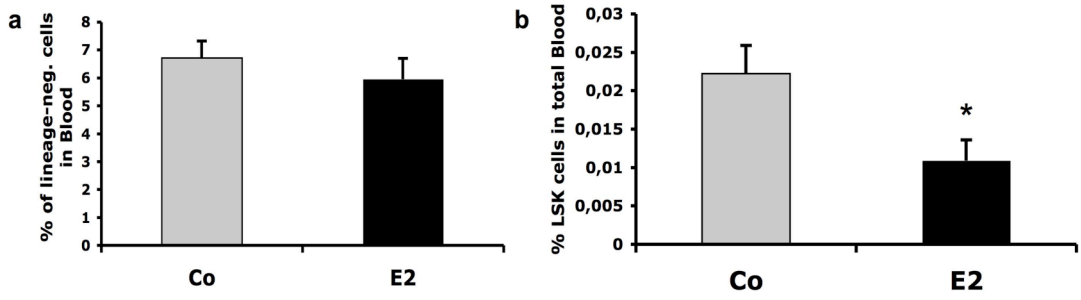


Figure 3.17: Percentages of lin^- and LSK cells in the blood. a) Percentage of lin^- cells ($B220^-$, $CD3^-$, $Gr\text{-}1^-$, $CD11b^-$, $Ter\text{-}119^-$) in the blood of control and 17-β-E_2-treated mice analyzed by flow cytometry (n=5). **b)** Percentage of LSK cells, representing the HSC fraction, in the blood of control and 17-β-E_2-treated mice analyzed by flow cytometry (n=5, $p<0.05$).

The FACS analysis revealed that lin^- cells were not significantly altered after 17-β-E_2 treatment. Surprisingly, the numbers of LSK cells in the peripheral blood were clearly decreased (Fig. 3.17). These results suggested an enhanced retainment of HSCs in the BM mediated by the surrounding niche cells.

3.1.8 17-β-E_2 regulates the mRNA levels of different adhesion molecules in HSC-supporting FBMD1 cells

Next we asked which 17-β-E_2-regulated genes expressed in cells of the niche might be involved in the HSC retainment.

Since we demonstrated that treatment of FBMD1 cells with 17-β-E_2 led to a more efficient support of HSCs, reflected by a higher number of cobblestone areas, we decided to analyze in these cells the alteration of gene expression due to 17-β-E_2 treatment. Total RNA of control and 10 day-17-β-E_2-treated FBMD1 cells were isolated and reverse transcribed to cDNA with an inserted oligo-dT-T7 primer. Via the T7 primer, the cDNA could then be transcribed to labeled cRNA, then hybridized on an Affymetrix 'A430' microarray chip to determine genome-wide mRNA expression. Bioinformatical processing and statistical analysis of the raw data using Affymetrix software led to the identification of upregulated and downregulated mRNAs in 17-β-E_2-treated FBMD1 cells (Table 3.1) Interestingly, several candidate genes were found which could be involved in the interaction of HSCs and their surrounding.

Symbol	Gene	Regulation	Function of the gene
CD34	CD34 antigen	+3.6	Single-pass transmembrane sialomucin protein, expression on early hematopoietic and vascular-associated tissue, Humans: HSC express CD34, Mice: progenitors and differentiated cells express CD34
Spon1	F-Spondin 1	+3.4	Highly expressed in rat neural tube, promotes neural cell adhesion, growth and guidance of axons
Dcn	Decorin	+3.2	ClassI-type small leucine-rich proteoglycan, non-collagen component of ECM, possibly involved in modulation of bioactivities of growth factors, cytokines (TGFß, TNFα, PDGF), expressed in various tissues including bones
Gsn	Gelsolin	+3.0	Component of ECM, proteoglycan, involved in cell migration and attachment, important in MSC-niche as ECM component, involved in cytoskeletal organization - regulated by integrin, involved in chondrocyte development
Prdx4	Peroxiredoxin 4	-2.7	Involved in removal of H_2O_2 in thyroid cells, abundantly expressed in many tissues, cytoplasmically distributed,reduction of H_2O_2-induced apoptosis
Epha5	Ephrin Receptor A5	-2.6	Largest subfamily of RTKs, transmembranous, involved in guidance and positioning of cells, modulation of cell morphology, involved in cancer

Table 3.1: Selected regulated genes from the microarray of 17-β-E_2-treated FBMD1 cells. Genes were selected for their fold-regulation and function, which could be conceivable in the interaction of HSCs and their special microenvironment.

17-β-E_2 induced expression of two of these six genes, CD34 and F-Spondin 1, as confirmed by real-time PCR analysis of cDNA from control and 17-β-E_2-treated FBMD1 cells (Fig. 3.18).

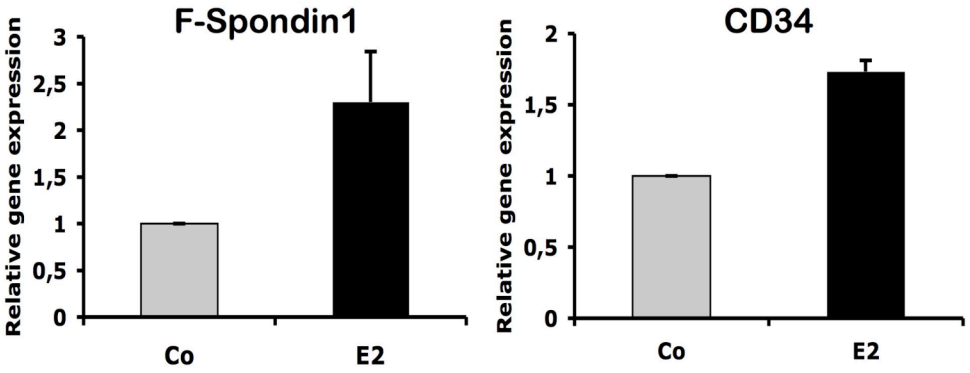


Figure 3.18: CD34 and F-Spondin 1 transcription levels in FBMD1 cells under 17-β-E_2 treatment. Relative gene expression levels of F-Spondin 1 and CD34 under 17-β-E_2 treatment determined by real-time PCR (n=3).

Both genes are involved in cell-cell adhesion, and F-Spondin 1 is also involved in the adhesion of cells to the extracellular matrix. CD34 is known as a HSC marker, preferably in humans, but it is also expressed on murine mesenchymal stem cells and their progeny (Copland et al. 2008). In conclusion, the upregulation of F-Spondin 1 and CD34 correlated with the increase of HSCs and also the efficient homing of HSCs under 17-β-E_2 treatment.

The higher expression of adhesion molecule F-Spondin 1 and CD34, involved in cell adhesion, would also implicate an increased retention of HSCs in the BM and a decreased release of HSCs into the blood. To test this hypothesis, we determined the presence of progenitor cells (lin^- cells) in the blood and LSK cells into the blood after 17-β-E_2 treatment. As shown in Fig. 3.17, lin^- cells were not changed, whereas HSCs, measured with LSK markers, were decreased in the blood. These results may indicate a stronger retention of HSCs in their niches provoked by 17-β-E_2 treatment, which is in agreement with efficient homing of HSCs of estrogen-treated mice and an increased expression of adhesion molecules by estradiol in HSC-supporting FBMD1 cells.

Taken together, our data clearly reflect an increase of progenitor cells and HSCs by a long-term treatment of 17-β-E_2 in the vascular niche. This effect is independent of the increase in bone mass, also resulting from 17-β-E_2 treatment. Beyond that, we proved that the higher numbers of HSCs are competent in reconstituting mice, and these cells perform even better in repopulating mice as analyzed by competitive reconstitution analysis. 17-β-E_2 increases the expression of distinct adhesion molecules, correlating with the increased HSCs. This upregulation of the adhesion molecules in combination

with the decreased numbers of HSCs in the peripheral blood suggests a stronger retention of HSCs in the vascular niche.

3.2 GH signaling in OBs increases HSC numbers and is modulated by STAT5

3.2.1 GH increases the number of HSCs in the vascular and endosteal niche of wildtype mice

GH influences bone mineral density via the OBs (de Boer et al. 1995). Furthermore, GH is a hematopoietic growth and differentiation factor, and it has been reported that mice overexpressing bovine transgenic GH and wildtype mice treated with human recombinant GH have larger numbers of LSK cells (Dorshkind and Horseman 2000; Carlo-Stella et al. 2004b; Pello et al. 2006). This effect is thought to be caused by increased HSC mobilization in the BM (Carlo-Stella et al. 2004b). To date, the underlying mechanism remains unsolved. We wondered if GH signaling in OBs contributes to an increase of HSC numbers by GH. One signal transduction pathway of GH action is the Jak/STAT signaling via STAT5a and STAT5b (Carter-Su and Smit 1998). Our aim was to investigate whether STAT5a/b in OBs contributes to the mediation of the GH effect on HSCs.

First, we tested the effects of GH on HSC numbers in wildtype mice. Therefore, we treated mice according to established protocols of the literature for five weeks by a daily injection of 55 μg recombinant human GH. We isolated the BM cells of the endosteal and vascular niche, and determined the fraction of $CD150^+/CD48^-$ cells by flow cytometry (Fig. 3.19).

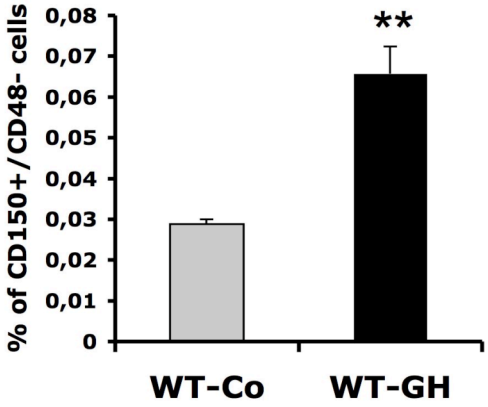


Figure 3.19: The percentage of CD150^{+}/CD48^{-} cells, representing HSCs, in the vascular niche is increased in wildtype mice after GH treatment. HSC numbers were determined by the analysis of surface markers (SLAM markers) with flow cytometry (n=5, p<0.01).

As expected, we confirmed that HSCs surrounded by stromal cells in the vascular niche are increased in number in wildtype mice under GH treatment. In addition, we showed for the first time that bone-adhered HSCs in the endosteal niche were increased in number by GH in wildtype animals (Fig. 3.20).

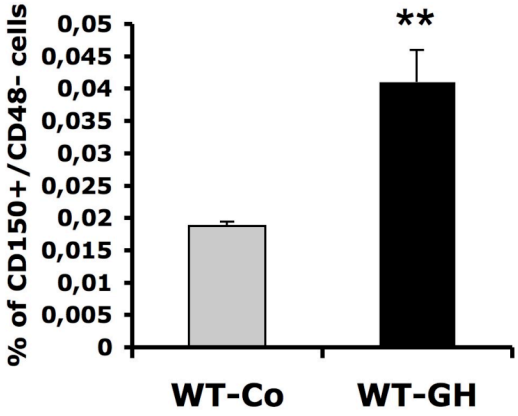


Figure 3.20: The percentage of CD150^{+}/CD48^{-} cells, representing HSCs, in the endosteal niche is increased in GH-treated wildtype mice. HSC numbers in the endosteal niche were investigated for surface markers (SLAM markers) by flow cytometry (n=5, p<0.01).

Therefore, we concluded that GH increased the number of HSCs in the vascular and endosteal niche.

3.2.2 STAT5 plays an important role in OBs and their interaction with HSCs

To address if STAT5 in the endosteal niche plays a role in GH effects on HSC numbers, we generated OB-specific STAT5-knockout mice. We used STAT5flox mice, where STAT5a and STAT5b genes are flanked by loxP sites (Cui et al. 2004). These mice were crossed with a transgenic mouse line carrying an inserted Cre recombinase under the control of the promoter Runx2 (Cbfa1), a transcription factor specifically expressed in OBs (Ducy et al. 1997). Genomic analysis of the offspring of these mice showed an efficient deletion of STAT5a and STAT5b in the bone and isolated OBs (data not shown). Therefore, these $STAT5^{OB}$ mice were suitable for the investigation of the contribution of STAT5 to GH signaling in OBs. We analyzed the $STAT5^{OB}$ mice, as shown in Fig. 3.21, and observed an increase in HSC numbers under GH treatment in the vascular niche, since this part of the niche is not affected in these knockout mice.

