lin$^-$Sca-1$^+$ Cells and Age-Dependent Changes of Their Proliferation Potential Are Reliant on Mesenchymal Stromal Cells and Are Leukemia Inhibitory Factor Dependent

Elisabeth Schraml [1,2], Christine Fehrer [3], Regina Brunauer [3], Günter Lepperdinger [3,*], Vera Chesnokova [4], Konrad Schauenstein [1,†]

[1] Institute of Pathophysiology and Immunology, Center of Molecular Medicine, Medical University of Graz, Heinrichstraße 31A, 8010 Graz, Austria

[2] present address: Institute of Applied Microbiology, University of Natural Resources and Applied Life Sciences Vienna, Muthgasse 18, 1190 Vienna, Austria

[3] Institute for Biomedical Aging Research, Austrian Academy of Sciences, Rennweg 10, 6020 Innsbruck

[4] Cedars-Sinai Medical Center, 8700 Beverly Blvd., Los Angeles, CA 90048, USA

† Deceased before completion of the manuscript

*** Corresponding author:** Günter Lepperdinger, Institute for Biomedical Aging Research, Austrian Academy of Sciences, Rennweg 10, A-6020 Innsbruck. Tel.: +43 512 583919 34, Fax: +43 512 583919 8; Email: guenter.lepperdinger@oeaw.ac.at

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Abstract

Aging as a process is paralleled by a variety of hematological alterations. Characteristic features are a diminished homeostatic control of blood cell production and a decline in immune functions. It is generally accepted that stromal cells play a basal role in hematopoiesis by providing survival and differentiation signals, by secreting cytokines, or through direct contact with hematopoietic stem cells, thereby supporting the generation and replenishment of hematopoietic progenitor cells (HPC). Here we demonstrated that HPC-related colony formation is positively influenced by mesenchymal stromal cells (MSCs) when grown in co-culture, in particular regarding the number of primary granulocyte/macrophage colony-forming units as well as with respect to the average size of the formed colonies. These effects were more pronounced when the MSCs originated from young donors than from old ones. Because leukemia inhibitory factor (LIF) plays an important role during hematopoiesis, properties of lin-- Sca-1+ cells and MSCs derived from LIF-deficient mice (LIF--/--) were determined both ex vivo and in vitro. LIF--/-- animals contain a significantly reduced number of lin-- Sca-1+ cells, nevertheless the replating capacity of LIF--/-- HPCs was found to be generally unchanged when compared to those from LIF+/+ animals. However, when cocultured with MSCs, LIF--/-- lin-- Sca-1+ cells exhibited comparable characteristics to HPCs derived from old wild-type animals.

Introduction

Bone marrow (BM) contains hematopoietic stem cells (HSCs) and progenitor cells (HPCs), osteoblasts, endothelial cells and nonhematopoietic multipotent stromal cells of mesenchymal origin, also called mesenchymal stromal cells (MSCs) [1]. BM MSCs participate in the formation of the BM microenvironment by providing growth factors and the appropriate extracellular matrix, both of which are intimately involved in the regulation of various intricate processes during hematopoiesis [2]. When provided as feeder cells in vitro, MSCs increase the expansion of HSCs and HPCs [3]. In these coculture systems, the stromal cells are generally considered to provide instructive signals that promote the expansion of HSCs through two major mechanisms: first through secretion of growth factors and cytokines [4, 5] that stimulate proliferation of HSCs and control the differentiation of their transiently proliferating progeny; second, stromal cells provide direct cell-cell contacts, which are again functionally relevant in terms of HSC proliferation and differentiation [6]. It is important to note in this context that MSCs are capable of inhibiting HPC differentiation and, to decrease the rate of apoptosis of cultured HPCs [7, 8]. MSCs isolated from aged donors contain a reduced number of rapidly self-renewing cells and the number of colony-forming units is decreased. Moreover, the number of MSCs which are enlarged in size and are thus considered degenerated due to stress-induced or replicative aging is markedly increased with elevated donor age. This implies that aging goes along with the gradual loss of proliferation capacity as well as with a decline in MSC fitness [9].

Cellular aging is also reflected by differential expression of specific marker genes. Senescence evasion factor (SNEV; hPrp19, hPso4, hNMP200), a multifaceted protein, which has been described only recently, appears to be a suitable marker in this respect. The human SNEV exhibits in vitro E3 ubiquitin ligase activity [10] that

directly binds to the β7 subunit of the 20 S proteasome [11]. Furthermore, SNEV is involved in DNA repair [12, 13] and pre-mRNA splicing [14], and is essential during mouse embryonic development [15]. SNEV was initially identified as being downregulated at replicative senescence in human umbilical vein endothelial cells (HUVECs) [16]. High levels of SNEV was shown to extend the lifespan of HUVECs by increasing their resistance to stress or by improving their DNA repair capacity [17]. On the other hand, senescent cells of different tissue origin were shown to express decreased SNEV transcript levels. Enhanced expression was found in several tumor cell lines [17]. Taking these observations together, SNEV appears to primarily have effects on the replicative potential of cells.

Leukemia inhibitory factor (LIF) is a multifunctional cytokine, which is expressed in many organs and tissues [18]. After BM has been irradiated, LIF is thought to act on the stromal cells, thereby promoting cytokine expression in the marrow, which in due course supports long-term repopulation of hematopoietic cells [19]. In competent cells, the factor binds to a heterodimeric membrane receptor, which is comprised of a low-affinity LIF-specific receptor and the gp130 receptor subunit. The latter also functions as a receptor subunit for IL-6, oncostatin M, cardiotrophin-1 and ciliary neurotrophic factor [20]. Only recently has it been demonstrated that LIF also influences the fate of MSCs [21]. Furthermore, LIF has also been suggested to play a role in controlling neural differentiation, as well as in regulating the maintenance of stem-cell-derived murine-spiral-ganglion neuron precursors [22]. When provided as a feeder cell layer, human embryonic lung fibroblasts over-expressing LIF efficiently support the self-renewal of human embryonic germ cells [23].