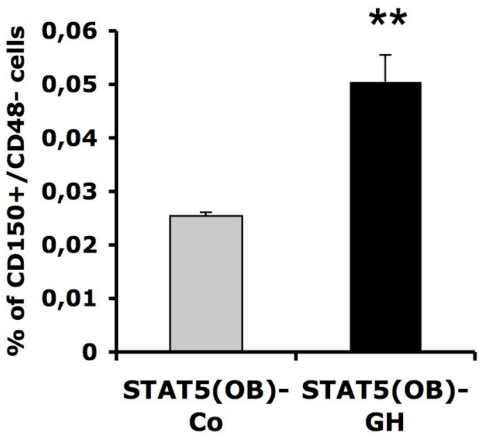


Figure 3.21: The percentage of $CD150^{+}/CD48^{-}$ cells, representing HSCs, in the vascular niche is increased in GH-treated $STAT5^{OB}$ mice. HSC numbers in the vascular niche were determined for surface markers (SLAM markers) by FACS analysis (n=5, $p<0.01$).

The analysis of the endosteal HSC niche revealed unexpected results. There were more bone-adhered HSCs in $STAT5^{OB}$ mice on a basal level compared to wildtype mice. Additionally, $STAT5^{OB}$ mice showed an even stronger increase (about four-fold in $STAT5^{OB}$ and two-fold in wildtype mice) of HSC numbers after GH administration in comparison to wildtype mice.

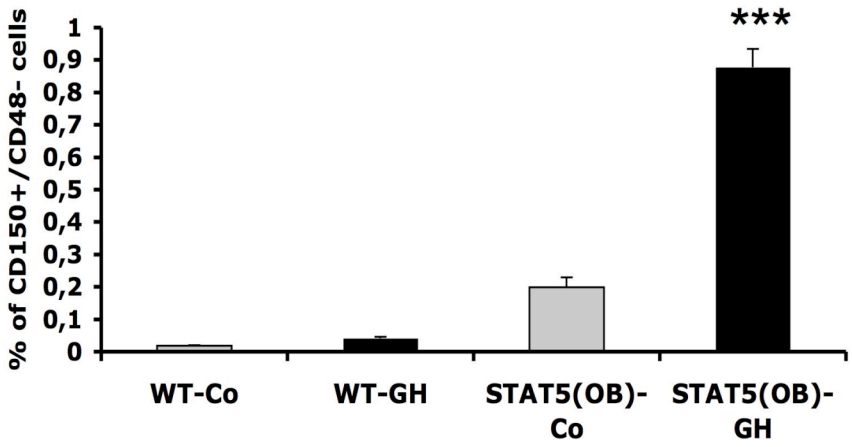


Figure 3.22: The percentage of CD150^{+}/CD48^{-} cells, representing HSCs, in the endosteal niche is increased in GH-treated wildtype and STAT5^{OB} mice. HSC numbers in the endosteal niche were determined for surface markers (SLAM markers) by flow cytometry (n=5, p<0.001).

We expected that removal of STAT5 in OBs would abrogate the mediation of GH signaling on bone-adhered HSCs. In contrast, we found a striking enhancement of this effect. Thus, the lack of STAT5 in OBs led to an increase in the number of HSCs in the endosteal HSC niche.

3.2.3 STAT5-knockout OBs increase the capacity of HSCs to form cobblestone colonies

We showed a clear increase of HSC numbers in the endosteal niche in wildtype and an even more striking increase in STAT5^{OB} mice under GH treatment (Fig. 3.22). In addition, we observed a basal enhancement of HSC numbers in the endosteal niche of STAT5^{OB} mice in the absence of GH treatment.

To test whether this effect is also mediated *in vitro*, we performed a CAFC assay using STAT5-deficient primary OBs as feeder cells, and determined the frequency of HSCs and thereby the support of HSCs.

Primary OBs from STAT5-knockout embryos at stage 18.5 were isolated and seeded as feeder cells in 96-well plates, and BM cells of untreated wildtype mice were seeded on top. After five weeks, cobblestone areas were counted and the frequency of HSC numbers calculated. STAT5-knockout OBs exhibited a strikingly higher frequency of long-term primitive HSCs than STAT5 heterozygous and wildtype OBs (Fig. 3.23). Thus, also *in vitro*, the deficiency of STAT5 in OBs augmented the maintenance of HSC numbers.

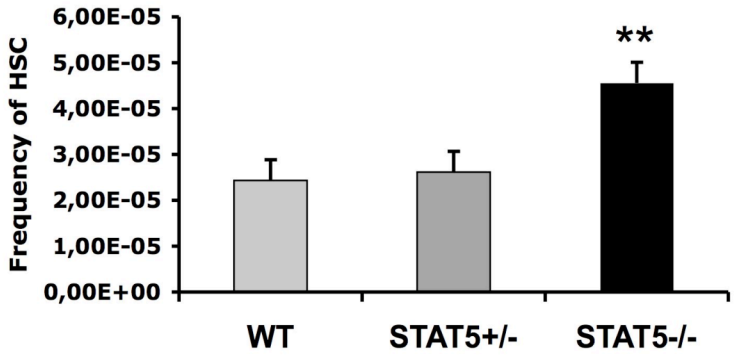


Figure 3.23: The frequency of HSCs in wildtype BM is increased when STAT5$^{-/-}$ OBs are used as feeder cells. The frequency of HSCs in wildtype BM supported by primary osteoblastic feeder cells was determined by the CAFC assay (n=3, $p<0.01$).

These results suggested that the lack of STAT5 in the OBs induced environmental alterations in the endosteal HSC niche, culminating in the increase of bone-adhered HSCs.

3.2.4 Activated STAT1 and STAT3 can compensate for the lack of STAT5 in OBs

HSC numbers were increased in the presence and absence of GH in mice lacking STAT5 in OBs. Furthermore, the cobblestone-formation capacity of HSCs was mediated by STAT5-knockout OBs *in vitro*. These data suggested a compensatory mechanism of other STAT proteins in the absence of STAT5. In the liver, it has been demonstrated that STAT1 and STAT3 are strongly activated and thereby compensate the loss of STAT5 (Clodfelter et al. 2006; Cui et al. 2007). To test whether this also occurs in OBs, we treated calvarial STAT5-knockout OBs with GH and analyzed the expression of the phosphorylated forms of STAT1 and STAT3. Western blot analysis of primary OBs treated with GH for 2 hours showed that wildtype OBs strongly increased STAT5 phosphorylation at residue tyrosine 694 (Fig. 3.24).

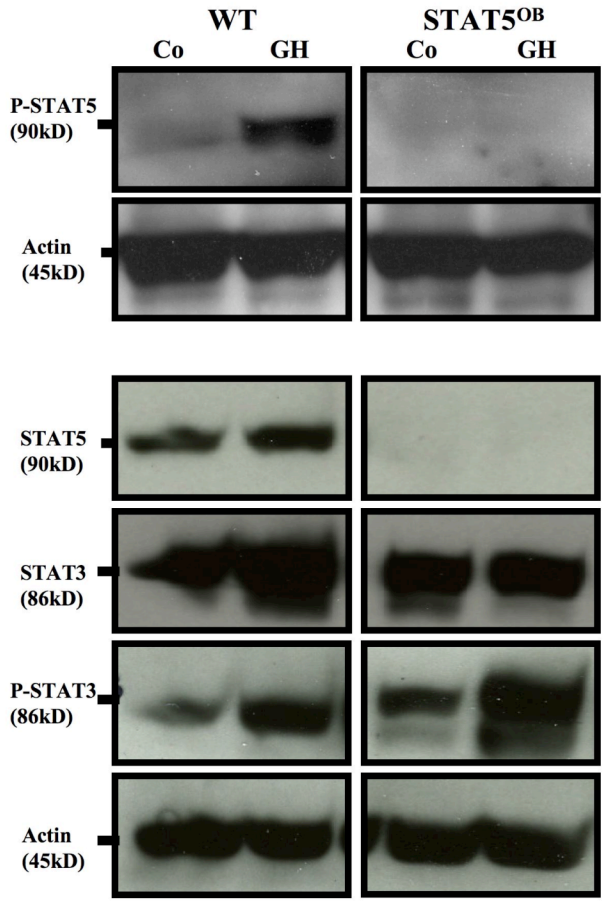


Figure 3.24: GH treatment activates STAT5 in wildtype OBs and STAT3 is strongly upregulated in the absence of STAT5. Western blot analysis of primary wildtype and STAT5-knockout OBs for expression levels of STAT5, STAT3 and their activated, phosphorylated forms. Actin was chosen as loading control.

As expected, STAT5 protein levels were not detectable in STAT5-deficient OBs. Levels of STAT3 protein were increased in wildtype OBs under GH treatment, whereas GH did not lead to an increase in STAT3 in $STAT5^{-/-}$ OBs. The activated, phosphorylated form of STAT3 (P-STAT3, phosphorylation at residue tyrosine 705) was increased under GH influence in wildtype cells and the increase was even more pronounced in $STAT5^{-/-}$ OBs.

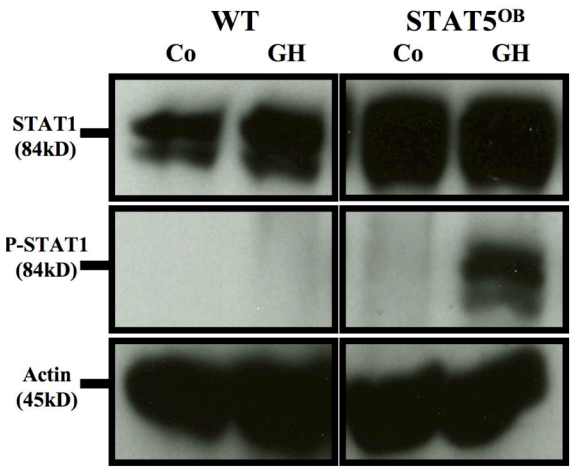


Figure 3.25: GH strongly activates STAT1 in the absence of STAT5. Western blot analysis of primary wildtype and STAT5-knockout OBs for expression levels of STAT1 and its activated, phosphorylated form. Actin was chosen as loading control.

STAT1 levels were not altered under GH influence in neither wildtype cells nor the $STAT5^{-/-}$ OBs (Fig. 3.25). However, the phosphorylated and thereby activated form of STAT1 (P-STAT1, phosphorylation at residue tyrosine 701) was not detectable in wildtype OBs in the presence or absence of GH. However, a strong expression of phosphorylated STAT1 was observed under GH treatment in the STAT5-deficient OBs. These results strongly suggested an elevated activation of STAT3 and preferably STAT1 in the absence of STAT5 and under stimulation by GH, which strongly correlated with the increased support of HSCs.

In summary, our data illustrate that GH increases the number of HSCs in the vascular and the endosteal HSC niche. We showed that OBs and the transcriptional activator STAT5 in the OBs play an important role in the mediation of the GH effect in the endosteal HSC niche. In wildtype mice, STAT5 is suggested to regulate the number of HSCs in the endosteal niche. If STAT5 is absent in OBs, this results in a compensatory activation of STAT3 and particularly STAT1, correlating with the increase of HSC numbers in the endosteal niche.

4 *Discussion*

4.1 Effects of 17-β-E_2 on the HSC niche

4.1.1 Long-term treatment of wildtype mice with 17-β-E_2 leads to an increase of bone mass but not to an increase of bone-adhered HSCs

It has been well established that long-term treatment with a pharmacological dose of 17-β-E_2 increases the bone mass, by increased activity of OBs and induced apoptosis in preosteoclasts (Liu and Howard 1991; Zecchi-Orlandini et al. 1999; Clodfelter et al. 2006). In 2003, Calvi et al. showed that genetically-altered osteoblastic cells, overexpressing activated parathyroid hormone/parathyroid hormone-related protein (PTH/PTHrP), are capable of regulating the HSC niche and increasing the HSC pool, mainly mediated via the upregulation of the Jagged1/Notch1 signaling pathway. Taking this into consideration, we suggested a possible regulatory role of increased levels of OBs on HSCs under 17-β-E_2 treatment. Due to direct interactions of specialized spindle-shaped OBs with HSCs, we assumed a regulation of bone-adhered HSCs by 17-β-E_2 (Zhang et al. 2003). Not only could the properties of osteoblastic cells to support HSCs be altered, but also an increased number of OBs and a larger endosteal surface after estradiol treatment could generate quantitatively more endosteal HSC niches, and thereby an increase of bone-adhered HSCs. We showed, however, by FACS analysis and CAFC assay (Figs. 3.2, 3.3 and 3.5) that there were no alterations in bone-adhered HSCs under 17-β-E_2 treatment. This leads to the conclusion that 17-β-E_2 treatment causes distinct changes in bone-forming cells, but not in the endosteal HSC niche. The supportive activity for HSCs is not altered by 17-β-E_2. Hence, the observed effects are not comparable to the effects of PTH treatment (Zhang et al. 2003).

Calvi et al. (2003) suggested an important role for the Notch signaling pathway in OB and HSC interaction, which is supposed to be altered under PTH treatment. However, we could not detect any changes in Jagged1 expression via real-time PCR analysis of 17-β-E_2-treated OBs (data not shown). Hence, we suggest that 17-β-E_2 influences OBs and OCs via molecular mechanisms which are completely independent of the Jagged1/Notch1 pathway. In line with this conclusion, we showed

that on the one hand the ERα in OBs was essential for the increase of bone mass by 17-β-E_2, but on the other hand did not lead to elevation of HSC numbers.

4.1.2 Long-term treatment of mice with 17-β-E_2 leads to an increase in competent HSCs in the vascular niche of the BM

In contrast to the results for the endosteal stem cell niche, we observed a striking increase in HSC numbers in the BM of the vascular niche harvested by flushing the BM as described previously (Sugiyama et al. 2006). The surface marker analysis for LSK and SLAM markers, the label-retention assay, the cobblestone colony-formation assay and the competitive repopulation analysis proved that fully-functional HSCs in the vascular niche were increased in numbers.

The enhanced number of $BrdU^+$ label-retaining cells under 17-β-E_2 treatment indicated higher numbers of quiescent cells located in the BM. The $BrdU^+$ slow-cycling cells in the BM that maintain the BrdU label for 70 days are believed to be mainly HSCs, because only stem cells are most of the time maintained in the G0/G1 phase of the cell cycle. However, it has recently been shown that BrdU label-retaining cells do not exclusively represent the most primitive HSCs (Kiel et al. 2007a). Therefore, it was essential to obtain further evidence of the increase of HSC numbers by 17-β-E_2 treatment. Therefore, we performed the gold standard test of primitive HSCs, the capacity of long-term engraftment in a limiting dilution assay and confirmed that 17-β-E_2 did indeed increase HSC numbers in the vascular niche (Figs. 3.13 and 3.14). In summary, we confirmed by four assays that 17-β-E_2 treatment induces a change in the vascular compartment of the HSC niche, resulting in elevated numbers of HSCs by a so far unknown mechanism. We assume that this effect is not mediated directly via the increased numbers of OBs, otherwise the bone-adhered HSCs would also be increased. However, we can not rule out a secondary effect exerted by osteoblastic cells on the vascular environmental cells, e.g. by secretion of cytokines or growth factors affecting the stromal cells.

4.1.3 Is the ERα or the ERβ the mediating molecule for the HSC number increase under 17-β-E_2 treatment?

Since most of the known effects of 17-β-E_2 are mediated by one or both of the known estrogen receptors, ERα and ERβ (Katzenellenbogen and Korach 1997; Enmark and

Gustafsson 1999; Nilsson et al. 2001a; Pettersson and Gustafsson 2001; Lindberg et al. 2002), we decided to investigate first of all if the mechanism for this phenomenon is estrogen receptor dependent. To date, there are only a few data regarding the role of ERα in the hematopoietic system. The only study investigating 17-β-E_2 treatment and HSCs showed that HSCs in ERα-knockout mice are not affected, by neither the mutation nor the pharmacological treatment (Thurmond et al. 2000). In this study, mice were treated with 5 mg 17-β-E_2/kg body weight for 10 days, and the impact of 17-β-E_2 on mice was only confirmed by the degeneration of the thymus. Additionally, we could not detect any changes in HSC numbers at this time-point (data not shown), but showed that at later time-points (up to four weeks) the HSC numbers were increased. To define the role of ERα in the effect of 17-β-E_2 on HSCs, we analyzed the ERα-knockout mice for the regulation of HSCs in the vascular niche under 17-β-E_2 treatment. The LDA of lethally-irradiated mice reconstituted with BM from treated or untreated wildtype mice and ERα-knockout mice (Fig. 3.11) indicated that the ERα is not the mediating molecule in this alteration of HSCs under 17-β-E_2 treatment.

In addition, we showed that ERα-knockout mice did not build up bone mass and did not limit the space in the BM for hematopoietic cells (Fig. 3.12). Flushing the BM from ERα-knockout mice under 17-β-E_2 treatment led to the same overall cell numbers as in untreated knockouts, whereas in wildtype mice we observed a decrease of BM cells. Since both wildtype and ERα-knockout mice had elevated HSC numbers upon 17-β-E_2 exposure, these data clearly indicated that the increased numbers of HSCs in wildtype mice do not result from the limited space in the BM cavity. Therefore, this increase is not caused by shifted hematopoietic populations, because HSCs were also increased when the BM cavity was unaffected.

The increase of HSC numbers, in mice lacking ERα, could also be mediated by ERβ. However surprisingly, the investigation of HSC surface markers from ERβ-knockout mice under 17-β-E_2 treatment showed comparable results to the wildtype mice. LSK cells were increased by 17-β-E_2 treatment in ERβ-knockout mice (Fig. 3.19). This experiment indicated that ERβ is also not responsible for the enhanced HSCs under estrogen influence. However, we can not exclude that in the absence of either ERα or ERβ, the presence of the other receptor mediates the estradiol effects. One possible way of investigating this would be the simultaneous treatment of mice with 17-β-E_2 and ICI 182,780, which is an antagonist for both ERs (Dauvois et al. 1993;

Parker 1993; Pink and Jordan 1996). Alternatively, the effects of 17-β-E_2 in mice lacking both receptors (ERα and ERβ double-knockout, DERKO) could be analyzed.
If nuclear receptors for estrogen are not involved, the recently discovered G-protein-coupled receptor GPR30 could exert the estradiol effects. GPR30 activates the epidermal growth factor receptor signaling pathway and is thereby able to switch on extracellular signal-regulated kinase, culminating in c-fos expression, which is estrogen receptor-responsive element independent (Maggiolini et al. 2004; Revankar et al. 2005; Vivacqua et al. 2006). To clearly define the role of this 'membrane-bound estrogen receptor' GPR30, it would be necessary to analyze the GPR30-knockout mouse (Wang et al. 2008) for the reaction of 17-β-E_2 treatment related to the HSCs in the vascular niche.

4.1.4 17-β-E_2 treatment of mice affects the environmental niche cells

The literature shows that various substances like hormones, e.g. PTH (Calvi et al. 2003), alter HSC numbers by influencing the HSC niches. Although a direct cell-autonomous effect of 17-β-E_2 on HSCs could not be ruled out, we have evidence that 17-β-E_2 treatment affected the stromal cells in the vascular HSC niche. We mimicked the vascular HSC niche *in vitro* and showed that pre-treatment of the stromal FBMD1 feeder cells for 14 days with 17-β-E_2 increased the support for HSCs, displayed by higher numbers of cobblestone-forming areas. A critical question in this regard is could we have observed a better support of HSCs because of a proliferative effect in the FBMD1 cells under the influence of 17-β-E_2. However, the protein content of a control and 17-β-E_2-treated FBMD1 culture was not altered (data not shown).
The modified CAFC assay was the first hint that 17-β-E_2 influences the environment of HSCs *in vitro*. To test whether this hypothesis is also appropriate *in vivo*, we performed the so-called 'homing assay', where CFSE-labeled lineage-negative sorted BM cells from wildtype mice were injected into sublethally irradiated control or 17-β-E_2-treated mice. Twelve hours post-transplantation, cells were traced by FACS analysis. Pre-treated wildtype mice showed that 17-β-E_2 is indeed influencing the surrounding cells of the HSC niche. Under 17-β-E_2 treatment, more $CFSE^+$ $lineage^-$ cells were detected in the vascular niche compared to control mice (Fig. 3.16). Although only a small population of BM cells was CFSE-positive, we saw a clear increase in this population in the 17-β-E_2-pre-treated recipients. First of all, 1×10^6 CFSE-labeled cells were injected per mouse. Twelve hours later, the whole BM from

the vascular niche was isolated including about 40×10^6 cells. It has been shown that approximately 60% of the injected cells locate first of all to the endosteal niche (Nilsson et al. 2001b). Therefore, the $CFSE^+$ fraction isolated from the vascular niche is rather small. Obviously, 17-β-E_2 treatment has advantageous effects on the vascular HSC niche, which lead to a more comfortable environment for HSCs and makes homing much more efficient. A possible explanation for this advantageous alteration in the niche could be a modification of vasculature. Increased vascularization would lead to a higher oxygen and nutrient supply in the vascular niche, positively affecting HSCs. Due to more vessels, the surface for homing in the vascular niche would increase as well. An enhanced vascularization will be detected by a trichrome staining of bone sections and the staining with the endothelial marker MECA32 (a pan-endothelial cell antigen), which also represents vascularization.

In addition, the CAR cells could be responsible for the observed effects. CAR cells strongly express CXCL12 and they have been shown to be indispensable in the HSC niche (Sugiyama et al. 2006). If CAR cells are the target of 17-β-E_2 in the vascular HSC niche, it could explain why the niche delivers an altered environment resulting in increased homing of stem and progenitor cells.

The effect in the vascular niche obviously leads to long-term changes in the stromal compartment of the niche, which is thereby better at supporting HSCs under the influence of 17-β-E_2. Further suggestions for this effect could be changes in gene expression levels, particularly in adhesion and cell-cell interaction molecules, e.g. Tie2/Ang-1 (Arai et al. 2004), N-Cadherin/β-Catenin (Zhang et al. 2003), CXCL12/CXCR4 (Sugiyama et al. 2006) or Jagged1/Notch1 Stier et al. 2002; Calvi et al. 2003). Furthermore, secreted molecules like G-CSF (Ju et al. 2007), Flt3 (Sitnicka et al. 2002), stem cell factor and IL11 (Brandt et al. 1992; Miller and Eaves 1997) have been shown to be involved in the HSC number increase and HSC/stromal cell interactions. Surprisingly, none of the mentioned adhesion molecules, Ang-1, N-Cadherin/β-Catenin, CXCL12/CXCR4 or Jagged1/Notch1, was regulated by 17-β-E_2 in FBMD1 cells detected by real-time PCR analysis (data not shown). Therefore, we concluded that these signaling pathways are not involved in increased HSC niche interactions under the influence of 17-β-E_2.

To investigate whether one of the ERs is involved in the mediation of 17-β-E_2 effects in the vascular HSC niche, we suggest a BM transplantation of wildtype BM in either ERα- or ERβ-knockout mice. This model could clarify if the receptors in the niche are essential for mediating 17-β-E_2 effects. However, the previous results showed that

both receptors, at least in their single action, are dispensable for the increase of HSC numbers in the vascular niche.

4.1.5 17-β-E_2 regulates the mRNA levels of different adhesion molecules in FBMD1 cells

Due to the fact that 17-β-E_2 did not alter the expression of typical candidate genes involved in HSC maintenance, we decided to investigate the gene expression in FBMD1 cells under 17-β-E_2 treatment by a microarray analysis. To circumvent unpredictable factors of influence like different cell types, which could show variable regulation for certain molecules, we decided to use the FBMD1 cells as the RNA source for the microarray. These cells increased the cobblestone colony formation under the influence of 17-β-E_2, and thereby the phenotype could be correlated to changes in gene expression. Table 3.1 represents the strongest regulated molecules, which were classified for a possible participation in HSC function. These six molecules were chosen for further investigation of their regulation via real-time PCR; only two of them could be confirmed.