Taking this into account, we investigated the functional role of MSCs and their age-associated effects with respect to purified BM lin-- Sca-1+ cells [24] derived from young (8--12 weeks of age) and old (1.5--2 years) C57BL/6 mice, as well as those

derived from from LIF-deficient mice. There were several reasons behind our decision to use lin-- Sca-1+ cells for our investigations.lin-- Sca-1+ One important factor was that the number of lin-- Sca-1+ c-kit+ cells, which are widely used, is very low (0.005% of BM) [25, 26], and therefore it would have been necessary to conduct detailed analysis to pool cells from individual animals. Because it is generally accepted in biogerontology that aged, inbred laboratory mice show individual phenotypic alterations and also become prone to a variety of age-associated diseases, we refrained from combining cells derived from different individuals. It is, furthermore, rather difficult to obtain old animals. Experimental results presented in this contribution suggest a role for LIF as an important factor controlling the proliferative potential of HSCs and their respective progenitor cells.

Materials and Methods

Experimental Animals

Young male C57BL/6 (n = 9) mice were purchased from the Institut für Labortierkunde und -genetik, Himberg, Austria. Aged C57BL/6 (n = 9) mice were provided by the Institute for Biomedical Aging Research, Austrian Academy of Sciences, Innsbruck, Austria. Young male LIF-deficient (LIF--/--, n = 9) and male wild-type (LIF+/+, n = 8) mice were provided by the Cedars-Sinai Medical Center, Los Angeles, Calif., USA and were maintained under specific pathogen-free conditions. LIF--/-- animals had been bred on a B6D2F1 genetic background [27] and diagnosed by PCR DNA analysis of biopsied tail tissue, as described previously [28]. Animals between 8 and 12 weeks of age were considered to be young and those between 1.5 and 2 years of age were considered to be old. All mice were kept and treated according to the institutional guidelines of the Medical University of Graz, and according to Austrian Law.

Bone Marrow Cell Isolation

For the preparation of cells, mice were first anesthetized by ether inhalation before being sacrificed by cervical dislocation. BM cells were flushed from the tibia, femur and humerus under sterile conditions. Cells were washed in phosphate-buffered saline (PBS) and subsequently centrifuged. After lysis of the erythrocytes in zapoglobin buffer (Beckman Coulter), the nucleated cells were counted using a Casy cell counter (Schärfe System, Germany). Cells were either centrifuged and adjusted to the desired cell concentrations in cell staining buffer [PBS supplemented with 3% fetal calf serum (PAA, Pasching, Austria) and 0.1% sodium azide] for subsequent cell sorting, or seeded at a density of 1.5 × 10e6 cells/well in a 24-well plate in RPMI 1640 medium supplemented with antibiotics and 20% fetal calf serum and cultivated at 37°C in a humidified atmosphere containing 5% CO_2 in air. The stromal cell layer was washed

after 24 h and cultivated for a further 6 days to establish an 80% confluent layer before commencing cocultures with HSCs.

Primary and Secondary Granulocyte/Macrophage Colony-Forming Unit Assay

Lineage negative (lin--) cells were isolated from BM with the aid of magnetic beads as provided in the Hematopoietic Progenitor Cell Enrichment Set – DM (BD Biosciences, San Diego, Calif., USA). After lin-- cells had been sorted with a FITC-labeled anti-Sca-1 antibody (BD Pharmingen, San Diego, Calif., USA) the purity of the cells (95--98%) was confirmed using FACS Scan (BD Biosciences). The cells were sorted by means of a FACS Vantage (BD Biosciences) into a 24-well plate containing 250 µl of methylcellulose media without erythropoietin (CellSystems, St. Katharinen, Germany) supplemented with cytokines (50 ng/ml rmSCF, 10 ng/ml rmIL-3, 10 ng/ml rhIL-6). After 4 days of incubation at 37°C in a humidified atmosphere containing 5% CO_2 in air, primary granulocyte/macrophage-forming colonies (CFU-GM) were scored and the size of each colony was measured using an ocular micrometer. Forty primary colonies, each consisting of at least 50 cells, were plucked individually. Single colonies were transferred separately to a 24-well plate containing 250 µl methylcellulose medium. By thoroughly mixing with methylcellulose, colonies were dispersed and yielded a single-cell suspension, which was subsequently incubated for 4 days at 37°C in humidified 5% CO_2 in air. For calculation of secondary colony forming ability we used the method described by Gordon and colleagues [29--31] with minor modifications. Briefly, as the distribution of the number of secondary colonies of a well was strongly skewed so that median, not mean, values are appropriate, counts were first transformed by adding 1 before taking the log 2. The arithmetic mean of the transformed numbers of secondary colonies of a well was the measure of the secondary replating capacity. We adopted this change from the originally described procedure for the following reasons: the

logarithm of 0 cannot be calculated, the logarithm of the complement of a distribution function has insufficient statistical properties and the area under the curve (AUC) of a distribution function is a linear transformation of the arithmetic mean and consequently does not change the usual test statistics. The proliferation index (AUC) was calculated using the trapezium rule [29--31] and reflected the capacity for self-renewal of CFU-GM. Furthermore, varying concentrations of purified mouse LIF (kindly provided by Maria Sibilia, Medical University, Vienna, Austria) were added. Each well was scored for the presence and number of GM-colonies consisting of more than 50 cells.

Quantitative Reverse-Transcriptase PCR

Total RNA was extracted with the aid of an RNeasy Mini Kit (Qiagen, Vienna, Austria), following the manufacturer's protocol. HPCs and MSCs were separated: first the HPCs were removed from the culture by carefully transferring them to RNA extraction buffer using a pipette. Then the remaining MSCs were trypsinized before commencing the RNA extraction procedure. RNA was digested with RQ1 RNase-free DNase (Promega, Mannheim, Germany). 50 ng and 100 ng RNA were reverse-transcribed using the Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen, Lofer, Austria). Quantitative PCR was performed with the aid of an iCycler (Biorad, Vienna, Austria). All samples were analyzed in duplicate and serial dilutions of cDNA from cells from primary control colonies were included as standards. Transcript levels were normalized for β-actin transcripts and the identities of PCR products were verified by melting curve analysis. The PCR reaction mixture contained 10 µl of iQTM SYBR Green Supermix (Biorad, Vienna, Austria), 5 pmol of gene-specific primers (LIF: forward 5□-GCTATGTGCGCCTAACATGAC-3□, reverse 5□-CGCTCAGGTATGCGACCAT-3□; SNEV: forward 5□-CAA GCC TCC CTC CGC CAC CAG-3□, reverse 5□-TTC TCG AGC AGC AGT GAC CT-3□; β-actin: forward 5□-

GGC TGT ATT CCC CTC CAT CG-3□, reverse 5□-CCA GTT GGT AAC AAT GCC ATG T-3□) and 1 or 2 µl of cDNA.