The first gene, the stem cell antigen CD34, is often used for the identification of HSCs, preferably in human BM since hHSCs, in contrast to mHSCs, are CD34-positive, and it has previously been shown that this molecule can act as a regulator of hematopoietic cell adhesion in mice (Healy et al. 1995; Okuno et al. 2002). Surprisingly, CD34-knockout mice do not show any impairment of self renewal, but CD34 plays a role in the differentiation of HSCs (Cheng et al. 1996). Furthermore, it is clear that CD34 expression on mHSCs is highly dependent of the developmental stage of mice (Ito et al. 2000; Matsuoka et al. 2001). Apart from its function on hematopoietic cells, it has been shown to be expressed on murine mesenchymal stromal cells (Copland et al. 2008). Therefore, CD34 could be involved in the interaction of HSCs and stromal cells in response to 17-β-E_2, which in turn would lead to a stronger attachment of HSCs in the niche and the retention of more undifferentiated HSCs in the niche.

F-Spondin 1 is partly functionally similar to CD34. This adhesion molecule is highly expressed in the rat floor plate of vertebrates, a small population of epithelial cells localized at the ventral mid-line of the neural tube (Schoenwolf and Smith 1990; Klar et al. 1992). The secreted molecule is necessary for the guidance of commissural axons at the floor plate and for the regulation of migration of neural crest cells (Burstyn-Cohen et al. 1999; Debby-Brafman et al. 1999). Furthermore, F-Spondin is

able to inhibit the outgrowth of embryonic motor axons and has a dual role in this system by inhibiting motor neurons and promoting the outgrowth of commissural neurons (Tzarfati-Majar et al. 2001). Although there are no data about a possible role for F-Spondin 1 in hematopoiesis or the interaction of HSCs and their microenvironment, this molecule could be involved in cell migration and growth in the BM.

Real-time PCR analysis showed that both genes were enhanced under 17-β-E_2 treatment in FBMD1 cells.

In summary, we discovered a new effect of 17-β-E_2 in the HSC niche. We strongly suggest that this effect is mediated via the microenvironmental cells of the vascular niche. We observed a clear upregulation of fully functional HSC numbers, shown by reconstitution of lethally-irradiated mice and the capacity to form cobblestone colonies. As possible mediators of this effect, we addressed two promising molecules which could be involved in the interaction of HSCs and their surrounding cells. To ascertain these possible mechanisms, we would perform a downregulation of these two distinct molecules by siRNA in FBMD1 cells. Due to the fact that siRNA only transiently alters the translation of a distinct mRNA into a protein, it would eventually be more reliable to use lentiviral stable transfections for this approach. The manipulated FBMD1 cells would be co-cultured with wildtype BM, and thereby investigated for their support of HSCs in the CAFC assay.

Furthermore, we showed that LSK cells were decreased in the peripheral blood of 17-β-E_2-treated mice. These data correlated with the upregulation of CD34 and F-Spondin 1. The upregulation of cell adhesion-involved molecules suggests a stronger retention of HSCs in the BM of the vascular niche.

In summary, our data contribute to shedding a little more light on the regulation of the HSC niche. Apart from this, we have to change our thinking about the role of estrogens in mammals. Estrogens are mostly related to the female reproductive tract and to bone physiology. However in addition to their familiar functions, we have to now consider their role in the regulation of HSCs. Therefore, we have to consider possible side-effects on the HSC niche under 17-β-E_2 therapy. Alternatively, one could also think of an adjusted 17-β-E_2 therapy in the distant future against HSC exhaustion during ageing. Our data contribute to an ongoing clarification of the regulation of HSCs and the interaction with their special microenvironment, which in turn leads to further elucidations in the questions about HSC exhaustion with ageing or even diseases like hematopoietic failure and cancer.

4.2 GH elevates HSC numbers in the endosteal niche via a STAT5-dependent mechanism

4.2.1 GH increases HSC numbers in the vascular and endosteal niche of wildtype mice

GH stimulates chondrocyte proliferation (Isaksson et al. 1982; Isaksson et al. 1985; Russell and Spencer 1985; Schlechter et al. 1986; Ohlsson et al. 1992), and the collagen production and proliferation of OBs (Morel et al. 1993; Kassem et al. 1994) by direct IGF-1-independent effects. Apart from its effects on bone-forming cells, the GHR is expressed in distinct leukocyte subpopulations, showing that GH is a hematopoietic growth and differentiation factor (Dorshkind and Horseman 2000). In addition, GH as well as overexpression of GH in bGH-transgenic mice lead to an increased number of HSCs measured as LSK cells. The same study also showed that the Jak/STAT signaling pathway is involved in this effect (Pello et al. 2006).
These effects led to the speculation that GH mediates its effect, at least in part, via the endosteal HSC niche.
First of all, we confirmed the results obtained in wildtype mice (Carlo-Stella et al. 2004a) under the influence of GH (Fig. 3.19). The subpopulation of CD150-positive/CD48-negative cells, representing the HSC fraction, was indeed increased after five weeks of GH treatment. We showed that the increase of HSC numbers affected both parts of the HSC niche. The effects on the vascular niche have previously been described (Pello et al. 2006). We showed for the first time that GH increased the number of HSCs located at the endosteal niche (Figs. 3.19 and 3.20).
It is suggested that GH acts via the CXCL12/CXCR4 axis through an upregulation of SOCS. SOCS blocks CXCR4 and results in HSC mobilization and release of HSCs in the peripheral blood (Pello et al. 2006). Therefore, the Jak/STAT signaling pathway is involved in this effect, based on the enhanced expression of the STAT target genes SOCS 1 and SOCS 3 (Yoshimura 1998; Krebs and Hilton 2000; Schluter et al. 2000). Furthermore, CXCR4 in combination with CXCL12 is able to activate Jak/STAT signaling independent of SOCS proteins. Whether this hypothesis holds true for the GH-mediated increase of HSC numbers we observed remains to be elucidated. In our experiments, mice were treated for five weeks with GH, in contrast to a 10 day-treatment in the earlier study. However, we showed a direct involvement of STAT5 in OBs for elevated HSC numbers, suggesting indeed a similar mechanism by Jak/STAT signaling.

4.2.2 STAT5 is important in OBs and their interaction with HSCs

To test whether STAT5 is essential in OBs for the GH effect on HSCs, we decided to disrupt the GHR signaling in OBs by deleting STAT5a/b in these cells, since conditional GHR-knockout mice are currently not available. We generated these $STAT5^{OB}$ mice for the first time, by specific deletion of STAT5 in OBs via the Cre recombinase under the control of the OB-specific Runx2 promoter (Rauch et al. 2009, submitted). The recombination was efficient in calvarial bone and long bone (data not shown); however, bone architecture was not altered (data not shown). Thus, under basal conditions STAT5 seems not to contribute to bone mass, which is in line with findings of earlier STAT5-knockout mice, which still express a hypomorph STAT5 protein (Sims et al. 2000).

$STAT5^{OB}$ mice had a normal response to GH regarding HSC numbers in the vascular niche. Since the vascular niche of $STAT5^{OB}$ mice is most likely not affected, these results are consistent with our expectation. All environmental cells as well as HSCs themselves should have normal STAT5 protein expression, except the OBs which are absent in this part of the niche. To test whether STAT5 is important in the vascular niche, further investigations of conditional knockout mouse models are required. These mice should lack STAT5 either in endothelial cells or mesenchymal cells.

Interestingly and unexpectedly, the disruption of STAT5 in OBs caused a stronger increase of HSC numbers after GH administration. In addition, HSC numbers of untreated $STAT5^{OB}$ mice were even increased.

We could confirm that the basal increase of HSC numbers in the endosteal niche of $STAT5^{OB}$ mice was caused by the lack of STAT5 in OBs by performing a CAFC assay with STAT5-deficient OBs and wildtype BM cells to investigate the cobblestone formation capacity (Fig. 3.23). In this assay, we showed that OBs isolated from STAT5-knockout embryos were better at supporting wildtype HSCs than heterozygous or wildtype OBs after five weeks of co-culture, represented by a higher frequency of HSCs.

Therefore, STAT5 deficiency did not lead to a decreased response to GH, rather to an enhanced reaction resulting in increased HSCs. Our data strongly suggest an action of GH via the environmental cells which support HSCs. To prove this hypothesis unequivocally, an OB-specific GHR-knockout mouse should be analyzed. However, such a mouse line has not yet been generated. To overcome this obstacle,

we intend to analyze GHR-knockout mice that have received wildtype BM in the near future (Zhou et al. 1997). In these mice, GH signaling is disrupted in the entire environment, but is intact in HSCs. We would expect an impaired response to GH reflected by unimpaired HSC numbers.

The increased HSC numbers in the endosteal niche of $STAT5^{OB}$ mice without GH treatment could have advantageous effects like resistance to myeloablative stress or HSC exhaustion with ageing (Ruzankina and Brown 2007). Both processes lead to decreased HSC numbers, which could result in hematopoietic failure. This conclusion is based on the hypothesis of the 'primordial HSC'. The primordial HSC is located in the endosteal niche under low oxygen conditions. When the HSC starts to differentiate, it leaves this part of the niche and migrates to the vascular niche, leading to further differentiation and a distinct lineage fate (Wilson et al. 2007). Our conditional mutants have more HSC at the endosteum, under basal conditions and the influence of GH. If these were primordial HSCs, it would protect them from HSC exhaustion with ageing or myeloablative stress. To address this, aged mice could be investigated via competitive repopulation assay to analyze if their HSCs in the endosteal niche are more competent in reconstituting lethally-irradiated mice. Furthermore, 5-fluorouracil treatment could show whether the $STAT5^{OB}$ mice are resistant to myeloablative stress.

One can also speculate about possible interactions of both niche parts. In our system, it would also be possible that the complete functional vascular niche, which is unaffected by the $STAT5^{OB}$ mutation, secretes secondary cytokines or growth factors under GH treatment. GH could even manipulate the named CXCL12/CXCR4 axis (Sugiyama et al. 2006, Pello et al. 2006) which in turn affects the endosteal niche, although there is an important part, STAT5, of the signaling pathway missing. However, we showed a vigorous increase of HSCs in the endosteal niche, which greatly exceeded the situation in wildtype mice, therefore this hypothesis can be refuted. Rather, the increased basal levels of HSCs in untreated $STAT5^{OB}$ mice suggest a vascular niche-independent effect, triggered by the manipulated Jak/STAT signaling pathway in the endosteal HSC niche.

4.2.3 Activated STAT1 and STAT3 can compensate for the lack of STAT5 in OBs

The unexpected enhanced HSC numbers by GH in the absence of STAT5 could be due to compensatory upregulation of other STAT factors mediating GH signaling.
Indeed, STAT5-knockout OBs showed enhanced phosphorylation of STAT3 and STAT1 by GH (Figs. 3.24 and 3.25). In contrast, wildtype OBs showed only moderate activation of STAT3 and no measurable induction of STAT1 phosphorylation. Furthermore, STAT5 was efficiently phosphorylated in wildtype OBs after GH treatment, indicating that under normal conditions STAT5 is an important mediator of GH signaling in OBs. Cui et al. (2007) recently showed that STAT3 and STAT1 also mediate GH effects in the liver of STAT5-deficient mice. It was shown that the compensatory mechanism is disrupted when liver-specific STAT5-mutant mice are crossed with STAT1-knockout mice. Compensatory upregulation has also been observed in human fibroblasts derived from patients with Laron syndrome, caused by mutations in the STAT5b gene, which display elevated STAT1 levels (Kofoed et al. 2003). To determine whether or not increased STAT1 activation is the cause of elevated HSC numbers in the endosteal niche of $STAT5^{OB}$ mice, we intend to cross $STAT5^{OB}$ mice with STAT1-knockout mice. These mice will show whether or not HSC numbers in the absence of STAT5 in OBs are corrected to normal levels when STAT1 is also abrogated.
A possible explanation for this effect is that in wildtype mice STAT5 is able to inhibit STAT3 and STAT1, whereas its absence leads to activation of STAT3 and STAT1, and thereby to an increase in HSC numbers. To date, there are no data available regarding a direct inhibitory effect of STAT5 on other STAT proteins. However, the STAT5-dependent expression of inhibitors of STAT signaling is a likely explanation. The STAT5 target genes SOCS 1 and SOCS 3 (Monni et al. 2001; Morales et al. 2002) are known to inhibit STAT1 phosphorylation and also STAT3 phosphorylation (Park et al. 2000; Vlotides et al. 2004). Whether SOCS 1 and SOCS 3 expression is reduced in STAT5-deficient OBs requires investigation. Also requiring investigation is whether overexpression of these factors diminishes elevated STAT1 and STAT3 activation in these cells.
In conclusion, the second part of this thesis contributes to the clarification of the HSCs and their interactions in the niche microenvironment. Furthermore, we have gained new insights into the molecular mediation of the GH effect on HSCs, as we now know that STAT5 in the OBs has an inhibitory effect on HSCs in the endosteal

stem cell niche. GH is used as standard therapy in individuals with GH deficiency (Dall et al. 2000; Drake et al. 2001) and it is also used for HSC mobilization (Arbona et al. 1998; Kroger et al. 2000; Carlo-Stella et al. 2004a). If one could prove that these HSCs are completely competent in reconstituting individuals with BM deficiency, GH would be an alternative for G-CSF-resistant BM donors (Lapidot and Petit 2002; Heim et al. 2003). Furthermore, GH actions on HSCs should be supervised when administered to GH-deficient patients. In conclusion, in the future GH could offer the possibility for a therapeutic approach against HSC exhaustion during ageing.