Statistics

Data were statistically analyzed using Student's t test, one-way ANOVA and one-way ANOVA followed by Dunn's method, as indicated. Analyses were performed with SigmaPlot 10.0 (SigmaPlot, Germany). The tests were two-sided with a probability of type 1 error of 0.05. Data are presented as mean values ± SEM.

Results

In vivo, HSC and HPC cells are considered to be in close contact with MSCs, and thus besides changes at the systemic level, age-dependent alterations in intercellular communication systems are thought to influence the steady state of self-renewal and the generation of progenitor cell types. We therefore examined the number of cells that are capable of growing into granulocyte/macrophage-forming colonies in vitro, as well as their respective secondary colony-forming ability. Both properties were found to be greatly decreased in animals of advanced age (fig. 1). In extension of these initial findings, we also observed that primary cultures of stromal cells derived from BM positively affect colony-forming ability and colony size of lin-- Sca-1+ cells (fig. 2a, b). In this particular context, we would like to point out that we observed no or only little physical contact between lin-- Sca-1+ colonies and MSCs, which most likely was due to the glutinous properties of the methylcellulose contained in the medium. Yet when lin-- Sca-1+ cells were cultured in the presence of MSCs as feeders, a significant increase of colony-forming ability, regardless of whether MSCs were derived from young or old animals became evident. In either situation, lin-- Sca-1+ cells derived from young animals formed more colonies (fig. 2c). With regard to the average size of the colonies, a significant increase was observed when young HSCs were grown in the presence of old MSCs as well as when old lin-- Sca-1+ cells were grown in culture together with young MSCs (data not shown). These observations were corroborated by monitoring the transcriptional level of SNEV, which is markedly downregulated during replicative senescence of HUVECs [17]. Moreover, we reported only recently that this factor is directly associated with the growth potential of HSCs within purified BM cell fractions [32]. Working along this line, we assessed the level of SNEV transcription in freshly isolated lin-- cells and found SNEV mRNA decreased in the

case of lin-- cells that had been derived from aged animals (fig. 3a), whereas cultured MSCs showed no significantly altered age-dependent change in SNEV expression (fig. 3b). Next we determined SNEV levels in lin-- Sca-1+ cells after growing into granulocyte/macrophage-forming colonies with and without MSCs. Elevated SNEV mRNA levels were observed in cells derived from primary colonies as compared to freshly isolated lin-- cells (fig. 3c), suggesting that induction of proliferation is paralleled by SNEV activation. In accordance with this assumption and with the aforementioned results, significantly lower levels of SNEV mRNA were present in lin-- Sca-1+ cells from both young and old donors when cultured together with MSCs, independent of donor age. This finding suggests that diffusible factors, most probably produced by MSCs, promote lin-- Sca-1+colony formation among lin-- Sca-1+ cells and increase their size. Therefore, the expression levels of various cytokines, such as LIF, IL-6, IL-7, IL-11, GM-CSF, G-CSF and c-kit ligand, were examined in young and old MSCs (data not shown). Only in the case of LIF transcript levels were found to be significantly decreased in cells derived from mice of advanced age (fig. 3d).

In light of these findings, and taking them together with previously published evidence that LIF plays a regulatory role in hematopoiesis [19], we next compared lin-- Sca-1+ cells derived from young LIF-deficient mice (LIF--/--) with those from corresponding wild-type animals (LIF+/+). As assessed by means of the CFU-GM assay, lin-- Sca-1+ cell frequency and their respective colony sizes were found to be significantly decreased in LIF--/-- mice when compared to those of wild-type mice (fig. 4a, b). Yet, secondary colony-forming ability remained unchanged (data not shown). In order to clarify whether LIF action is sufficient to rescue the decline in HSC colony formation, recombinant murine LIF was added to LIF--/-- lin-- Sca-1+ cells, which underwent the CFU-GM assay. 25 units per ml of LIF resulted in a significant increase in colony-forming capacity and colony size (fig. 4c, d).

In order to test whether it is LIF, secreted by MSCs, that supports lin-- Sca-1+ cell colony formation, lin-- Sca-1+ cells were cultured in the presence of LIF--/-- or LIF+/+ MSCs. Interestingly, lin-- Sca-1+ cells of either genotype formed increased numbers of colonies, irrespective of whether MSCs had been derived from LIF-deficient or wild-type animals. Also in this case, lin-- Sca-1+ cells from wild-type mice formed more and larger colonies (fig. 5). Only when LIF--/-- lin-- Sca-1+ cells and LIF--/-- MSCs had been cocultured, the average colony size was comparable to lin-- Sca-1+ cells that had been grown alone, indicating that a cell system which lacks LIF secretion is incapable of enhancing the growth of lin-- Sca-1+ cells. (fig. 5d). This finding was again substantiated by monitoring SNEV expression levels in normal, LIF-deficient lin-- cells and cultured lin-- Sca-1+ cells in the presence of MSCs. Lin-- cells, which had been derived from LIF--/-- mice, expressed SNEV at a lower level than wild-type lin-- cells (fig. 6). After growth of primary and secondary colonies, the cells showed significantly higher SNEV expression. However, in primary and secondary colonies of both mutant and wild-type mice, only little change in SNEV transcription level was observed. In accordance with the results described above, SNEV expression was also found to be unchanged when cells of either genotype had been grown in coculture with MSCs. The expression levels of GM-CSF, G-CSF and c-kit ligand were found to be similar in LIF--/-- and LIF+/+ MSCs, while IL-6, IL-7 and IL-11 transcription appeared slightly, yet not significantly, increased (data not shown).

Taken together, lin-- Sca-1+ cells derived from old animals and from young LIF--/-- animals displayed similar properties when compared to control animals (table 1), with a single exception: secondary colony forming capacity was found to be comparable in LIF--/-- and wild-type animals.