Finally, although this thesis addresses two different osteogenic hormones, acting via distinct signaling pathways, there are interactions between the two pathways. It has previously been shown that particularly in adolescent children during puberty there is a strong relationship between sex steroids and GH (Veldhuis 1996; Veldhuis et al. 2000; Coutant et al. 2004). We investigated the impact of two hormones both involved in bone metabolism and HSC maintenance. GH as well as 17-β-E_2 upgrade bone quality, which is the main component of the endosteal HSC niche. The loss of estrogen or GH results in impaired bone growth or even osteoporosis and could affect the endosteal HSC niche. This study critically investigated the independent effects of GH and 17-β-E_2 on HSCs and their specialized microenvironment. In summary, we showed that both hormones show striking effects in the HSC niche. Furthermore, we have contributed to the clarification of the molecular mechanisms behind these effects. Ultimately, we can not exclude a related effect of the investigated osteogenic hormones, which identifies new possibilities for the ongoing clarification of the HSC niche.

5 Materials and methods

5.1 Materials

5.1.1 Materials

Cell culture flasks	Greiner Bio-One
Cell culture products, except cell culture flasks	Becton Dickinson
Cell strainers (40 μm)	Becton Dickinson (BD)
Cryovials for cell culture	Sarstedt
FACS tubes	Becton Dickinson
Mice strains	C57/Bl6 Ly 5.1 (in C57/Bl6 background) 129 SvEV 129 SvEv/Ola
Micro-glass syringe for loading SDS-PAGE gels	Hamilton
Needles (27/26/24/23 gauge)	Roth
Optical tapes for covering real-time PCR plates	Biorad
Reaction tubes (0.5/1.5/2.0 ml)	Roth
Sterile filters for blue-cap flasks	Millipore
Sterile filters for syringes	Millipore
Syringes, single use (1/2/5/10 ml)	Roth
Thermo fast 96-well plates for real-time PCR	Peqlab

Table 5.1: List of materials used for experiments.

5.1.2 Chemicals

α-MEM	Gibco-Invitrogen
β-Mercaptoethanol for cell culture	Gibco-Invitrogen
17-β-Estradiol pellets	Innovative Research of America
17-β-Estradiol water soluble (cyclodextrin encapsulated)	Sigma
Agarose	Biomol
Agilent RNA 6000 nano reagents part I	Agilent
Ammonium persulfate	Merck
Biotaq	Bioline
BrdU-Flow kit	Becton Dickinson

5(6)-Carboxyfluorescein-diacetate-N-succinimidyl-ester (CFSE)	Sigma
Collagenase A	Roche
Dispase II	Roche
1,4-Dithiothreitol (DTT)	Roth
DMEM	Gibco
dNTPs used at a concentration of 2.5mM, unless otherwise stated	Bioline
ECL	Pierce
ECL-Plus	GE Healthcare
Ethanol p.A. and denatured	Roth
Ethylenediaminetetraacetic acid (EDTA)	Roth
Fetal calf serum (FCS)	Gibco
First strand buffer (with additional DTT)	Qiagen
Formaldehyde (37%, acid free)	Roth
Growth hormone (human)	Merck-Serono
Horse serum	PAA
Hydrocortisone	Sigma
IMDM	Gibco
Immobuffer for immolase	Bioline
Immolase	Bioline
Isopropanol	Roth
Lineage-cell depletion kit (# 130090858)	Myltenyi Biotec
Lysis buffer for proteins (Ras assay kit)	Pierce
Lysis buffer from EZ-Detect™ Ras activation kit	Pierce
Magnesium chloride for rt-PCR	Bioline
NH_4 buffer for biotaq	Bioline
Non-essential amino acids (NEAA)	PAA
Nonidet P40	Roth
Penicillin/streptomycin	PAA
Phosphate-buffered saline (PBS)	PAA
Platinum SYBR Green qPCR Super-Mix-UDG	Invitrogen
Probidium iodide (1 mg/ml)	BD Pharmingen
Protease-Inhibitor complete mini	Roche
Proteinase K	Gerbu
Reverse Transcriptase	Qiagen
RNAse A (100 KU/mg)	Invitrogen
RNeasy mini kit	Qiagen
RNAse Out	Qiagen
Rotiphorese 10xSDS-PAGE mix	Roth
SeeBlue Plus2 pre-stained standard (protein standard)	Invitrogen
Sensi mix for real-time PCR	Quantace
Sodium dodecyl sulfate (SDS)	Roth
SYBR-Green I	Quantace
Tetramethylethylenediamine (TEMED)	Roth
Trypsin/EDTA	PAA
Tween 20	Roth

Table 5.2: List of chemicals used for experiments.

5.1.3 Buffers and solutions

All buffers were made with deionized distilled water, unless otherwise stated.

10x Running buffer for SDS-PAGE pH 8.3	For 5 l: 151.4 g Tris base 721 g Glycine 50 g SDS pH 8.3
10x Transfer buffer for Western blotting	For 5 l: 151.4 g Tris base 750.7 g Glycine
1x Transfer buffer for Western blotting	For 5 l: 500 ml 10x Buffer 500 ml Ethanol 4 l ddH_2O
2x Sample buffer	For 40 ml: 16 ml 10% SDS 5 ml 1M Tris pH 6.8 4 ml Glycerol (86%) 12.6 ml ddH_2O Bromphenol blue (~25 mg)
50x TAE	2M Tris base 1M Acetic acid 50mM EDTA pH 8.0
Erythrocyte lysis buffer 10x stock solution (prepare 1x solution fresh each time)	89.9 g NH_4Cl 10.0 g $KHCO_3$ 370.0 mg EDTA (pH 8.0) Dissolve in 1 l H_2O Adjust pH to 7.3, store at 4°C (tightly closed)
FACS buffer	PBS 2% FCS
MACS buffer	PBS 2% BSA 50mM EDTA

Stripped serum (covered with dextran-treated charcoal (DCC) for the removal of steroids)	FCS or HS was heat inactivated for 30 min at 56°C. Afterwards, serum was mixed with a pellet of an equivalent amount of DCC slurry (0.05% dextran, 0.5% charcoal in 50mM Tris buffer pH 7.4, stirred at 4°C overnight). The mixture was incubated for 45 min at 45°C, followed by centrifugation (20 min, 4500 g, 4°C). The supernatant was transferred to a fresh DCC pellet and the procedure was repeated. After sterile filtration, the DCC-stripped serum was stored at -20°C.
Stripping buffer for removing antibodies from proteins on nitrocellulose membranes	2% SDS 62mM Tris HCl pH 6.8 100mM β-Mercaptoethanol
Tail buffer	50mM Tris HCl pH 8.0 100mM EDTA pH 8.0 100mM NaCl 20% SDS
TBS-T	20mM Tris HCl pH 7.5 137mM NaCl 0.1% Tween 20
TE buffer pH 7.5	10mM Tris HCl 0.5M EDTA

Table 5.3: List of laboratory-prepared buffers and solutions used for experiments.

5.1.4 Media for cell culture

FBMD1 cells	IMDM 10% FCS 5% HS 1% NEAA 1% Pen/Strep 10^{-5}M Hydrocortisone 50μM β-Mercaptoethanol
Primary OBs	α-MEM 10% FCS 1% NEAA 1% Pen/Strep
CAFC assay	IMDM 10% FCS 5% HS 1% NEAA 1% Pen/Strep 10^{-5}M Hydrocortisone 50μM β-Mercaptoethanol

Medium for E_2 treatment of FBMD1 cells	IMDM 10% DCC-FCS 5% DCC-HS 1% NEAA 1% Pen/Strep 10^{-5}M Hydrocortisone 50μM β-Mercaptoethanol

Table 5.4: List of media used for experiments.

5.1.5 Primers for genotyping

Cbfa1-Cre Primer 2.5	5´-TGG-CTT-GCA-GGT-ACA-GGA-G-3´
Cbfa1-Cre Primer 24	5´-CCA-GGA-AGA-CTG-CCA-GAA-GG-3´
Cbfa1-Cre Primer 30	5´-GGA-GCT-GCC-GAG-TCA-ATA-AC-3´
ERα-Primer 539	5´-TAG-GCT-TTG-TCT-CGC-TTT-CC-3´
ERα-Primer 540	5´-CCC-TGG-CAA-GAT-AAG-ACA-GC-3´
ERα-Primer 541	5´-AGG-AGA-ATG-AGG-TGG-CAC-AG-3´
GHR In3+1	5´-CCT-CCC-AGA-GAG-ACT-GGC-TT-3´
GHR In4-1	5´-CCC-TGA-GAC-CTC-CTC-AGT-TC-3´
GHR Neo-3	5´-GCT-CGA-CAT-TGG-GTG-GAA-ACA-T-3´
STAT1 P1	5´-CAG-ATA-ATT-CAC-AAA-ATC-AGA-GAG-3´
STAT1 P2	5´-CTG-ATC-CAG-GCA-GGC-GTT-G-3´
STAT1 P3	5´-TAA-TGT-TTC-ATA-GTT-GGA-TAT-CAT-3´
STAT5-Primer 1685	5´-GAA-AGC-ATG-AAA-GGG-TTG-GAG-3´
STAT5-Primer 1686	5´-AGC-AGC-AAC-CAG-AGG-ACT-AC-3´
STAT5-Primer 1709	5´-CCC-ATT-ATC-ACC-TTC-TTT-ACA-G-3´
STAT5-Primer 1842	5´-AAG-TTA-TCT-CGA-GTT-AGT-CAG-G-3´
Tie2Cre I	5´-CGG-TCG-ATG-CAA-CGA-GTG-ATG-AGG-3´
Tie2Cre II	5´-CCA-GAG-ACG-GAA-ATC-CAT-CGC-TCG-3´

Table 5.5: List of primers used for genotyping.

5.1.6 Primers for real-time PCR

Actin fwd	5´-AGA-GGG-AAA-TCG-TGC-GTG-AC-3´
Actin rev	5´-CAA-TAG-TGA-TGA-CCT-GGC-CGT-3´
CD34 fwd	5´-GCA-GGA-AAG-TGG-CAT-CTC-TT-3´
CD34 rev	5´-ACC-ACA-ACT-TGA-CCC-AAA-GG-3´
Decorin fwd	5´-TCA-GTC-CAG-AGG-CAT-TCA-AA-3´
Decorin rev	5´-TTG-GTG-ATC-TTG-TTG-CCA-TC-3´
ERα fwd	5´-TGA-ACA-CAG-TGG-GCT-TGC-T-3´
ERβ fwd	5´-TCG-TTT-CGC-ATT-CCT-ACC-TC-3´
ERα rev	5´-CCA-TGA-CCA-TGA-CCC-TTC-AC-3´
ERβ rev	5´-ATG-AAG-GCC-TGA-AGC-TGT-GT-3´
F-Spondin fwd	5´-GGT-CCC-AGT-GGT-CTG-AAT-GT-3´
F-Spondin rev	5´-CTG-CTC-ACT-CCT-CCT-GCT-CT-3´
Gelsolin fwd	5´-GAC-TGT-GCA-GCT-GAG-GAA-TG-3´
Gelsolin rev	5´-TGA-AGT-AGC-CGG-AGA-AGG-TG-3´
Peroxiredoxin 4 fwd	5´-CCC-ACT-GGA-TTT-CAC-CTT-TG-3´
Peroxiredoxin 4 rev	5´-CCC-CAG-TCC-TCC-TTG-TCT-T-3´

Table 5.6: List of real-time PCR primers.