Discussion

In adult animals, the BM is the major site of blood production. It is now generally accepted that the sole way in which most mature blood cells are produced is through determination and differentiation of progenitors that originate from HSCs. These intricate processes are thought to occur in the extravascular spaces close to the bone surface and blood sinuses, and they appear to be intimately guided by BM stromal cells, which are closely associated within a scaffold composed of well-organized extracellular matrix [33]. The relationship between these two closely associated, yet distinct, cell types and the maintenance of the stem cell characteristics of HSCs, as well as proliferation and differentiation of hematopoietic progenitors, has recently been investigated in great detail using mutant mouse models. It is important to note in this context that mice which exhibit major defects in bone development and/or bone remodeling may also show a partially dysfunctional hematopoiesis. These findings together resulted in the conceptual framework that within BM, osteoblasts and/or osteoclasts are constantly morphing the primary HSC niche, and furthermore support the notion that these cells exert distinct functional roles during hematopoietic cell differentiation [34--36]. In mice, distinct age-dependent changes within the HSC compartment have been identified. Most notably, HSC numbers increase while homing efficiency is markedly decreased. This is paralleled by a skewing of myeloid differentiation potential [37]. In particular, in full-grown C57BL/6 (B6) mice, HSCs on average make up 0.004% of the mononuclear cells within the BM [38]. The HSC pool size may vary to some extent between common strains of laboratory mice that are regularly used for this type of study, as shown by de Haan et al. [39, 40], who have investigated strain-to strain differences in 5 particular strains (C3H/He, CBA/J, DBA/2, BALB/c and C57BL/6), using a cobblestone-area-forming cell assay. In addition, age-related changes ~~to~~ of the proliferative activity of primitive cells could be observed.

These are heavily strain-dependent and, most interestingly in this context, these changes appear to be related to the maximal lifespan of the mouse strain. For example, in C57BL/6, a long-lived strain which we used in the present study, individual animals of advanced age showed 2- to 3-fold more primitive cells than their younger counterparts.

MSCs are adherent cells of nonhematopoietic origin. It is believed that MSCs are capable of giving rise to a variety of terminally differentiating cell types, such as osteogenic precursors. Furthermore, it has been suggested that these cells regulate localization, self-renewal and differentiation of HSCs, in particular by secreting cytokines and growth factors, such as a variety of ILs, GM-CSF and LIF, as well as distinct extracellular matrix proteins. These factors establish a prevalent microenvironment, which is instructive to primitive hematopoietic cells, in particular regarding their clonogenic capacity, as well as many other functional characteristics [41]. Only recently has it been demonstrated in an elegant series of experiments that soluble, bioactive factors, which are secreted by MSCs, are sufficiently capable of sustaining hematopoiesis. These observations further nurtured the notion that direct cell-to-cell contacts are not required for proper hematopoiesis in vitro [42].

The aging of a biological system corresponds well to two major criteria. Firstly, mortality rate increases with the age of the organism, and secondly, characteristic phenotypic changes within an individual emerge and are manifested during later stages in life [43]. Features of HSCs are their clonogenicity, their capacity to greatly proliferate, and the potential of their progeny to differentiate into multiple cell lineages in the hematopoietic system during organism's lifetime [44, 45]. It is, therefore, interesting in the context of organism aging to consider whether alterations in intercellular communication between HSCs/HPCs and MSCs emerge in an age-dependent manner. We analyzed the basic properties of lin-- Sca-1+ cells, such as

their colony-forming and replating abilities when brought in conjunction with MSCs. Working along this line, we could for the first time show that regardless of whether lin-- Sca-1+ cells had been obtained from young or old animals, MSCs positively influenced lin-- Sca-1+ cells in culture to form colonies and enhancing their corresponding proliferation rates. These findings could be further substantiated by measuring the transcriptional levels of SNEV in the context of lin-- Sca-1+ cells forming primary and secondary colonies. This marker has previously been recognized, because it is strongly downregulated in cells that have undergone irreversible growth arrest at the point of replication senescence. Interestingly, high levels of SNEV appear to extend the life span of HUVEC by increasing their resistance to stress, most probably by enhancing the cells' capacity to repair DNA [17]. Here we demonstrate that SNEV expression is decreased when lin-- Sca-1+ cells had been grown in the presence of MSCs. This suggests that MSCs may relieve pressure on lin-- Sca-1+ cells, thereby exerting a protective function.

To elucidate the molecular nature of signals that support the growth and fitness of lin-- Sca-1+ cells, we searched for signaling molecules that are secreted by MSCs and thus may exert an instructive influence on the proliferation of lin-- Sca-1+ cells in culture. One putative candidate, LIF, was found to be differentially regulated in MSCs when derived from aged animals. Next, we showed that lin-- Sca-1+ cells numbers were significantly decreased in LIF--/-- mice, while the addition of recombinant LIF supported the colony-forming ability of lin-- Sca-1+ cells in culture. A second important finding was that in the presence of MSCs, the colony-forming ability of lin-- Sca-1+ cells appeared to be independent of LIF. However, irrespective of whether LIF originated from lin-- Sca-1+ cells or MSCs, the size of the colonies was increased. This finding strongly supports the notion that the proliferative capacity of lin-- Sca-1+ cells relies on LIF.

These data closely fit with the recent observation that the injection of LIF into mice leads to increased numbers of megakaryocytes and platelets [46, 47]. In line with these findings, murine hematopoietic progenitor cells have been shown to markedly expand in the presence of a combination of LIF-SCF-IL-1β or LIF-SCF-IL-3 [48]. Furthermore, the addition of LIF to purified CD34+thy-1+ cells and AC6.21, a murine BM-derived stromal cell line, has resulted in the expansion of CD34+ thy-1+ cells without gross phenotypic changes [49, 50].

Conclusion

Age-dependent hematological alterations that eventually lead to a decline in immune functions may be causally related to survival and differentiation signals derived from stromal BM cells. And indeed, HPC-related colony formation was found to be positively influenced by MSCs. Interestingly, lin-- Sca-1+ decreased the levels of the novel aging and cellular stress marker SNEV, when grown in the presence of MSCs. This is indicative for MSCs being protective, because they provide HPCs with a proper niche. In addition to this finding, it became apparent that MSCs in coculture with lin-- Sca-1+ cells support colony formation in a LIF-independent manner. LIF--/-- animals contain a significantly reduced number of lin-- Sca-1+ cells, which, however, exhibited comparable characteristics to HPCs derived from old wild-type animals. In the analyses presented here, lin-- Sca-1+ cells from old animals and from young LIF--/-- animals behaved similarly, both with respect to HPC frequency and proliferative capacity. In conclusion, our data greatly strengthen the assumption that LIF plays a pertinent functional role during the complex cellular interplay of hematopoiesis in vivo.