5.1.7 Western blot antibodies

Antibody	**Source**	**Company**	**Specificity**	**Product size**
β-Actin (I-19): sc-1616	goat	Santa Cruz	mouse, rat, human	42 kDa
P-STAT1	rabbit	Cell Signaling	mouse, rat, human	84, 91 kDa
P-STAT3	rabbit	Cell Signaling	mouse, rat, human	79, 86 kDa
P-STAT5	rabbit	Cell Signaling	mouse, human	90 kDa
STAT1	rabbit	Cell Signaling	mouse, rat, human	91, 84 kDa
STAT3	rabbit	Cell Signaling	mouse, rat, human	79, 86 kDa
STAT5 (C17): sc-835	rabbit	Santa Cruz	mouse, rat, human	92 kDa

Table 5.7: List of Western blot antibodies.

5.1.8 FACS antibodies

Antibody	Source	Company	Specificity	Conjugate
B220	rat	eBioscience	mouse, human	PE, FITC
CD117 (c-Kit)	rat	eBioscience	mouse	APC
CD11b	rat	eBioscience	mouse, human	APC, FITC, PE
CD11c	hamster	eBioscience	mouse	APC
CD150	rat	eBioscience	mouse	PE
CD19	mouse	eBioscience	mouse	APC
CD244 (2B4)	rat	eBioscience	mouse	FITC
CD3	hamster	eBioscience	mouse	PE, APC, FITC
CD4	rat	eBioscience	mouse	PE, FITC, APC
CD48	hamster	eBioscience	mouse	APC
CD8a	rat	eBioscience	mouse	FITC, PE, APC
FC-Block (CD16/CD32)	rat	eBioscience	mouse	pure
GR-1	rat	eBioscience	mouse	PE, FITC
MHCII	rat	eBioscience	mouse	PE
Sca1 (Ly6A-E)	rat	eBioscience	mouse	PE, biotinylated
Secondary Streptavidin conjugated APC-Cy7	-	eBioscience	biotin	APC-Cy7
Ter-119	rat	eBioscience	mouse	FITC, PE

Table 5.8: List of FACS antibodies.

5.1.9 Investigated knockout mice

The STAT5-loxP mice and the STAT5-knockout mice were kindly provided by Lothar Hennighausen at the National Health Institute in Bethesda, USA (Cui et al. 2004).
The GHR-knockout mice were kindly provided by John J. Kopchick at the Edison Biotechnology Institute and Department of Biomedical Sciences, College of Osteopathic Medicine, Ohio University in Athens, Ohio, USA (Zhou et al. 1997).
The STAT1-knockout mice were kindly provided by the Department of Veterinary Molecular Genetics and Biotechnology, Head: Mathias Müller, at the Institute of Animal Breeding and Genetics, Vienna, Austria (Durbin et al. 1996).
The Tie2Cre-transgenic animals were kindly provided by the laboratory of Bernd Arnold from the DKFZ in Heidelberg, Germany (Constien et al. 2001).
The ERα- and ERβ-knockout mice were kindly provided by the laboratory of Pierre Chambon at the Institute for Genetics and Cellular and Molecular Biology in Strasbourg, France (Lubahn et al. 1993; Krege et al. 1998).

The ER-loxP animals were kindly provided by the laboratory of Günther Schütz at the DKFZ in Heidelberg, Germany (Wintermantel et al. unpublished).
All transgenic animals were bred either in C57/Bl6 ($STAT5^{OB}$, STAT5 knockout, STAT1 knockout, Tie2Cre, $ER\alpha^{Runx2cre}$), 129 SvEv (ERα knockout and ERβ knockout) or 129 SvEv/Ola (GHR knockout) background.

5.2 Methods

5.2.1 Isolation of DNA from mouse tail biopsy for genotyping

The tip of a mouse tail was digested with 600 µl tail buffer and 20 µl proteinase K for 2 h at 56°C and 2,000 rpm, until the tissue was completely pyrolyzed. Two hundred and fifty µl of a 6M NaCl solution was added, mixed thoroughly and centrifuged at 16100 g for 7 min at RT. The supernatant was transferred into a new 1.5 ml reaction tube, and 500 µl isopropanol was added to precipitate the DNA. The tube was shaken thoroughly (to optimize the precipitation, the tube can be incubated for at least 30 min at -20°C), and centrifuged again for 10 min at 16100 g and RT. The supernatant was removed carefully, and the pellet washed with 70% ethanol for 30 min at RT. Following centrifugation for 10 min at 16100 g (RT), the supernatant was removed, and the pellet dried for 15 min under the fume hood. The pellet was diluted according to its size in 20–100 µl TE buffer for 2 h at 37°C. The DNA solution was stored at -20°C.

5.2.2 PCRs for genotyping

PCR is a method to amplify DNA from individual gene loci. All PCR-mastermixes were prepared on ice. Genomic DNA (isolated from tail biopsy) was added. The mixture was incubated according to the specialized prototcol for each PCR.

ERα PCR

Mastermix:

PCR buffer (NH_4 buffer)	2.5 µl

$MgCl_2$	0.75 µl
dNTPs	2.0 µl
Primer mix (0.6 pmol)	1.0 µl
Taq (biotaq)	0.2 µl
H_2O	17.55 µl
	24 µl (0.5 µl template)

Table 5.9: Mastermix for ERα genotyping.

Program and expected fragments:

<table>
<tr><td colspan="2">94°C (3´)</td><td rowspan="3">Results:
WT: 360 bp
Floxed: 497 bp
Deletion: 431 bp</td></tr>
<tr><td>94°C (20´´)
58°C (20´´)
72°C (1´)</td><td>35 cycles</td></tr>
<tr><td colspan="2">72°C (7´)
10°C forever</td></tr>
</table>

Table 5.10: Program and expected results for ERα genotyping.

Runx2Cre PCR

Mastermix:

PCR buffer (immobuffer)	2.5 µl
$MgCl_2$	2.0 µl
dNTPs	2.0 µl
Primer 24	0.75 µl
Primer 30	0.75 µl
Primer 2.5	0.1 µl
Taq (immolase)	0.4 µl
H_2O	15.5 µl
	24 µl (0.5 µl template)

Table 5.11: Mastermix for Runx2Cre genotyping.

Program and expected fragments:

94°C (2´)		Results: WT: 780 bp Transgene: 600 bp
94°C (20´´) 59°C (30´´) 65°C (40´´)	40 cycles	
65°C (10´) 10°C forever		

Table 5.12: Program and expected results for Runx2Cre genotyping.

STAT5-loxP PCR

Mastermix:

PCR buffer (NH_4 buffer)	2.5 µl
$MgCl_2$	1.0 µl
dNTPs	2.0 µl
Primer 1685	0.5 µl
Primer 1686	0.5 µl
Primer 1842	0.5 µl
Taq (biotaq)	0.2 µl
H_2O	16.8 µl
	24 µl (1 µl template)

Table 5.13: Mastermix for STAT5-loxP genotyping.

Program and expected fragments:

94°C (3´)		Results: WT: 450 bp Deletion: 350 bp Floxed: 200 bp
55°C (30´´) 72°C (2´+30´´) 94°C (20´´)	35 cycles	
72°C (10´) 10°C forever		

Table 5.14: Program and expected results for STAT5-loxP genotyping.

STAT5-null PCR

Mastermix:

PCR buffer (NH_4 buffer)	2.5 µl
$MgCl_2$	1.0 µl
dNTPs	1.0 µl
Primer 1686	0.5 µl
Primer 1709	0.5 µl
Taq (biotaq)	0.1 µl
H_2O	18.4 µl
	24 µl (1 µl template)

Table 5.15: Mastermix for STAT5-null genotyping.

Program and expected fragments:

<table>
<tr><td colspan="2">94°C (3´)</td><td rowspan="3">Results:
WT: no product
Deletion: 570 bp</td></tr>
<tr><td>55°C (30´´)
72°C (2´+30´´)
94°C (20´´)</td><td>35 cycles</td></tr>
<tr><td colspan="2">72°C (10´)
10°C forever</td></tr>
</table>

Table 5.16: Program and expected results for STAT5-null genotyping.

STAT1 PCR

Mastermix:

PCR buffer (NH_4 buffer)	2.0 µl
$MgCl_2$	1.2 µl
dNTPs	2.0 µl
Primer 1	0.75 µl
Primer 2	0.1 µl
Primer 3	0.75 µl
Taq (biotaq)	0.1 µl
H_2O	12.5 µl
	18 µl (0.5 µl template)

Table 5.17: Mastermix for STAT1 genotyping.

Program and expected fragments:

95°C (5´)		Results: WT: 140 bp Mutant: 340 bp Heterozygous: both fragments
95°C (30´´) 55°C (40´´) 72°C (40´´)	40 cycles	
72°C (5´) 10°C forever		

Table 5.18: Program and expected results for STAT1 genotyping.

GHR PCR

Mastermix:

PCR buffer (immobuffer)	2.5 µl
$MgCl_2$	2.0 µl
dNTPs	2.0 µl
In3+1	0.5 µl
In4-1	0.5 µl
Neo-3	0.5 µl
DMSO	1.0 µl
Taq (immolase)	0.4 µl
H_2O	14.6 µl
	24 µl (0.5 µl template)

Table 5.19: Mastermix for GHR genotyping.

Program and expected fragments:

95°C (2´)		Results: WT: 390 bp Mutant: 220 + 290 bp Heterozygous: all three
95°C (15´´) 58°C (20´´) 72°C (30´´)	40 cycles	
72°C (10´) 10°C forever		

Table 5.20: Program and expected results for GHR genotyping.

Tie2Cre PCR

Mastermix:

PCR buffer (immobuffer)	2.5 µl
$MgCl_2$	2.0 µl
dNTPs	2.0 µl
Primer Cre I	0.5 µl
Primer Cre II	0.5 µl
Taq (immolase)	0.4 µl
H_2O	16.1 µl
	24 µl (0.5 µl template)

Table 5.21: Mastermix for Tie2Cre genotyping.

Program and expected fragments:

<table>
<tr><td colspan="2">94°C (3´)</td><td rowspan="3">Results:
WT: no product
Transgene: 600 bp</td></tr>
<tr><td>55°C (30´´)
72°C (2´+30´´)
94°C (20´´)</td><td>39 cycles</td></tr>
<tr><td colspan="2">72°C (10´)
10°C forever</td></tr>
</table>

Table 5.22: Program and expected results for Tie2Cre genotyping.

5.2.3 RNA isolation from primary cells and cell lines

The isolation of RNA was performed with the RNeasy Mini Elute Cleanup Kit from Qiagen. The protocol is included in the kit or ready for download at http://www1.qiagen.com/literature/handbooks/literature.aspx?id=1000290&r=1833.

5.2.4 Digestion of DNA in RNA samples

DNA potentially remaining after RNA isolation was digested with RNase-Free DNase according to protocols from Qiagen (http://www1.qiagen.com/literature/Default.aspx?Term=DNAse&Language=EN&LiteratureType=4%3b8%3b9%3b10&ProductCategory=0).

5.2.5 Determining the quantity and quality of isolated RNA

For the estimation of the quantity and quality of isolated RNA, the Agilent RNA 6000 nano labchip kit was used, followed by measurement of the sample chips in the Agilent 2100 bioanalyzer (Agilent).

5.2.6 cDNA synthesis from RNA samples using reverse transcription

Reverse transcriptions started with the incubation of 1 µg RNA (in up to 10 µl volume) with 1 µl oligo-dT primers for 5 min at 70°C. This mix was kept on ice until the mastermix was added.

Mastermix	**Amount in µl per 9 µl test sample**
5x First strand buffer	4.0
100mM DTT	2.0
10mM dNTP	1.0
RNaseOUT	0.4
SuperScript II reverse transcriptase	1.0
DEPC-water	0.6

Table 5.23: Mastermix for reverse transcription of RNA to cDNA.