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Table 1.: Overview of functional properties of HPCs regarding cells derived from old wildtype C57BL/6 or cells from $LIF^{-/-}$ animals in comparison to cells derived from young C57BL/6 or cells from $LIF^{+/+}$ animals; decreased (↓), increased (↑), or no differences (↔).

	old C57BL/6	**LIF$^{-/-}$** B6D2F1
GM-CFU assays		
$lin^-Sca\text{-}1^+$ frequency	↓	↓
replating (self-renewal) capacity	↓	↔
$lin^-Sca\text{-}1^+$ cells + young $LIF^{+/+}$ MSC	↑	↑
$lin^-Sca\text{-}1^+$ cells + old $LIF^{-/-}$ MSC	↑	↑
colony size of cultivated $lin^-Sca\text{-}1^+$		
without MSC	↓	↓
with + young $LIF^{+/+}$ MSC	↑	↑
with + old $LIF^{-/-}$ MSC	↑	↑
SNEV expression level		
lin^-cells	↓	↓
MSC	↓	↓
$lin^-Sca\text{-}1^+$ cells + MSC	↓	↓

Figure Legends

Figure 1: Age-dependent properties of lin$^-$Sca-1$^+$ from C57BL/6 mice.

(A) Frequency of lin$^-$Sca-1$^+$ cells was significantly decreased in old mice. (B) lin$^-$Sca-1$^+$ cells from old mice, which had been re-plated, form significantly less secondary colonies. (C) Numbers of secondary colonies were further validated using the method described by Gordon et al. 1998 indicating a reduction of self renewal capacity (AUC, area-under-the-curve). Data (from 9 animals, assays performed in ten replicates) were statistically analysed applying Student´s *t* test.

Figure 2: Co-culture of lin$^-$Sca-1$^+$ cells with MSC.

(A) HPC colony-formation and (B) colony size for ten replicates regarding lin$^-$Sca-1$^+$ cells derived from 9 mice were found to be significantly increased in the presence of MSC after 4 days of cultivation. Representative examples of photomicrographs of individual cultures are depicted below the graphs in A and B. Data were statistically analysed using Student´s *t* test. (C) Young and old HSC were co-cultured as quatruplicates in the presence of a MSC layer derived from either young or old animals (N=9); in any case colony-forming ability increased in the presence of MSC. Data were statistically analyzed using one-way ANOVA.

Figure 3: Expression of SNEV and LIF in different cells types derived from BM

(A) The transcript level of SNEV was found decreased in ex vivo explanted lin$^-$ cells of old animals, while SNEV expression was found comparable in cultivated MSC of either age (B). Data were statistically analysed using Student´s *t* test. (C) SNEV mRNA expression increased significantly in lin$^-$Sca-1$^+$ cells after formation of primary colonies but decreased when cells had been grown in the presence of MSCs. This observation was independent of donor age. (D) LIF mRNA expression levels were

found significantly decreased in MSC derived from old donors compared to young donors. Data were statistically analysed using a one-way ANOVA followed by the Dunn´s method. SNEV copy numbers were standardized according to *α*-actin levels.

Figure 4: Colony formation of lin$^-$Sca-1$^+$ cells derived from LIF$^{-/-}$ mice.

(A) Colony number (B) and size of lin$^-$Sca-1$^+$ cells derived from LIF$^{-/-}$ animals (N=9, ten replicates) were found significantly decreased as compared to LIF$^{+/+}$ (N=8, ten replicates).

In the presence of exogenous LIF (C) colony number (D) and colony size formed by lin$^-$Sca-1$^+$ cells, which had been derived from either 4 LIF-deficient or 4 wild type mice were found increased after addition of 25 units/mL of recombinant LIF (ten replicates). Data were statistically analysed using Student´s *t* test.

Figure 5: Properties of LIF$^{-/-}$ lin$^-$Sca-1$^+$ cells co-cultured with MSC.

(A and B) MSC support colony formation of lin$^-$Sca-1$^+$ cells, both from LIF$^{-/-}$ (N=9) or wild type animals (N=8). Assays were carried out in quatruplicates. (C and D) LIF$^{-/-}$ lin$^-$ Sca-1$^+$ cells formed smaller colonies than lin$^-$Sca-1$^+$ cells derived from LIF$^{+/+}$, whereas the size of primary colonies was found to be increased when lin$^-$Sca-1$^+$ cells had been grown in the presence of MSCs, regardless of whether the latter had been derived from LIF$^{-/-}$ or wild type animals. Data were statistically analysed using a one-way ANOVA.

Figure 6: Expression of SNEV in hematopoietic progenitor cells after co-culture with MSCs.

Freshly isolated lin$^-$ cells from individual LIF$^{-/-}$ animals (N=9) showed significantly less SNEV mRNA expression in comparison to cells derived from LIF$^{+/+}$ animals (N=8). SNEV mRNA expression increased significantly in lin$^-$Sca-1$^+$ cells derived from LIF$^{-/-}$

animals after formation of primary and secondary colonies, yet greatly decreased when the cells had been grown in the presence of MSC for 4 days, regardless of whether these had been derived from $LIF^{+/+}$ or $LIF^{-/-}$ mice. Data were statistically analysed using a one-way ANOVA followed by the Dunn´s method. SNEV copy numbers were normalized according to *α*-actin mRNA levels.