Per sample, 9 µl of mastermix was added and incubated at 50°C for 1 h under mixing of the samples every 20 min. The reaction was inactivated by heating the reaction to 65°C for 15 min. Eighty µl RNase-free water was added to the synthesized cDNA.

5.2.7 cDNA check using β-actin PCR

Chemicals	**Amount in µl per 25 µl test sample**
PCR buffer	2.5
$MgCl_2$	1.0
dNTP	1.0
β-Actin fwd	1.0
β-Actin rev	1.0
Taq	0.1
H_2O	17.4
cDNA	1.0

Table 5.24: Mastermix for β-actin PCR for checking correct reverse transcription of RNA to cDNA.

Program and expected fragments:

<table>
<tr><td colspan="2">95°C (3´)</td><td rowspan="3">Results:
One band at 150 bp</td></tr>
<tr><td>95°C (15´´)
57°C (20´´)
72°C (30´´)</td><td>27 cycles</td></tr>
<tr><td colspan="2">72°C (7´)
10°C forever</td></tr>
</table>

Table 5.25: Program and expected results for actin PCR.

5.2.8 Selection of primers for real-time PCR

All primers for real-time PCR were picked with the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The sequences of the chosen genes were found in the Ensembl Genome Browser (http://www.ensembl.org/index.html). To make sure that the primers would only align on the chosen gene, the primer sequences were tested with the BLAST program for nucleotide-nucleotide interactions from NCBI (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE=Nucleotides&PROGRAM=blastn&MEGABLAST=on&BLAST_PROGRAMS=megaBlast&PAGE_TYPE=BlastSearch&SHOW_DEFAULTS=on).

5.2.9 Real-time PCR

Chemicals	Amount in µl per 15 µl test sample
Sensi mix	10.0
SYBR-Green	0.2
Forward primer	0.4
Reverse primer	0.4
H_2O	4.0
DNA	5.0 (1:12 diluted)

Table 5.26: Mastermix for real-time PCR using chemicals from Quantace.

Chemicals	Amount in µl per 15 µl test sample
Platinum SYBR Green qPCR Super-Mix-UDG	9.3
Forward primer	0.4
Reverse primer	0.4
H_2O	4.9
DNA	5.0 (1:12 diluted)

Table 5.27: Mastermix for real-time PCR using chemicals from Invitrogen.

Every sample was investigated in triplet. In the analysis of every gene, a standard curve (single test) was included. Therefore, a cDNA (from the pool of the investigated samples) was diluted in seven steps, each with 1:5. Starting with the undiluted cDNA, the dilution factor at the 7^{th} step was 0.0064.

The analysis of real-time PCR was performed with the software Bio-Rad iQ 5.0. The threshold cycles (T_c) were automatically calculated by the software and were then included in the calculation of the relative gene expression as follows:

$$\text{Ratio} = (E_{GOI})^{\Delta CP(\text{control-sample})} / (E_{HKG})^{\Delta CP(\text{control-sample})}$$

This formula referred to the efficiency of the PCR, which was calculated from the slope by the following term:

$$E = 10^{(-1/\text{slope})}$$

The slope was again automatically calculated by the software via the specific threshold cycles of the standard curve.

5.2.10 Microarray analysis of FBMD1 cells after 17-β-E_2 treatment

Microarray analysis was performed by Markus Hildner from the Institute of Vascular Medicine (Head: Andreas Habenicht, FSU Jena) on an Affymetrix chip '430 A' for a Mus musculus genome-wide screen representing approximately 14,000 well-characterized genes. Therefore, total RNA of control and 10 day-17-β-E_2-treated FBMD1 cells were isolated and cRNA was hybridized on an Affymetrix 'A430' microarray chip to determine genome-wide mRNA expression. Bioinformatical processing and statistical analysis of the raw data using Affymetrix software led to the identification of upregulated and downregulated mRNAs. Further information and a detailed description of the system is available at http://www.med.uni-jena.de/ivm/.

5.2.11 Magnetic sort of cell populations with the autoMACS

This method is one possible method to sort cells due to the expression of specific cell surface molecules. Thereby, the cells were incubated with magnetic (ferric conjugates) antibodies and sorted as positive (magnetic) or negative (non-magnetic) selection. A magnetic column in the autoMACS device was then able to perform the selection. Protocols for handling and staining were included with the specific antibodies from Myltenyi Biotec.

5.2.12 FACS due to cell surface molecules

FACS is a method for analyzing and sorting cell populations due to the expression of specific cell surface molecules. The whole experiment was performed on ice. A single cell suspension was prepared by pushing the investigated tissue samples through a 40 μm cell strainer. After centrifuging the cells for 5 min at 583 g and RT, 10 μl FC block (CD16/32 in dilution 1:16 in FACS buffer) was added for 15 min at RT to every sample, to avoid non-specific binding of the antibodies. Afterwards, 50 μl of the fluorescent conjugated antibody was added to the pellet for 25 min. Unless otherwise stated, all antibodies were diluted 1:100 in FACS buffer. The cells were washed twice after staining and diluted in FACS buffer. The measurement of the samples was performed with the FACSCalibur or the FACSCanto II. Data analysis was performed with FlowJo 8.0.

5.2.13 SDS-PAGE and Western blot analysis

To analyze the proteome of cells, proteins can be isolated by lyzing the cells and detecting the proteins with specific antibodies. Unless otherwise stated, in all experiments for each sample, the cells of one 10 cm (diameter) cell culture dish were used to isolate proteins. The confluent cells were washed twice with cold PBS on ice, and 500 µl lysis buffer (including protease inhibitors) was added. The cells were scraped with a silicon scraper, and this suspension was pipetted into a 1.5 ml reaction tube. Samples were incubated for at least 5 min on ice and centrifuged for 10 min at 4°C and maximum speed 16100 g. The supernatant was separated into a new tube, and pellet and supernatant were stored at -20°C.

For separation of proteins, SDS-PAGE followed by Western blot analysis was used as a standard procedure (Laemmli 1970). For loading the samples, 50 µl of protein lysate, 50 µl 2x sample buffer and 10 µl DTT were mixed, heated at 94°C for 3 min, and centrifuged for 10 min at 16100 g and RT. Approximately 80 µl of the supernatant was loaded on the collecting gel.

The size of the investigated protein determines the percentage of the separating gel; in principle, the smaller the protein the higher the concentration of the separating gel.

Acrylamide concentration (%)	6	8	10	12	15
Separation range (kD)	50–200	30–95	20–80	12–60	10–43

Table 5.28: Separation range of SDS gels.

Separation of proteins was performed at 40 mA for approximately 3.5 h. Afterwards, proteins were transferred to a nitrocellulose membrane for 3 h at 85 V (or 20 V o.n.) via Western blotting. After blotting, the nitrocellulose membrane was washed in TBS-T and was cut if necessary to detect several proteins of different size in one experiment. Due to the specificities of the antibodies, the membrane was blocked with either milk powder or BSA to avoid non-specific binding. Blocking of the membrane was followed by incubation with the first antibody according to its specific protocol (datasheet of specific antibody). After washing 3 x 15 min, the secondary antibody conjugated with HRP was added onto the membrane for 1 h at RT. For detection, ECL or ECL-Plus was used. The membrane was incubated with the reagent for 5 min, then the chemiluminescence was detected with an x-ray film.

5.2.14 Isolation of primary OBs

For the isolation of OBs, calvariae of wildtype mouse embryos were isolated from mice not older than PND5. For STAT5$^{-/-}$ OBs, embryos were taken at E18.5. The whole preparation was conducted in sterile conditions under the laminar air-flow box. With a pair of fine scissors, the skin around the upper head of the embryo was removed and the calvaria was cut out with a cut around the head above the ears, eyes and nose. The calvariae were washed in PBS and the outer parts of neck or nose tissue were removed. Calvariae were digested in 1 ml 0.1% collagenase/0.1% dispase mix in α-MEM for 10 min at 37°C and 900 rpm shaking. After this first 10 min of digestion, the supernatant was discarded and 1 ml new digestion mix was added. The incubation time for digestion was always 10 min at 37°C and 900 rpm. After the second digestion, the supernatant was collected on ice and this procedure was repeated until the fifth digestion. The supernatants from the same calvaria were pooled, and after five digestions, centrifuged at 583 g for 5 min (RT). The supernatant was removed and the pellet was plated for each calvaria onto a 5 cm cell culture dish at 37°C, 5% CO_2 in α-MEM with 10% FCS, 1% pen/strep, 1% NEAA. Cells were split at confluency, but not more than twice. To achieve typical osteoblastic phenotype, cells were differentiated at confluency by adding 10mM β-glycerophosphate and 50 μg/ml ascorbic acid to the medium.

5.2.15 Treatment of primary OBs with GH

Unless otherwise stated, the cells were treated with 200 μg of GH for 2 h.

5.2.16 Culture of stromal cell line FBMD1

The FBMD1 cell line was established from primary BM and can be differentiated into adipocytes. Cells were cultured in IMDM with 10% FCS, 5% HS, 1% pen/strep, 10^{-5}M hydrocortisone and 10^{-5}M β-mercaptoethanol at 37°C and 5% CO_2.

5.2.17 The CAFC assay

This co-culture assay allows the numbers of HSCs in the BM of an organism to be estimated. FBMD1 cells were cultured in the inner 60 wells of a 96-well plate until they were confluent. The outer wells were filled with sterile water for optimal humidification. The BM was seeded onto the feeder layer in six different dilutions. Starting with the dilution of 4.05×10^5 cells/ml, this suspension was diluted five times

each 1:2, and thereby ending in dilution six with 1,667 cells/ml. To obtain a statistically reliable result, 20 wells were filled with BM cells from the same dilution.
The feeder cells for the CAFC assay can be varied, primary OBs or primary BM stromal cells can also be used. To ensure that the feeder cells survived during the duration of the assay (5–7 weeks), the 96-well plates were pre-coated with 0.5% gelatine before seeding the feeders.
After 35 days with a weekly medium change, every well was observed for cobblestone area-forming cells. Formation of these special colonies in a feeder cell-submitted surrounding is a unique feature of HSCs. The investigated wells were scored as 'positive' if there were cobblestone-forming areas or 'negative' if there were no cobblestone-forming areas. Due to the number of negative wells in a certain dilution and the distinctive cell number of the dilution, a frequency of HSC was calculated using Poisson statistics.

5.2.18 Isolation of vascular and endosteal HSCs

After killing mice with either CO_2 or cervical dislocation, the hindlimbs and the upper arms were prepared and all muscles were removed. HSCs from the vascular niche were harvested by flushing the BM with a 1 ml syringe, filled with IMDM medium containing 2% FCS and 1% pen/strep. To harvest the tightly attached HSCs of the endosteal HSC niche, the BM was first flushed out with medium, as described above. The empty bones were cut into very small pieces and these pieces were digested with a 0.1% collagenase/0.1% dispase mix in IMDM without supplements for 2 h at 37°C at 900 rpm shaking. After digestion, the mix was filtered over a cell strainer, centrifuged at 583 g for 10 min (RT), and the cell pellet was diluted in CAFC medium. The cell suspension was then used in the CAFC assay or FACS analysis.

5.2.19 LDA – *in vivo* analysis via BM transplantation into lethally-irradiated mice

This method allows the determination of whether or not a HSC is able to repopulate a lethally-irradiated mouse. To distinguish between the investigated cells and the innate cells of the irradiated mouse, two specific mouse strains were used, which differ only in the isoform of a cell surface alloantigen named CD45. All recipient mice had the isoform CD45.1, all mice serving as donors for transplantation had the isoform CD45.2. Mice were lethally irradiated with two times 5.5 Gy with time lag of

at least 2 h to keep the destruction of the intestine low. For transplantation, the mice were injected i.v. with the mixture of compromised BM cells and the test cells. Thereby, the test cells were applied in three different dilutions to estimate the number of HSCs among the test cell population, whereby the amount of compromised cells was constant for every transplanted mouse. The compromised cells guaranteed on the one hand the survival of the lethally-irradiated mice and performed selective pressure on the tested cells on the other hand.

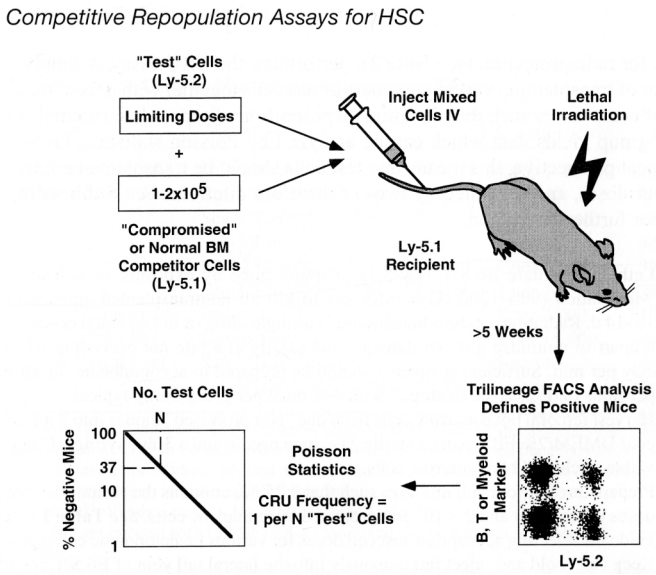


Figure 5.1: Performance of the LDA in CD45.1 (Ly 5.1) mice. Figure from Klug and Jordan 2004, "Hematopoietic Stem Cell Protocols".

Fifteen weeks post-transplantation, the transplanted mice underwent a blood analysis. After lyzing the erythrocytes with NH_4Cl solution according to standard protocols, leukocytes were stained for B cells (B220), T cells (CD3e), granulocytes (GR1, CD11b) and CD45.2 with fluorescent conjugated FACS antibodies, also according to standard protocols. Only if in every cell population (T cells, B cells and granulocytes) at least 5% CD45.2-positive cells were detected, the mouse was successfully transplanted and thereby scored as 'positive'. According to Poisson statistics, the number of 'negative' (not successfully transplanted) mice and the number of transplanted test cells led to a frequency of HSCs after statistical analysis.

5.2.20 Establishment of compromised BM cells for *in vivo* LDA

Compromised BM cells are essential for the survival of mice in transplantation experiments after lethal irradiation, and they simultaneously perform selective

pressure on the test BM cells. For the establishment of CCs (Compromised BM cells), a small group of CD45.2 mice (six to eight animals) were lethally irradiated with 2 x 5.5 Gy. Following each of these, mice received intravenously 5×10^6 freshly isolated BM cells from a healthy CD45.2 mouse. Four to six weeks post-transplantation, these mice were killed and their BM was isolated. The isolated BM was then in turn injected intravenously into the tail veins of a new group (10–12 CD45.2 mice) of lethally-irradiated animals, with 5×10^6 cells per mouse. Six to 12 weeks after the second transplantation, the BM of the transplanted CD45.2 mice was applied as compromised cells in the LDA. Unless otherwise stated, every mouse always received 2×10^5 compromised cells together with its individual number of CD45.1-positive test cells.

5.2.21 'Homing assay' with CFSE-labeled BM cells

HSCs directly interact with their environment. To investigate if and how efficient this special surrounding is in supporting the HSCs, the homing assay with CFSE-labeled cells was applied. A cohort of mice (four to 10) was sublethally irradiated with 8 Gy. The mice were left for four days to ensure myeloablation, and thereby to clear space for the labeled transplanted cells in the BM. BM cells were isolated from wildtype mice, lineage depleted (lineage cell depletion kit from Myltenyi Biotec), and labeled for 10 min with 10μM CFSE in PBS with 0.5% FCS at 37°C. The staining was stopped with PBS including 20% FCS. After centrifugation for 7 min at 350 *g* (RT), cells were washed with IMDM containing 1% pen/strep and 2% FCS. The sorted and labeled cells were injected intravenously into the irradiated mice with 1×10^6 cells per mouse. Twelve hours after transplantation, the BM was analyzed by FACS analysis with the FACSCanto II for CFSE-positive ($CFSE^+$) cells in the BM.

5.2.22 Von Kossa - staining

Lumbar vertebral bodies (L3–L5) and one tibia of each mouse were dehydrated in ascending alcohol concentrations and embedded in methylmethacrylate as described previously (Amling et al. 1999). Sections of 5 µm were cut in the sagittal plane on a Microtec rotation microtome (Techno-Med, Munich, Germany). These sections were stained by toluidine blue and by the van Gieson/von Kossa procedure as described (Amling et al. 1999).

5.2.23 Animal breeding and husbandry

Animal husbandry was performed by the animal care of the Leibniz Institute for Age Research, the IVTK of the FSU Jena and the IVTK at the clinical center of the FSU Jena, according to current guidelines of the German Animal Protection Law.
Wildtype animals (C57/Bl6) and Ly5.1 mice were delivered from Jackson Laboratories.

5.2.24 Applications on mice

GH was injected into the peritoneum (i.p.) with a daily dose of 2.5 mg/kg for five weeks (five days a week).
Transplantations with BM were injected intravenously (i.v.).
Long-term applications of 17-β-E_2 were achieved with 17-β-E_2 pellets (0.36 mg/60 day-release). The pellets were implanted under the skin of the back of anesthetized mice, and the wound was closed with a metal clamp (9 mm).

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7 Abbreviations

α-ERKO	Estrogen Receptor α - knockout
α-MEM	Minimal essential media with α-modification
5-FU	5-Fluorouracil
17-β-E_2	17-β-estradiol
AlP	Alkaline Phosphatase
APC	Allophycocyanin
APC-Cy7	Allophycocyanin - anti-cyanine 7
APS	Ammonium persulfate
BM	Bone Marrow
BMD	Bone Mineral Density
BrdU	Bromdesoxyuridin
BSA	Bovine serum albumin
°C	degree celsius
CA	Cobblestone Area
CAFC	Cobbblestone-Area-Forming-Cell assay
CFSE	5(6)-Carboxyfluorescein diacetate N-succinimidyl ester
C-terminal	Carboxy-terminal
DCC	Dextran-treated charcoal
DCC-HS	Dextran-treated charcoal horse serum
DCC-FCS	Dextran-treated charcoal fetal calf serum
DKFZ	Deutsches Krebsforschungs Zentrum, Heidelberg
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
DTT	Dithiothreitol
E	Embryonic day
ECM	Estracellular Matrix
ECL	Enhanced chemiluminescence
EDTA	Ethylen-diamin-tetra-acetate
e.g.	example given
ERE	Estrogen Responsive Element,
ERK	Extracellular-Signal Regulated Kinase
et al.	And others
f.k.a.	Formerly known as
FACS	Flourescence Activated Cell Sorting
FCS	Fetal calf serum
FITC	Fluorescein isothyocyanate
FLI	Fritz-Lipmann-Institute
FLI	Fritz-Lipmann-Institute, Leibniz-Institute for Age Research
Flt 3	fms-like Tyrosine Kinase 3, also flk 2
FSU	Friedrich-Schiller-University Jena
GH	Growth hormone
GHD	Growth Hormone Deficiency
GPR 30	G-coupled protein Receptor 30
hHSC	human Hematopoietic Stem Cells
HS	Horse serum
HSC	Hematopoietic Stem Cells
HSC	Hematopoietic Stem Cell
IGF1	Insulin-like growth factor 1
IL	Interleukin
IMDM	Iscove´s modified Dulbecco media
IVTK	Institut für Versuchstierkunde (Institute for experimental animals Jena, belonging to the FSU)
kb	Kilobase
kD	Kilodalton
ko	Knockout
LDA	Limiting-Dilution-Analysis

LSK	Lineage-negative, Sca1-positive, cKit-(CD117)positive cells
LT	Long-term
LT-HSC	Long- term Hematopoietic stem cells
mA	Milli-Ampere
mHSC	Murine Hematopoietic Stem Cells
NEAA	Non-essential amino acid
NFκB	Nuclear factor κ B
N-terminal	Amino-terminal
OPG	Osteoprotegrin
OPN	Osteopontin
PAGE	Polyacrylamideelectrophorese
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
Pen/Strep	Penicillin / Streptomycin
PI3K	Phophatidylinositol 3-kinase
PND	Post-natal day
PY	Pyronin Y
RNA	Ribonucleic acid
RT	Room Temperature
rt-PCR	Real-time polymerase chain reaction
Runx2	Also known as cbfa1, essential TF in osteoblasts and chondrocytes
SDS	Sodiumdodecylsulfate
siRNA	Small interfering ribonucleic acid
SLAM	Signaling lymphocyte activation molecule
SOCS	Suppressor of cytoline signaling
ß-ERKO	Estrogen Receptor ß - knockout
STAT	Signal transcucer and activator of transcription
$STAT5^{End}$	Conditional knockout mouse for STAT5 only in endothelial cells
$STAT5^{OB}$	Conditional knockout mouse for STAT5 only in osteoblasts
TBS-T	Tris-buffered saline with Tween 20 (polysorbate detergent) as detergent
TF	Transcription Factor
TF	Transcription Factor
TNF	Tumor necrosis factor
V	Volt
WT	Wild type

8 Table of figures

9 Selbständigkeitserklärung

Hiermit erkläre ich, diese Arbeit selbständig verfasst und keine anderen als die angegebenen Hilfsmittel verwendet zu haben. Wörtliches oder indirekt übernommenes Gedankengut wurde nach bestem Wissen als solches gekennzeichnet.
Mit ist die geltende Promotionsordnung der FSU Jena bekannt. Ich habe die Dissertation selbst angefertigt und habe alle von mir benutzten Hilfsmittel, perönliche Mitteilungen und Quellen in meiner Arbeit angegeben. Ich habe keine Hilfe eines Promotionsberaters in Anspruch genommen und Dritte haben weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten die im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen.
Gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung wurden von mir bei noch keiner anderen Hochschule eingereicht.

Anett Illing, Dezember 2008

10 Lebenslauf

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Werdegang

Derzeitige Tätigkeit seit 12/2004	Doktorarbeit am FLI Jena zum Thema „Wirkung von osteogenen Hormonen auf Hämatopoetische Stammzellen“
Hochschulstudium 10/1999 – 11/2004	Studium der Biologie an der TU Dresden mit Abschluss zur Diplombiologin, Hauptfächer: Zoologie, Genetik, Biochemie Diplomarbeit am Institut für Zoologie, Thema: „Wirkung von *Cimicifuga racemosa* auf die Genexpression im Uterus von DA/Han-Ratten und auf die Fähigkeit von immortalisierten Säugerzellen / Uterus-Karzinomzellen im Soft Agar Kolonien zu bilden“
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Realschulabschluss 09/1986 – 06/1996	Besuch der Grund- und Mittelschule Rittersgrün, Abschluss: Mittlere Reife

Außeruniversitäre Aktivitäten während des Studiums

Praktikum am MPI für Anthropologie Leipzig 03/2003-04/2003	Molekularbiologische Tätigkeit in der Gruppe „Molekulare Anthropologie und Populationsgeschichte“ zum Thema „Genetische Differenzierung zwischen Deutschland und Polen anhand von SNP-Markern“
01/2002 – 12/2003 Freie Mitarbeiterin im „Deutschen Hygienemuseum Dresden“	Zuarbeiten für den Projektleiter des „Gläsernen Labors“, Betreuung von molekularbiologischen Projekttagen für Schüler

EDV-Kenntnisse

- Sehr gut vertraut mit PC und Apple Systemen
- Microsoft Office, Endnote, Photoshop, Adobe Reader und Illustrator, Canvas
- iQ 5.0 zur Real-time PCR-Analyse, FACS-Diva, Agilent bioanalyzer
- Diverse Datenbanksysteme zur Katalogisierung von Mauszuchten

Sprachen

- Englisch (sehr gut)
- Französisch (Grundkenntnisse)

11 Poster, Vorträge, Veröffentlichungen

Posterpräsentationen	FLI Klausurtagungen 2006 (18. - 20.06) und 2007 (03. - 05.07) Spetses International Summer School 2007 (31.08 - 09.09) Tagung „Genetics and Aging“ 2007 in Jena Titel der Poster: „Effects of osteogenic hormones on hematopoietic stem cells and their environment“
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