Fig.: 1

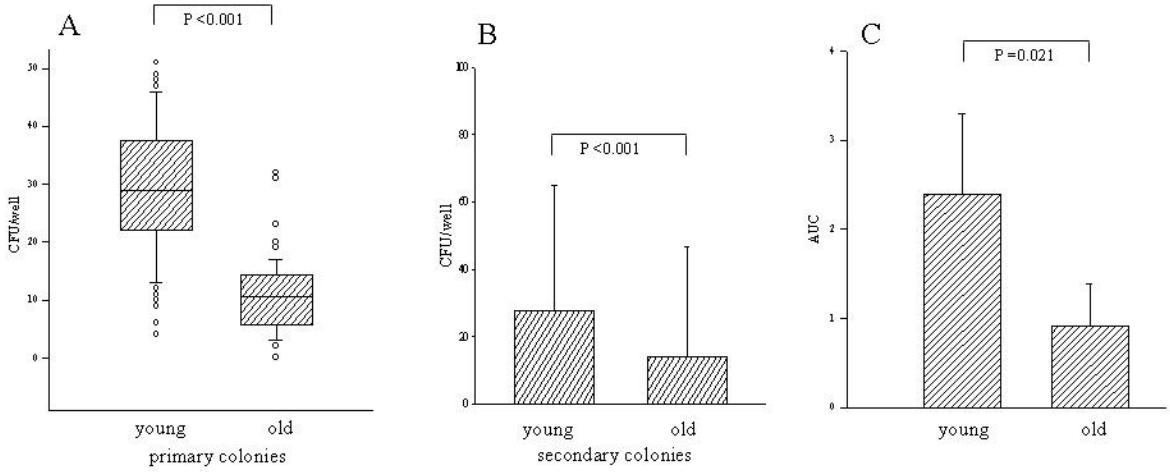

A
P <0.001
CFU/well
young
old
primary colonies
B
P <0.001
CFU/well
young
old
secondary colonies
C
P =0.021
AUC
young
old

Fig.: 2

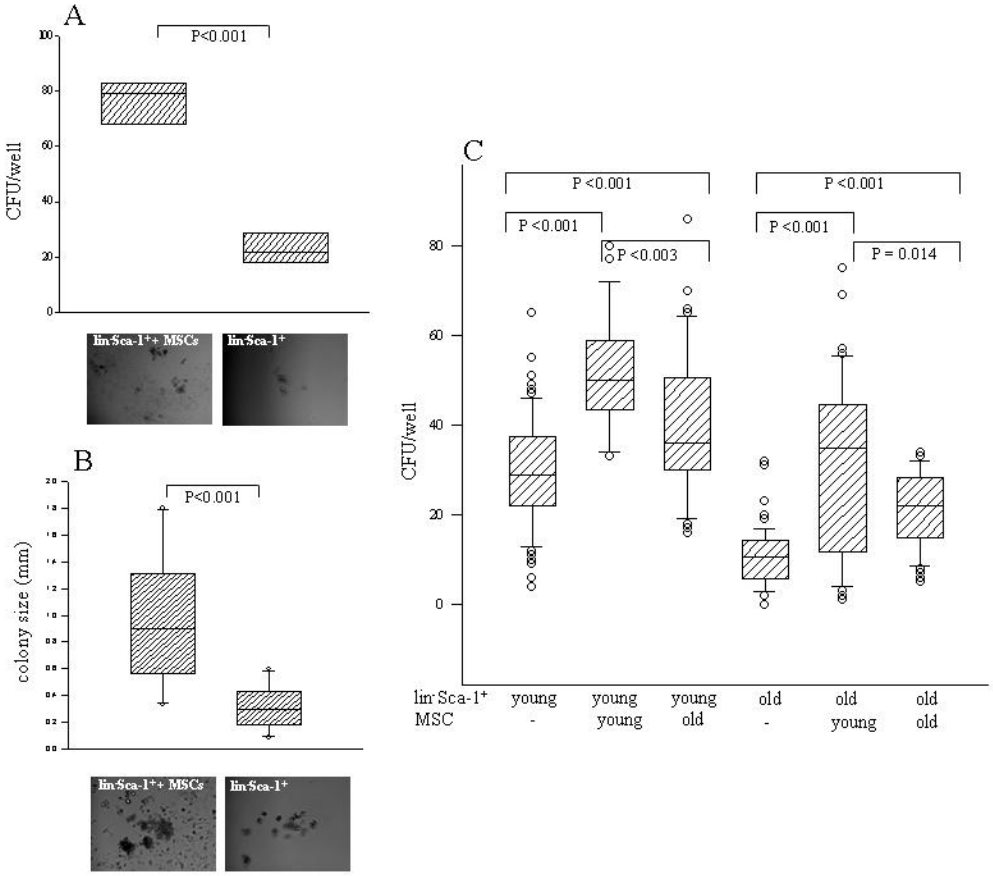

A
P<0.001
CFU/well
lin-Sca-1++ MSCs
lin-Sca-1+
B
P<0.001
colony size (mm)
lin-Sca-1++ MSCs
lin-Sca-1+
C
P <0.001
P <0.001
P <0.001
P <0.001
P <0.003
P = 0.014
CFU/well
lin-Sca-1+ young young young old old old
MSC - young old - young old

Fig.: 3

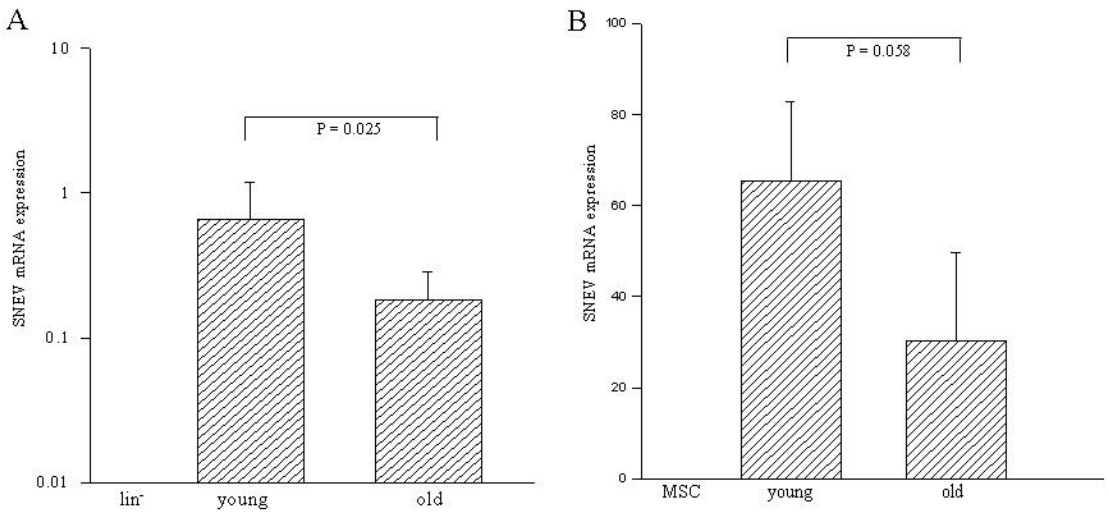

A
SNEV mRNA expression
10
1
0.1
0.01
P = 0.025
lin-
young
old
B
SNEV mRNA expression
100
80
60
40
20
0
P = 0.058
MSC
young
old

Fig.: 3

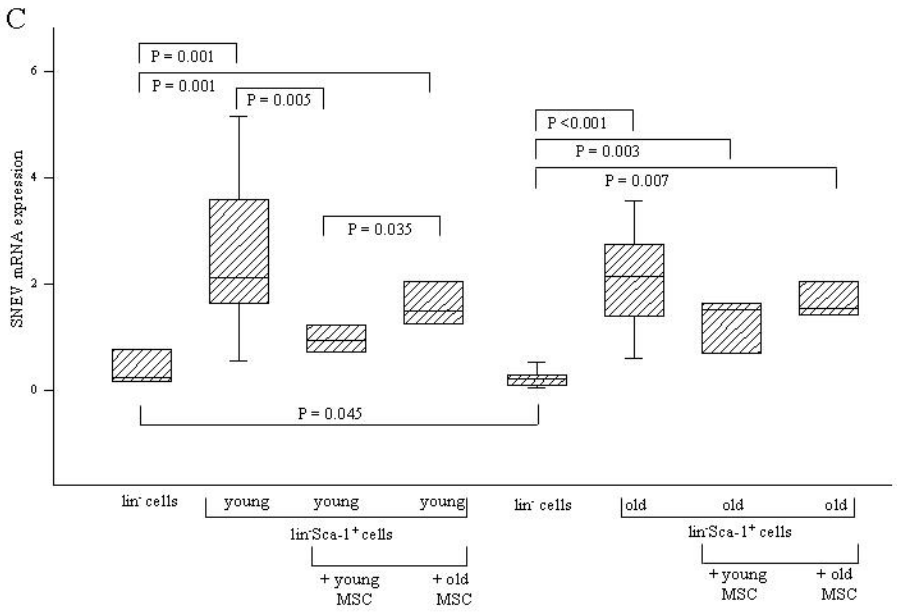

C
SNEV mRNA expression
6
4
2
0
P = 0.001
P = 0.001
P = 0.005
P = 0.035
P = 0.045
P <0.001
P = 0.003
P = 0.007
lin- cells
young
young
young
lin-Sca-1+ cells
+ young MSC
+ old MSC
lin- cells
old
old
old
lin-Sca-1+ cells
+ young MSC
+ old MSC

Fig.: 3

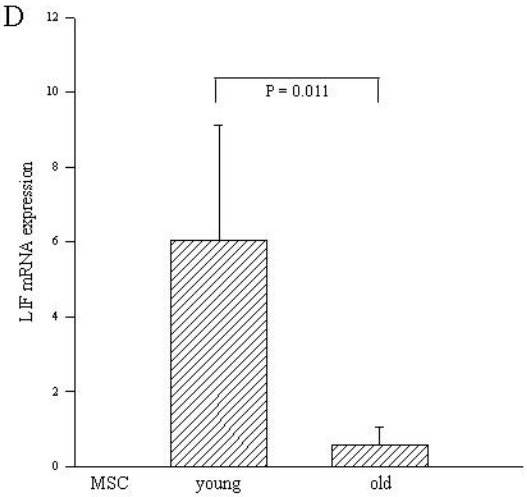

D
P = 0.011
LIF mRNA expression
MSC
young
old

Fig.: 4

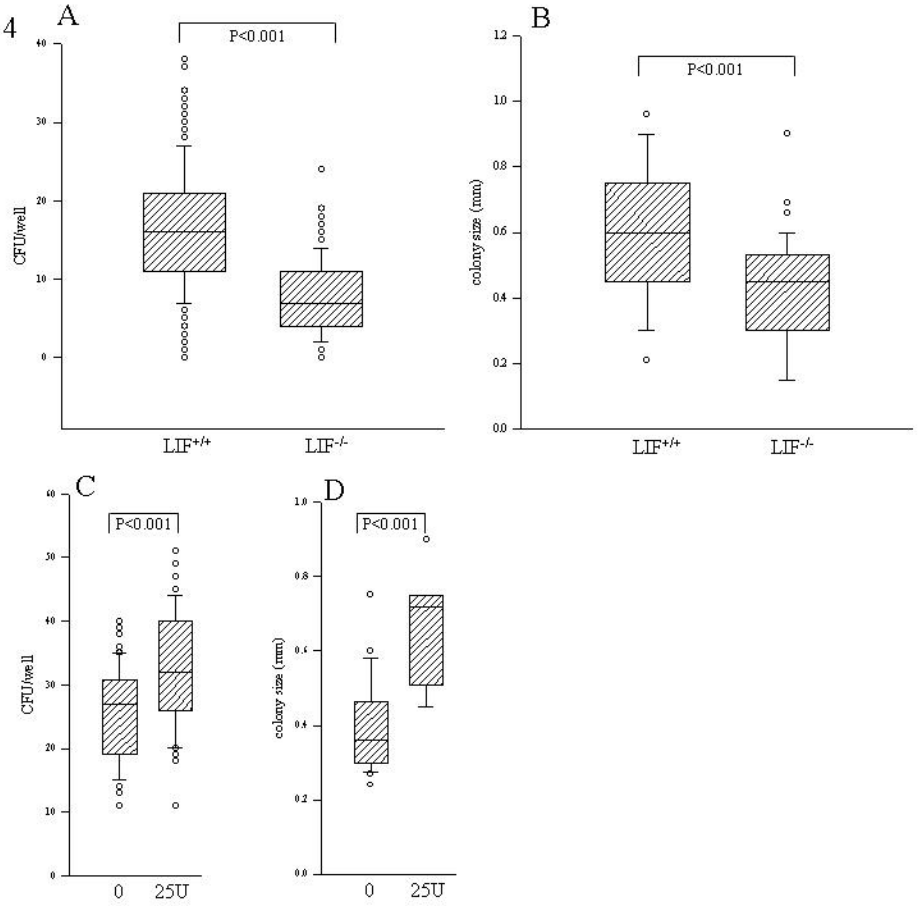

A
P<0.001
CFU/well
LIF+/+
LIF-/-
B
P<0.001
colony size (mm)
LIF+/+
LIF-/-
C
P<0.001
CFU/well
0
25U
D
P<0.001
colony size (mm)
0
25U

Fig.: 5

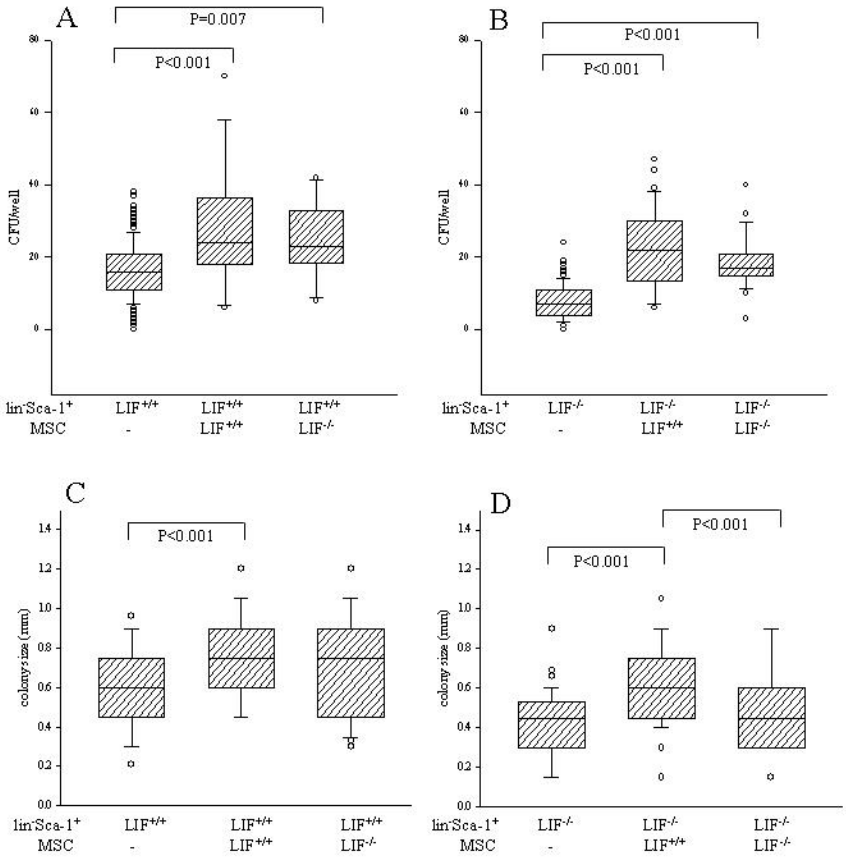
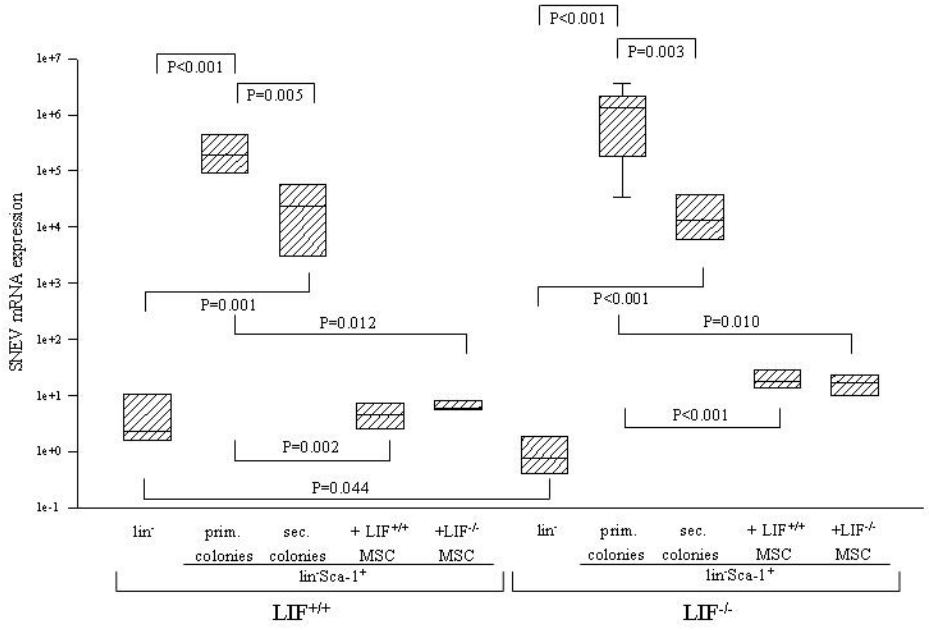

A
P=0.007
P<0.001
CFU/well
lin⁻Sca-1⁺ LIF⁺/⁺ LIF⁺/⁺ LIF⁺/⁺
MSC - LIF⁺/⁺ LIF⁻/⁻
B
P<0.001
P<0.001
CFU/well
lin⁻Sca-1⁺ LIF⁻/⁻ LIF⁻/⁻ LIF⁻/⁻
MSC - LIF⁺/⁺ LIF⁻/⁻
C
P<0.001
colony size (mm)
lin⁻Sca-1⁺ LIF⁺/⁺ LIF⁺/⁺ LIF⁺/⁺
MSC - LIF⁺/⁺ LIF⁻/⁻
D
P<0.001
P<0.001
colony size (mm)
lin⁻Sca-1⁺ LIF⁻/⁻ LIF⁻/⁻ LIF⁻/⁻
MSC - LIF⁺/⁺ LIF⁻/⁻

Fig.: 6


SNEV mRNA expression
P<0.001
P=0.005
P=0.001
P=0.012
P=0.002
P=0.044
P<0.001
P=0.003
P<0.001
P=0.010
P<0.001
lin⁻ prim. colonies sec. colonies + LIF⁺/⁺ MSC +LIF⁻/⁻ MSC
lin⁻Sca-1⁺
LIF⁺/⁺
LIF⁻/⁻